Samples of aggrecan chondroitin sulfate, isolated from normal human knee cartilages of individuals from fetal to 72 years of age, were digested with chondroitin lyases. The products were analyzed by fluorescence-based anion exchange high performance liquid chromatography to separate and quantitate nonreducing terminal structures, in addition to internal unsaturated disaccharide products. The predominant terminal structures were the monosaccharides, GalNAc4S and GalNAc4,6S as they were present on 85–90% of all chains. The remaining chains terminated with the disaccharide GlcA GalNAc4,6S as they were present on 5–9% of all chains. Disulfated in aggrecans from adults (22–72 years of age). First, terminal GalNAc residues were almost exclusively 4-sulfated in aggrecan from fetal through 15 years of age, but were ~50% 4,6-disulfated in aggrecans from adults (22–72 years of age). Second, the terminal disaccharide GlcA GalNAc4S was on ~7% of chains on aggrecan from fetal through 15 years of age, but on only ~3% of chains on adult aggrecan. In contrast, the proportion of chains terminating in GlcA GalNAc6S, ~9%, was unchanged from fetal to 72 years of age. This terminal disaccharide is proposed to be recognized by the widely used monoclonal antibody 3B3. However, chemical quantitation of the structure together with solid phase 3B3 (~) immunoassay of fetal and adult aggrecans showed that the content of the terminal disaccharide does not necessarily correlate with immunoreactivity of the proteoglycan, as chain density and presentation on the solid phase are critical factors for recognition of chain terminals by 3B3. The quantitative results obtained from chemical analyses of all nonreducing termini of aggrecan chondroitin sulfate chains revealed important changes in chain termination that occur when cellular activities are altered as adult articular cartilage is formed after removal of growth cartilage. These findings are discussed in relation to specific enzymatic steps that generate the nonreducing termini of chains in the biosynthesis pathway of chondroitin sulfate proteoglycans and their modulation in tissue development and pathology.
is known about the quantitative relationship between content of such chain termini in a PG and its reactivity with 3B3(−), and no information is available about the binding affinity of the antibody for the saturated mimotope compared with the affinity for the unsaturated epitope (25).

A range of studies have identified distinct nonreducing terminal residues present on CS synthesized in vitro by cells in culture (27, 28) and in isolated microsomal preparations (29), but data on the composition of nonreducing termini on CS from proteoglycans extracted from tissues are not available. It is therefore largely unknown if the mechanism for chain termination proposed from biosynthetic experiments in vitro operates in vivo, and whether it is altered in tissue development, maturation, or pathologies. Particularly in this context, it remains to be determined if immunosassays for the nonreducing terminal structures, such as 3B3(−), are reliable for detection of changes in the chain termination reactions.

We report here, using a newly developed highly sensitive fluorotag HPLC method (26) that distinct patterns exist in the nonreducing terminal residues of CS on aggrecan isolated from human cartilages of different ages. Based on these results it is proposed that distinct enzymatic steps operate at the CS chain termini, depending on the maturation stage of the tissue. Since only limited information is available by which quantifiable biochemical parameters can be selected that characterize the changeover from a transient growth cartilage to a stable adult cartilage, the nonreducing terminal structures of CS chains may provide sensitive indicators for modulations in extracellular matrix production during the development and growth of cartilage as well as other connective tissues.

**EXPERIMENTAL PROCEDURES**

β-Glucuronidase (bovine liver), monoclonal antibody 3B3 acetics fluid, and rat anti-mouse IgM were from ICN. Nitrocellulose membranes (0.2 µm) were from Bio-Rad. Hybond N+ membranes (0.2 µm), the ECL Western blotting reagent, and Hyperfilm were obtained from Amersham Corp. All other chemicals were obtained as described in Plass et al. (26).

Preparation of Special Materials—Macroscopically normal cartilage was excised from the hips of 59- and 72-year-old women undergoing replacement surgery following femoral neck fractures. Aggrecan was purified by dissociative CsCl density gradient centrifugation from 4 µm guanidine HCl extracts of the cartilages. The high buoyant density fractions (ρ > 1.59 g/ml, the D1 fractions) were dialyzed exhaustively against water, lyophilized, dissolved in water, and assayed for dimethylmethylene blue reaction (30). Preparations were stored at −20 °C in 1 mM cysteine at 60 °C for 16 h. Papain was inactivated by addition of iodoacetamide to a final concentration of 10 mM. β-galactosidase. Chains were then liberated by β-elimination in 50 mM NaOH, 1 M NaBH4 for 24 h at 37 °C. The solutions were then acidified on ice with 15% (w/v) acetic acid, but were not further analyzed, as recovery of 35S-labeled rat chondrosarcoma aggrecan was dilute with Tris-buffered saline to final concentrations of 0.78–100 µg/ml of total GAG. Aliquots (25 µl) of each dilution were applied to membranes in a 96-well dot blot apparatus (Bio-Rad). Macromolecules were adsorbed to the membranes for 1 h at room temperature before removing buffer and any unbound aggrecan by PBS wash and filtration. Individual dots were punched out with a normal puncher and washed with 5% (w/v) milk powder in PBS. Total radioactivity bound to the membranes was determined by scintillation counting. At concentrations between 0.78 and 50 µg/ml, a constant proportion of the applied sample (30 ± 5%), remained immobilized to either nitrocellulose or nylon N+. However, at higher concentrations, both membranes became saturated within a maximum of 350–400 ng of CS immobilized per dot area.

The binding of papain generated CS peptides and of intact aggrecan membranes was determined with a quantitative toluidine blue assay (31). For nylon N+ membranes, CS chains and aggrecan gave almost identical staining intensities at each concentration, for the entire concentration range used for immunosassay. On the other hand, only aggrecan, but not CS peptides, bound to nitrocellulose.

**CS Mapping of Membrane Immobilized Macromolecules—**Four dots for each concentration of immobilized 35S-labeled rat chondrosarcoma aggrecan (nitrocellulose and nylon N+) or papain generated 32P-labeled CS peptides (nylon N+) were incubated for 6 h at 37 °C in 200 µl of 50 mM sodium acetate, pH 7.5, with 1 milliunit each of chondroitinase ABC and ABCII. The amounts of radioactivity released by the enzymes were determined by scintillation counting. More than 90% of the radioactivity was released as Δdisaccharides from nitrocellulose membranes, and under identical conditions, only 30–40% of the radioactivity was removed from nylon N+ membranes. Moreover, papain-generated CS peptides immobilized on nylon N+ were essentially insensitive to chondroitinase digestion, with only ~5% of bound radioactivity released. This was in agreement with the data on the cationic membrane surface resulting in only limited access to the enzyme, or alternatively, some of the digestion products may remain bound to the cationic membrane. Intact aggrecan or CS peptides can be removed from the cationic membrane after a brief incubation with 2 M guanidine HCl, but were not further analyzed, as recovery of 35S-macromolecules was less than 10%.

**Assessment of Binding of Aggrecan and CS Chains to Nitrocellulose and Nylon N+ Membranes—**Quantitation of aggrecan bound to nitrocellulose and nylon N+ membranes was done as follows. 35S-Labeled rat chondrosarcoma aggrecan was diluted with Tris-buffered saline to final concentrations of 0.78–100 µg/ml of total GAG. Aliquots (25 µl) of each dilution were applied to membranes in a 96-well dot blot apparatus (Bio-Rad). Macromolecules were adsorbed to the membranes for 1 h at room temperature before removing buffer and any unbound aggrecan by PBS wash and filtration. Individual dots were punched out with a normal puncher and washed with 5% (w/v) milk powder in PBS. Total radioactivity bound to the membranes was determined by scintillation counting. At concentrations between 0.78 and 50 µg/ml, a constant proportion of the applied sample (30 ± 5%), remained immobilized to either nitrocellulose or nylon N+. However, at higher concentrations, both membranes became saturated within a maximum of 350–400 ng of CS immobilized per dot area.

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expressed as gray scale units of pixel density per dot as determined with the NIH 1.57 Image Analyses software. The film response was linear between 10 and 150 units for all exposure times. The 30-4 exposure time was chosen to give the optimum range for assaying aggrecan populations of low and high 3B3(-) reactivity.

RESULTS

Fluorescent HPLC Analyses of Chondroitinase Digests from Human Aggrecan CS—CS chains, released from aggrecans from normal knee joint cartilages, were chromatographed on Superose 6 (Fig. 1). Chains from adult (>22 years) aggrecan were hydrodynamically smaller ($K_{av}$ 0.62, molecular mass $\approx 18$ kDa) than those from fetal ($K_{av}$ 0.40, molecular mass $\approx 45$ kDa) or juvenile (newborn to 1 year) ($K_{av}$ 0.46, molecular mass $\approx 35$ kDa) aggrecans. Moreover, CS chains from the 15- and 17-year-old donors (shown for the 17-year sample, Fig. 1) eluted as a broader peak, indicating a greater heterogeneity in hydrodynamic sizes of the chain population in cartilages of individuals who are in the final stages of skeletal maturation.

We examined the possibility that the marked change in the average hydrodynamic size of CS on aggrecan deposited into the cartilages at different stages of development and maturation may be accompanied by changes in the composition of the nonreducing chain terminals. Ion exchange HPLC analyses of fluorotagged chondroitinase digestion products was done under conditions in which all internal $\Delta$disaccharides and nonreducing terminal monosaccharides and disaccharides are measured (26). The majority of the fluorospectroscopic results in the digests of human aggrecan represented the sulfated $\Delta$disaccharides (Table I), and in agreement with previous reports (3, 6, 7), the ratio of A$\Delta$6S:A$\Delta$4S was about 0.75 in aggrecan CS from cartilages up to 1 year of age, but increased to a much higher level, $\sim$25, in samples from individuals older than 15 years of age.

The nonreducing termini of CS chains (GalNAc4S, GalNAc6S, GlcA-GalNAc4S (Di4S) and GlcA-GalNAc6S (Di6S), Fig. 2), identified in the digests, constituted about 1.5, 2.3, and 6.7% of the total chondroitinase digestion products in fetal, young (newborn to 1 year), and adult aggrecan samples (22–72 years), respectively. The number averaged repeating disaccharides for each chain population was calculated from the ratio of interior $\Delta$disaccharides to nonreducing termini (Tables I and II). This indicated that fetal CS chains contained $\sim$66, young juvenile chains (newborn to 1 year) $\sim$42, and adult chains (22–72 years) of $\sim$14 repeats. The number averaged molecular masses calculated from these results were $\sim$32 kDa for fetal, $\sim$20 kDa for juvenile, and $\sim$8 kDa for adult CS. These number averaged molecular mass values were smaller than molecular masses estimated by the $K_{av}$ elution positions on gel filtration chromatography (45, 35, and 18 kDa, respectively) (Fig. 1). It is interesting to note that Midura et al. (28) reported for the analyses of metabolically labeled CS chains, isolated from rat chondrosarcoma aggrecan, an average molecular mass of chains determined after gel chromatography of $\sim$24 kDa, compared with the calculated number averaged molecular mass from chemical analyses of $\sim$18 kDa. In the current study, the discrepancy in size estimations may be explained, in part, if the dimethylmethylene blue dye accentuates detection of long chains, such that the contribution of shorter chains to the chromatographic profile would be underscored. In addition, the hydrodynamic size of CS on gel filtration columns was found to be influenced by sulfation isomer composition of the chains (5). Therefore, uniform sulfation, as seen with adult CS, may result in a different conformation than the copolymeric 4- and 6-sulfation, seen in CS from younger subjects.

The HPLC analyses further showed that sulfated GalNAc residues were the most abundant termini on human aggrecan CS, representing 85–90% of the total termini (Fig. 2A and Table II). The terminal GalNAc was always 4-sulfated, but after 15 years of age, a large portion was in the 4,6-disulfated form (Fig. 2A) such that $\sim$45% of CS chains on adult (22–72 years) aggrecan terminated with GalNAc6S. A small proportion of CS chains from all the aggrecan samples terminated with GlcA, as shown by the presence of the saturated disaccharides Di4S and Di6S in chondroitinase digests (Fig. 2B). Both sulfation isomers were present at all ages, with a 65:35 ratio of about 0.9 for the fetal and juvenile samples which increased to $\sim$3 in the adult samples (Table II). Together both saturated disaccharides represented about 14% of the terminals in fetal and young juvenile aggrecan (fetal to 15 years), and 10% in

* Calculated using an equation derived on Superose 6 calibrated with hyaluronan oligosaccharides of defined sizes (36).
indicated on the panel B with GalNAc4S (residues were quantitated, and the percentage of chains terminating 2-aminopyridine and analyzed by HPLC (26). Nonreducing terminal digested with chondroitinases, and products were derivatized with 10-chondroitin sulfate. Determined by capillary zone electrophoresis (5).

**TABLE I**

| Age       | n  | Percent of digestion products | Percent of ΔDi4S | ΔDi6S/ΔDi4S  
|-----------|----|-------------------------------|------------------|------------
| Fetal     | 2  | 98.31 ± 0.21                 | 1.0 ± 1.1        | 0.73 ± 0.03 |
| Juvenile (newborn to 1 year) | 3  | 97.67 ± 0.35                | 7.33 ± 0.58      | 0.77 ± 0.07 |
| 15 yr     | 1  | 95.8                         | 2.0              | 23         |
| 17 yr     | 1  | 94.3                         | 2.0              | 31         |
| Adult (22–72 years) | 4  | 93.33 ± 0.33                 | 1.50 ± 0.58      | 25.75 ± 2.75 |

* The values shown for the individual ages represent the means of three independent analyses for each sample.
* Determined by fluorescent derivitization and anion exchange HPLC (26).
* Determined by capillary zone electrophoresis (5).

**FIG. 2.** Nonreducing terminal composition of human aggrecan chondroitin sulfate. 10-μg portions of the purified aggrecan were digested with chondroitinases, and products were derivatized with 2-aminopyridine and analyzed by HPLC (26). Nonreducing terminal residues were quantitated, and the percentage of chains terminating with GalNAc4S (panel A) and GalNAc4,6S (panel B) was determined. The age ranges of cartilage specimen are indicated on the x axis and represent “growth cartilages” from fetal, young juvenile (2 months to 1 year), and juvenile (15 and 17 years) donors and “adult cartilage” from donors 22–72 years of age.

The lower abundance of these terminals in the adult was due to a reduction in the number of chains terminating with Di4S, while the proportion of CS chains terminating with Di6S remains essentially constant (7.0 ± 0.5%) over the entire age range (Fig. 2B). Thus, for all aggrecan samples about 1 CS chain out of every 14 terminates in Di6S, the postulated mimotope for 3B3(−) reactivity.

**Role of Nonreducing Terminal GlcA-GalNAc6S in 3B3(−) Reactivity**—It has been reported that aggrecan in developing embryonic cartilages reacts with 3B3(−), but aggrecan in adult cartilages does not. It has become generally accepted that the 3B3(−) mimotope, i.e. nonreducing terminal Di6S, represents a unique chain termination mechanism that is a feature of fetal CS, but not adult CS (15, 38). This conclusion is, however, not supported by the chemical analyses of nonreducing termini (Fig. 2B), which showed that the proportion of CS chains with this terminus is the same in both fetal and adult aggrecans. We therefore examined the relationship between 3B3(−) reactivity and the content of Di6S in fetal and adult human aggrecan CS using solid phase immunoassays, often employed in such studies.

Equivalent amounts (based on total GAG contents, determined by dimethylmethylen blue reactivity) of the two aggrecans were immobilized to nitrocellulose membranes. When normalized to total GAG content per membrane area, it is important to note that bound samples of adult aggrecan contain almost 3 times more CS chains than those of fetal aggrecan, because of the difference in their sizes (Fig. 3). Hence, immobilized adult aggrecan will contain about 3 times more Di6S terminals per area of membrane than will fetal aggrecan. Indeed, when the immobilized aggrecans were exposed to the monoclonal antibody 3B3(−), the adult sample had considerably higher reactivity per mass of CS than did the fetal sample (Fig. 4A, left-hand panels). Identical dots were incubated with chondroitinase to recover the bound CS as disaccharides and nonreducing terminal products (see “Experimental Procedures” for details). After mercuric acetate treatment of the digests, which eliminates all the unsaturated disaccharides, Di4S and Di6S could be quantitated (Fig. 4A, right-hand panel). This showed that both types of aggrecan contained similar amounts of Di4S (peak 1), but the adult sample had a significantly higher content of Di6S (peak 2). Thus, dots with immobilized adult aggrecan contained about 1.9 ng of Di6S, and those with fetal aggrecan contained about 0.4 ng of Di6S. This 4.5-fold difference is within the range predicted by the calculations shown in Fig. 3 and is consistent with the higher 3B3(−) reactivity of the adult sample.

Additional evidence to support that Di6S termini are required for 3B3(−) reactivity in this immunoassay was obtained by pretreating aggrecan with β-glucosidase, an exoglycosidase that specifically hydrolyzes nonreducing terminal GlcA residues (26, 28). This enzyme completely eliminated immunoreactivity in both fetal and adult aggrecan (Fig. 4B, left-hand panel). Subsequent analyses for the nonreducing terminals Di4S and Di6S in the treated, immobilized aggrecans (Fig. 4B, right-hand panel) showed a specific reduction in these peaks (indicated by arrowheads) with no change in the GalNAc4,6S in the adult (Fig. 4B, peak 3). Furthermore, in similar dot-blot experiments with aggrecan isolated from the rat chondroscarcinoma cell lines Rx and Ng (data not shown) virtually no 3B3(−) was detected. About 15% of the CS chains in these aggrecan preparations terminate with Di4S (Rx) or with Di6S (Ng), while less than 0.5% of the chains terminate with Di6S. Altogether, these results would be entirely consistent with Di6S being necessary for 3B3(−) reactivity.

**Reactivity of Fetal and Adult Aggrecan with 3B3(−)**—We examined if the abundance of CS chains terminating with Di6S can be quantitated using a solid phase 3B3(−) immunoassay. A range of GAG concentrations of fetal and adult aggrecans were...
immobilized to nitrocellulose and visualized with 3B3(−) (Fig. 5A, left-hand panel). Over this range of GAG concentrations, immunoreactivity was measurably lower for fetal than for adult aggrecan (Fig. 5B, left-hand panel). However, when the difference in Di6S content was taken into consideration (see Fig. 3), both aggrecans gave comparable reactivities per Di6S content (Fig. 5C, left-hand panel). Thus over a narrow substrate range (0.2–1 μg of GAG), 3B3(−) is a good measure for the abundance of Di6S termini on aggrecan CS immobilized on nitrocellulose.

Fetal and adult aggrecan were also assayed after immobilization to nylon N, an increasingly popular substrate for binding proteoglycans (15, 32, 33). With this membrane, the assay range for 3B3(−) was greater (0.05 ng to 1 μg of GAG) than on nitrocellulose (0.2–1 μg) (Fig. 5, A and B, right-hand panels). However, the mimotopes from the two aggrecans, when normalized for Di6S contents, displayed different reactivities with the antibody (Fig. 5C, right-hand panels). For example, 0.5 ng of fetal Di6S compared with 0.21 ng of adult Di6S was required for 100 units of pixel density. Therefore, ~2.5 times more mimotope from fetal aggrecan had to be bound to generate a reactivity that was equal to that seen for the adult aggrecan.

These results suggest that mimotope presentation and accessibility are particularly important when 3B3(−) immunoreactivity is determined on cationic membranes. Immobilization of PGs to these membranes takes place via the anionic GAGs and may involve the entire chain, including the nonreducing terminus. This was supported by the findings that papain-generated CS peptides do not bind to nitrocellulose, but bind quantitatively to nylon N membranes. However, when bound, the CS chains are neither digestible with chondroitinase nor reactive with 3B3(−) (data not shown). The interaction of chains with the cationic membrane will be more restricted when they

### Table II
Compositional analyses of nonreducing terminal residues of human aggrecan CS

| Age            | n | Percent of (Di4S+Di6S) | Di6S/Di4S | GalNAc4,6S/4S |
|----------------|---|------------------------|-----------|---------------|
| Fetal          | 2 | 14.5 ± 0.70            | 0.92 ± 0.01 | 0             |
| Juvenile       | 3 | 14.0 ± 1.0             | 0.89 ± 0.08 | 0             |
| 15 yr          | 1 | 15.0                   | 2.9        | 0             |
| 17 yr          | 1 | 11.0                   | 3.2        | 0.7           |
| Adult (22–72 yr) | 4 | 9.50 ± 0.58            | 3.02 ± 0.17 | 1.80 ± 0.05   |

* The values shown for the individual ages represent the means of three independent analyses for each sample.

The values shown for the individual ages represent the means of three independent analyses for each sample. Determined by fluorescent derivitization and anion exchange HPLC (26). This represents the abundance of GlcA nonreducing terminal residues as a proportion of all detected terminal residues.

### Fig. 3.
Schematic representation of fetal and adult aggrecan structure. A representation of fetal and adult aggrecan molecules, with emphasis on the length of the CS chains and the abundance of the Di6S termini, is shown. The number of CS chains per mass of total GAG (determined by dimethylmethylene blue reactivity) for the two aggrecans was determined from the Di6S content per mass of GAG (see "Experimental Procedures" for details) and the number averaged molecular masses based on the known number of repeating disaccharide units of the CS chains. The Di6S content was determined from the fluorescent HPLC analyses (see Fig. 2B). The PGs also contain keratan sulfate chains that are not shown. The adult aggrecan is shown truncated, as the average size of the molecules are reduced by limited proteolysis in the C-terminal core protein regions with time in the tissue (37).

### Fig. 4.
Effect of β-glucuronidase on 3B3(−) reactivity of adult human aggrecan. Fetal and adult human aggrecans were incubated in the absence (A) or presence (B) of β-glucuronidase (see "Experimental Procedures"). Portions (1 μg of GAG, as determined by dimethylmethylene blue reactivity) were immobilized in quadruplicate on nitrocellulose membranes and reacted with antibody 3B3(−) (left-hand panels). Equivalent dots were digested with chondroitinases (see "Experimental Procedures"). Products pooled from four dots were derivatized with 2-aminopyridine, exposed to mercuric acetate to remove the Δdisaccharides (26), and chromatographed on anion exchange HPLC. The figures show the position in the chromatogram (12–26 min) in which the nonreducing terminal structures Di4S (peak 1, 16.2 min), Di6S (peak 2, 17.1 min), and GalNAc4,6S (peak 3, 24.6 min) elute. Those peaks that decreased after β-glucuronidase digestion are indicated by arrowheads in panel B. The GalNAc4,6S peak in the fetal sample appears as a result of the ΔDi6S degradation by mercuric acetate and is not a reducing terminus in these aggrecans. The positions of nonspecific reaction peaks, generated by incubation of the nitrocellulose membranes in chondroitinase buffer alone, are indicated by an asterisk (*).
FIG. 5. 3B3(-) immuno-dot-blot assay of fetal and adult aggrecan on nitrocellulose and cationic nylon N+ membranes. Fetal and adult aggrecans were immobilized to nitrocellulose (left-hand panel) or nylon N+ membranes (right-hand panel) and developed with 3B3(-) (panel A). Immunoreactivity of fetal (□) and adult (■) aggrecan was quantitated by densitometric scanning of the dots and expressed per µg of GAG loaded (panel B). Based on the amount of GAG bound (see "Experimental Procedures") and the measured content of Di6S per µg of GAG, the calculated values for reactivity per ng of mimotope are shown in panel C. The amounts of fetal or adult aggrecan required to generate 100 area units of pixel density are indicated in panels B and C.

are attached to core proteins such that after immobilization some chains will come into contact with the membrane substrate, whereas others will remain in the solution phase where they can react with the antibody. The ease with which chains interact with the membrane may be further influenced by the degree of flexibility of the individual chains, and this could contribute to the differences in reactivities observed for fetal and adult aggrecan. Thus, fetal chains are about 3 times as long as adult chains, and their termini are further away from the core protein (Fig. 3). The collapsed structure of the fetal aggrecan therefore will distribute chains over approximately 3 times the area on the membrane, allowing more of the fetal CS termini to come into contact with the membrane, which would make them inaccessible to the antibody. In addition, it is possible that factors, such as cooperative binding to multiple sites on the IgM, require clustering of chains containing the Di6S termini. Differences in the arrangement of such chains along the CS attachment regions may also contribute to the variation in mimotope reactivities seen with the two aggrecans.

DISCUSSION

Application of the highly sensitive fluorotag HPLC method (26) to quantitate products in chondroitinase digests of human aggrecan, isolated from the femoral condyle cartilages of individuals from fetal age to 72 years, supports previous reports (3, 6, 7) on age-related changes in the fine structure of CS chains. Thus, a decrease in the average chain size occurred between fetal and early postnatal ages (up to 1 year). At skeletal maturity (>15 years) as growth cartilage is removed and replaced by adult cartilage, the average aggrecan CS chain size was decreased from 20 kDa to about 8 kDa, and the ratio of 6- to 4-sulfation on interior disaccharides is increased from ~0.77 to ~2 in the adult.

The nonreducing termini on human aggrecan CS were also isolated and quantitated in these analyses. These were GalNAc4S, GalNAc4,6S, GlcA-GalNAc4S, and GlcA-GalNAc6S (Fig. 2), and the relative abundance of the different termini was almost identical for CS from fetal cartilage to cartilage 15 years of age, but was distinctly different for CS in adult cartilages. Developmentally or pathologically induced alternations at the nonreducing termini CS have been proposed previously from immunological studies using the monoclonal antibody 3B3(-) (15, 19–24, 38). Our results confirm that this monoclonal antibody reacts with the nonreducing terminal sequence GlcA-GalNAc6S, since only aggrecans containing CS with this terminus showed a positive reaction. This was destroyed after a brief β-glucuronidase digestion to selectively eliminate the nonreducing terminal GlcA residues. The additional quantitative chemical data, however, suggest that the content of this nonreducing terminal disaccharide on PGs can be quite independent of their overall 3B3(-) reactivity. Specifically, concentration and presentation of the CS chains are important factors that need to be considered in the interpretation of 3B3(-) immunochemical assays. It may be particularly important to consider when comparing aggrecans by immunohistochemistry (21, 23, 38) or Western blotting after agarose acrylamide composite gels (15, 20, 22, 24). For example, we have demonstrated that in nitrocellulose-based assays (Fig. 5C) the threshold for 3B3(-) detection is very steep, followed by only a narrow dynamic range (0.2–0.8 ng), and this may easily result in an "all or none" reactivity. Moreover, it has been shown that, after electrophoretic separation on composite gels, aggrecan from fetal cartilage migrated as a single narrow band, whereas aggrecan from adult cartilage migrated more diffusely in a broad band or multiple closely spaced bands (3, 33). The concentration of CS chains would be expected to be higher in the tight band of the fetal aggrecan than in the diffuse bands of the adult aggrecan, and following transblot, only the fetal sample would be above the threshold concentration of Di6S required for a positive reaction with 3B3(-). Hence, structural properties of aggrecan that determine the electrophoretic migration on composite gels may be important confounding factors not often considered for 3B3(-) Western blot assays. In summary, the studies presented above clearly indicate that immunochimical assays, with other anti-CS antibodies (14, 25, 40) to report structural alterations within the CS chains, may also need to be validated with chemical analyses similar to those described here.

The data derived from the chemical analyses of the nonre-
The chain terminal residues of adult cartilage CS are clearly different. Most significantly, after formation of a nonreducing terminal GalNAc4S (step 1), this residue can be 6-sulfated (step 6), to form GalNAc4S6S, after which it is no longer a substrate for addition of GlcA. The reduction in the proportion of the GalNAc4S6S termini in the adult CS, with concomitant increase in GalNAc4S,6S, suggests that a separate 6-sulfotransferase activity, with specificity for nonreducing terminal GalNAc4S, appears after skeletal maturity and may be related to the GalNAc4S,6S transferase that was partially purified from human serum (39). Most notably, such an enzyme activity can also be detected in rat chondrosarcoma cells, that produce CS chains, with virtually no interior 6-sulfated disaccharides but with ~30% of the chains containing GalNAc4S,6S on their nonreducing terminus (28). The terminal disaccharides generated by extension of GalNAc with GlcA (step 2) were largely 6-sulfated in the adult, in keeping with the high level of 6-sulfation of internal disaccharides in adult cartilage CS (Table 1), and the observed reduction in the proportion of Di4S terminals in the adult CS (Fig. 2B) would follow from the very low activity of 4-sulfotransferase for internal sulfation (step 4). However, the 6- to 4-sulfation ratio in the terminal disaccharides was significantly lower (~3) than that of the internal disaccharides (~25) (Table 1). This suggests that extension of GalNAc4S by addition of a single GlcA (step 5) may generate a significant proportion of the Di4S terminals during CS production in adult cartilages.

In conclusion, this study showed for the first time the presence of age-related patterns in sulfation of the nonreducing terminal mono- and disaccharides on aggrecan CS and suggests that distinct modifications take place at these positions during CS biosynthesis. Further characterization of the enzymatic and cellular mechanisms underlying these biosynthetic steps and examination of these modifications in other CS PGs should provide new insights into the posttranslational modification pathways of PG core proteins during extracellular matrix production in a range of cells as well as during different stages of development, maturation, or pathology of various connective tissues (14, 25, 41, 42).

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