Animal cell mutants defective in glycosaminoglycan biosynthesis

(Chinese hamster ovary cells/replica plating/proteoglycans/sulfation/xylosyltransferase)

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ABSTRACT   We have obtained Chinese hamster ovary cell mutants defective in the biosynthesis of glycosaminoglycans by screening replicate colonies immobilized on polyester cloth. Depending upon the strain, the mutants accumulated less ^{35}S-labeled glycosaminoglycans per μg of cell protein by a factor of 6–60 compared to the wild type. Some of the mutants incorporated [6-^{3}H]glucosamine into glycosaminoglycans to the same extent as the wild type, suggesting that sulfate addition was specifically altered. In contrast, five strains failed to generate ^{3}H-labeled glycosaminoglycans normally. In four of these, the initiation of glycosaminoglycan assembly was specifically altered, since the addition of p-nitrophenyl-β-xyloside restored sulfation to normal. Enzymatic assay of the xylosyltransferase in extracts prepared from these mutants revealed that one of the strains, S745, contained less enzyme activity by a factor of 15 than the wild type. This mutant provides genetic evidence that the xylosyltransferase assayed in vitro is responsible for the initiation of chondroitin sulfate and heparan sulfate biosynthesis in vivo.

Proteoglycans are ubiquitous components of mammalian cells and tissues (1–3). To a large extent, their chemical and physical properties are determined by the glycosaminoglycan chains attached glycosidically to serine residues of core proteins (Fig. 1). For example, in cartilage, the chondroitin sulfate proteoglycans produced by chondrocytes provide a hydrated, viscous gel of high compressibility (1). In other tissues, cells are surrounded by an extracellular matrix composed of proteoglycans, collagen, and other macromolecules, which together may influence cell adhesion, shape, and possibly locomotion (4–6). In hepatocytes, membrane-intercalated heparan sulfate proteoglycans are thought to bridge the extracellular matrix and the intracellular cytoskeleton, whereas proteoglycans bound to endothelial cell plasma membranes facilitate the binding of circulating macromolecules (4–6). The presence of proteoglycans in secretory granules and lysosomes (4) suggests that they may also play a role in fundamental processes of the cell, such as secretion and endocytosis.

Tissues and cells vary tremendously in the array of proteoglycans present (7), and the arrangement of repeating disaccharide units containing ester sulfate, N-sulfate, N-acetyl groups, D-glucuronic acid, and L-iduronic acid suggests that enormous molecular heterogeneity of the glycosaminoglycans may exist (Fig. 1). Undoubtedly, the composition of glycosaminoglycans is determined biosynthetically (2), but the factors that control their assembly and distribution remains unclear. An important approach to these problems is the isolation of mutants altered in specific biosynthetic steps.

In this report we demonstrate a screening technique (8, 9) that permits the identification of Chinese hamster ovary (CHO) cell mutants bearing mutations in different stages of glycosaminoglycan biosynthesis. One of these mutants has a defect in the xylosyltransferase (2), the first sugar transfer reaction in glycosaminoglycan formation (Fig. 1).

EXPERIMENTAL PROCEDURES

Materials. Na^{35}SO$_4$ (25–40 Ci/mg; 1 Ci = 37 GBq) and D-[6-^{3}H]glucosamine HCl (25–30 Ci/mmol) were obtained from Amersham. UDP-[1-^{3}H]xylose was purchased from New England Nuclear. All nonradioactive chemicals were generally obtained from Sigma. Chondroitinase AC from Arthrobacter aurescens (Sigma), chondroitinase ABC from Proteus vulgaris (Sigma), and heparitinase from Flavobacterium heparinum (Miles) were stored in 50 mM Tris·HCl (pH 7.0) at −20°C.

Cell Cultures. CHO cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61) and grown in an atmosphere of 5% CO$_2$ in air and 100% relative humidity in Ham's F12 growth medium (GIBCO) supplemented with 100 μg of streptomycin sulfate per ml, 100 units of penicillin G per ml, and 10% (vol/vol) fetal bovine serum (HyClone, Logan, UT). Growth medium lacking sulfate was prepared from individual components (10), substituting chloride salts for sulfate salts and omitting streptomycin sulfate. Defined medium was supplemented with 10% (vol/vol) fetal bovine serum dialyzed 10^6-fold against phosphate-buffered saline (P$_i$/NaCl) (11).

Mutageneses were performed with ethylmethane sulfonate as described (8). After outgrowth at 33°C and storage in liquid nitrogen, an aliquot of cells was revived and tested for the incidence of strains resistant to 1 mM ouabain. Mutagen-treated stocks in which the frequency of ouabain-resistant strains was greater than 1/10^4 viable cells were used for the mutant screenings described below.

Polyester Cloning. The immobilization of CHO cells on 17-μm polyester discs was performed as described (8, 9), with two layers of cloth per dish. The isolation of the mutants is described in detail in the legend of Fig. 2. Glass beads and polyester cloth were prepared as described (8, 9).

Xylosyltransferase Assay. Measurement of xylosyltransferase activity using silk fibroin as substrate (12) was performed in extracts prepared from cells grown at 37°C in normal growth medium. Typically, cells in the exponential phase of growth were harvested in 0.25 M sucrose containing 20 mM Tris·HCl (pH 7.4), 1 μg of leupeptin per ml, 0.5 μg of pepstatin A per ml, 20 μM phenylmethylsulfonyl fluoride, and 10 mM sodium azide by scraping with a rubber policeman. The cell suspensions were centrifuged at 1100 × g at 4°C for 5 min and resuspended at a protein concentration of 7–10 mg/ml as measured by the method of Lowry et al. (13). After brief sonication, the extracts were stored at −20°C. Assay conditions optimized for CHO cells are provided in the legend of Table 4. Enzyme activity was not detectable in the supernatant of the low-speed centrifugation used to harvest the cells.
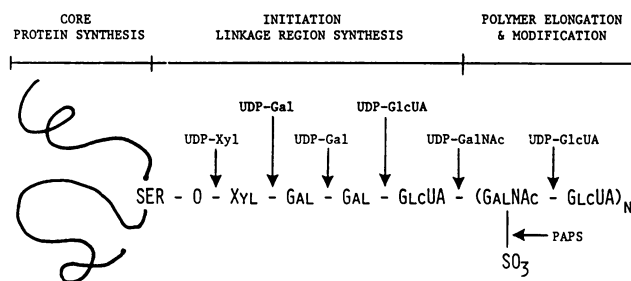

Abbreviation: CHO, Chinese hamster ovary.

FIG. 1.   Biosynthesis of a chondroitin sulfate proteoglycan in mammalian cells. PAPS, 3'-phosphoadenyl-5'-phosphosulfate.

FIG. 2.   Visualization of mutants defective in $^{35}SO_4$ incorporation by autoradiography. Mutagen-treated cells were placed in 100-mm (diameter) tissue culture dishes containing 15 ml of complete growth medium to yield ≈300 colonies per plate at 33°C. After 1 day, the attached cells were overlaid with two layers of polyester cloth (17-μm pore diameter) fitted to the dish and glass beads were added (8, 9). The cells were incubated at 33°C for another 16 days to permit the formation of colonies. At this time the medium was aspirated and the beads were decanted from the dish. The polyester cloth was removed and placed at 33°C in a 100-mm (diameter) bacterial culture dish containing 15 ml of growth medium lacking sulfate. After 2 days, the discs were shifted to 40°C for 16 hr. Next, they were placed in 5 ml of the same growth medium supplemented with $^{35}SO_4$ (10 μCi/ml). Four hours later, the discs were treated with 10% trichloroacetic acid, which resulted in the precipitation of ^{35}S-glycosaminoglycans linked to protein, while unincorporated radioactive sulfate was removed by washing each disc in a Buchner funnel under reduced pressure with ≈100 ml of 2% trichloroacetic acid. After drying the discs at room temperature overnight, they were exposed to Kodak XAR-5 x-ray film for ≈24 hr. The discs were then stained with 0.05% Coomassie brilliant blue G in methanol/water/acetic acid, 45:45:10 (vol/vol), and destained in methanol/water/acetic acid as described (8, 9). Throughout these manipulations the original master plate was stored at 28°C under otherwise normal growth conditions in medium supplemented with 10 units of nystatin per ml and 2.5 μg of Fungizone (GIBCO) per ml. A piece of Whatman no. 42 paper and glass beads were used to prevent the formation of satellite colonies during storage. Mutants identified as blue-stained colonies on the disc lacking an autoradiographic halo (the arrow indicates the position of mutant S745) were retrieved with glass cloning cylinders and trypsin (19) from the master plates. All mutants were passed through the above cloning procedure until only mutant colonies were present at the final repurification. (*A* and *B*) The stained disc and corresponding autoradiogram, respectively, from the original mutant screening. (*C* and *D*) Results obtained from the first repurification of mutant S745, indicated by the arrow in *A* and *B*.

Composition of Radioactive Glycosaminoglycans in CHO Cells.

CHO cells were labeled with 50 μCi of $^{35}SO_4$ per ml for 3 days at 37°C in growth medium lacking inorganic sulfate. The cells were harvested by adjusting the medium in the dish to 0.1 M NaOH. After neutralization with acetic acid, the mixture was digested at 40°C for 16 hr with 2 mg of Pronase (Calbiochem) per ml in 0.32 M NaCl/40 mM sodium acetate, pH 5.5. The released glycosaminoglycans were collected in the presence of 2 mg of chondroitin sulfate (mixed isomers) per ml by precipitation with cetylpyridinium chloride and ethanol according to established procedures (14). Portions of the radioactive material were treated for 8 hr at 40°C with 5 milliunits of chondroitinase AC or ABC (15) or 0.5 unit of heparitinase (16). Radioactive products were quantitated after treatment with 1% cetylpyridinium chloride in the presence of 0.32 M NaCl as described by Oldberg *et al.* (17).

RESULTS

Autoradiographic Detection of Mutant Colonies Defective in Sulfate Incorporation. CHO cells, like other mammalian cells (3, 18), incorporate $^{35}SO_4$ preferentially into glycosaminoglycans. Analysis of ^{35}S-labeled glycosaminoglycans (^{35}S-glycosaminoglycans) obtained from wild-type CHO cells revealed that 55% ± 5% of the radioactive material was susceptible to chondroitinase AC (or ABC) digestion, and 40% ± 5% was cleaved by heparitinase (*Experimental Procedures*). Although sulfated glycosaminoglycans are soluble in trichloroacetic acid, their attachment to protein in intact cells renders them partially precipitable with acid. This suggested the possibility of detecting glycosaminoglycan biosynthesis *in situ* in CHO colonies immobilized on polyester cloth by autoradiography (Fig. 2; refs. 8 and 9). In preliminary experiments, we observed that wild-type CHO cells incorporated sulfate (as measured by autoradiography) in proportion to the protein content of the colony (as measured by the Coomassie blue G staining intensity). In contrast, mutagen-treated colonies grown at 33°C yielded an occasional variant that failed to incorporate $^{35}SO_4$ into acid-precipitable material at 40°C (arrow, Fig. 2 *A* and *B*). Putative mutants identified in this way were harvested and passed through the screening procedure again to confirm their phenotypes (Fig. 2 *C* and *D*). Mutant colonies, defective in $^{35}SO_4$ incorporation, were purified by multiple cycles of cloning and autoradiography, expanded in monolayer culture, and stored in liquid nitrogen. The incidence of colonies that failed to incorporate $^{35}SO_4$ was approximately 1/500 in mutagen-treated populations and $<10^{-4}$ in the absence of mutagenesis.

Growth Characteristics of the Mutants. All of the mutants were obtained under conditions in which potentially lethal mutations in essential genes might be rescued by growth at low temperature (33°C). Analysis of the ability of the mutants to form colonies under permissive (33°C) and restrictive conditions (40°C) revealed that three of the mutants (S719, S729, and S739) were temperature-sensitive for
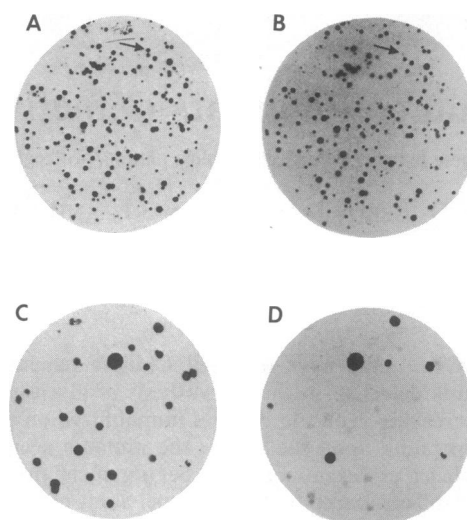
growth at 40°C (data not shown). The other variants proliferated normally at 33°C and 40°C, suggesting that there was no necessary correlation between reduced sulfate incorporation, as measured by autoradiography, and the temperature-sensitive growth phenotype. At 37°C all of the strains gave rise to colonies normally and no obvious alterations in adhesion, trypsin sensitivity, and gross morphology were noticeable (data not shown).

Defective Incorporation of $^{35}SO_4$ in the Mutants. To examine the extent to which sulfate uptake was reduced, we incubated cells with $^{35}SO_4$ at 33°C and 40°C under conditions that label the glycosaminoglycans to constant specific radioactivity (Table 1). As shown, the incorporation of $^{35}SO_4$ relative to cell protein was less in strain S803 by a factor of 6 and less in strain S757 by a factor of 60 compared to wild-type cells. The defects were independent of the temperature at which

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Table 1. Content of sulfated glycosaminoglycans in mutant and wild-type cells at 33°C and 40°C

	^{35}S cpm/μg of cell protein			
	33°C			40°C
Strain	Cells	Medium	Total	(total)
Wild-type CHO-K1	1800	4000	5800	6500
Mutant				
S20	30	190	220	230
S719	120	300	420	470
S729	90	260	350	360
S733	50	90	140	290
S739	30	120	150	80
S745	40	150	190	390
S757	20	70	90	110
S761	140	250	390	470
S785	270	600	870	1130
S803	380	780	1160	1060
S813	30	120	150	450

Multiple 60-mm (diameter) tissue culture dishes were each seeded with $\approx 1 \times 10^5$ cells in 5 ml of growth medium lacking inorganic sulfate and containing 10 μCi of ^{35}SO$_4$ per ml. After 2 days of incubation at 33°C some of the dishes were shifted to 40°C. After overnight incubation the medium was removed, and the cell monolayer was washed five times with P$_i$/NaCl at 4°C. Treatment of the cells with 1 ml of 0.1 M NaOH resulted in their solubilization and an aliquot was assayed for protein content (13). Another portion was neutralized with acetic acid and mixed with an equal volume of 4 mg of Pronase per ml in 0.64 M NaCl/80 mM sodium acetate, pH 5.5. A portion of the spent medium was treated under similar conditions. Carrier chondroitin sulfate (mixed isomers) was included at 2 mg/ml. After incubation at 45°C for 16 hr, the liberated glycosaminoglycans were collected by precipitation with 1% cetylpyridinium chloride in 0.3 M NaCl on Whatman 3MM paper as described by Wasteson *et al.* (20). Radioactivity was quantitated by liquid scintillation spectrometry using 10 ml of Aquasol (New England Nuclear), and the values obtained were corrected for the amount of cell protein digested. Each value is the average of at least two determinations. The difference in ^{35}SO$_4$ incorporation at 33°C and 40°C in the mutants is within the limits of experimental error.

the cells were grown (Table 1), providing additional evidence that the temperature-sensitive growth phenotype of mutants S719, S729, and S739 was caused by a mutation unrelated to the defect in sulfate incorporation. Both mutant and wild-type cells secreted about two-thirds of ^{35}S-glycosaminoglycans into the growth medium, suggesting that none of the defects was the result of abnormal secretion. Similar results were obtained when the cetylpyridinium chloride–glycosaminoglycan complexes were collected in the presence of 0.1 M NaCl, by direct ethanol precipitation of the Pronase digests, and when the labeling was performed in the presence of 0.1 mM sodium sulfate (data not shown).

Glucosamine Incorporation as a Measure of Carbohydrate Backbone Formation. Because sulfate incorporation is dependent upon the synthesis of the carbohydrate backbone (Fig. 1), we labeled cells simultaneously with ^{35}SO$_4$ and [6-^{3}H]glucosamine, a precursor of the hexosamine moieties of the chains (Table 2). Initial difficulty in obtaining adequate radioactive glucosamine incorporation was overcome by reducing the glucose content in the growth medium to 1 mM. Incorporation of ^{35}SO$_4$ under these conditions was not dramatically altered in parental wild-type cells and the mutants (Table 2). Quantitation of the ^{3}H-labeled glycosaminoglycans revealed two classes of mutants in the collection. In the first, ^{3}H labeling occurred to the same extent as observed in wild-type cells (mutants S20, S733, S739, S757, S785, and S813), whereas in the second class, uptake of [^{3}H]glucosamine was reduced by a factor of 4–6 (S719, S729, S745, S761, and S803). These results suggested that the glucosamine-negative strains contained lesions in chain initiation

Table 2. Content of glycosaminoglycans labeled with ^{35}SO$_4$ and [6-^{3}H]glucosamine in mutant and wild-type cells at 37°C

	cpm/μg of cell protein	
Strain	^{35}SO$_4$	[6-^{3}H]Glucosamine
Wild-type CHO-K1	3800	2100
Mutant		
S20	70	1800
S719	600	490
S729	280	530
S733	240	1500
S739	120	1800
S745	70	390
S757	230	1100
S761	180	510
S785	510	1500
S803	960	570
S813	120	2300

Multiple 100-mm (diameter) tissue culture dishes were each seeded with $\approx 2 \times 10^5$ cells in 15 ml of sulfate-free growth medium containing 1 mM glucose and 10 μCi each of [6-^{3}H]glucosamine and ^{35}SO$_4$ per ml. After incubation at 37°C for 3 days, aliquots of the medium and the cells were treated as described in the legend of Table 1. Liberated glycosaminoglycans containing ^{3}H and ^{35}S radioactivity were quantitated by liquid scintillation spectrometry. After correction for $\approx 5\%$ spillover of ^{3}H and $\approx 20\%$ spillover of ^{35}S radioactivity into the opposite windows, the values were normalized to the amount of cell protein treated. Shown are the average values from duplicate determinations of the radioactivity recovered from the medium plus the cells.

or polymerization, whereas the other mutants most likely were unable to add sulfate to newly synthesized chains. The reduction of glucosamine incorporation was not as great as the decrease in sulfation due to the incorporation of glucosamine into other complex carbohydrates (unpublished results).

Sorting Chain-Initiation Mutants with β-xyloside. To examine whether the reduced [^{3}H]glucosamine incorporation observed in some of the mutants (Table 2) might reflect altered core protein synthesis or chain initiation (Fig. 1), we measured the ability of wild-type CHO cells to use β-xylosides (21) as primers for glycosaminoglycan chain formation (Table 3). In control experiments, wild-type cells were briefly incubated with ^{35}SO$_4$ with and without cycloheximide and p-nitrophenyl-β-xyloside. Treatment with cycloheximide

Table 3. Incorporation of ^{35}SO$_4$ into glycosaminoglycans in the presence of p-nitrophenyl-β-xyloside

	^{35}S cpm/μg of cell protein	
Strain	Without β-xyloside	With β-xyloside
Wild-type CHO-K1	1200	1400
+ Cycloheximide	40	1100
Mutant		
S719	30	1400
S729	30	1450
S745	10	1500
S761	40	1050
S803	230	490

Sixty-millimeter (diameter) dishes were each seeded with $\approx 1 \times 10^5$ cells in 5 ml of growth medium lacking sulfate. After 3 days of incubation at 37°C, the medium was replaced with 5 ml of sulfate-free medium containing 10 μCi of ^{35}SO$_4$ per ml and 1 mM p-nitrophenyl-β-xyloside. Some cultures containing wild-type cells also received 100 μg of cycloheximide per ml 2 hr before and during the labeling. After 4 hr at 37°C, aliquots of the medium and the cells were treated as described in the legend of Table 1, and ^{35}S-glycosaminoglycans were collected in the presence of 0.1 M NaCl. Shown are the average values of the cells plus medium from two determinations.

alone reduced the incorporation of $^{35}SO_4$ from 1200 cpm/μg of cell protein to 40 cpm/μg of cell protein, suggesting that the majority of glycosaminoglycans generated in CHO cells is normally linked to protein. In the presence of cycloheximide and 1 mM β-xyloside wild-type cells incorporated nearly the same amount of sulfate as compared to the control without cycloheximide (1100 cpm/μg of cell protein vs. 1400 cpm/μg of cell protein, respectively), demonstrating that β-xylosides will substitute for xylosylated core proteins in *de novo* glycosaminoglycan synthesis. When the five glucosamine-negative mutants (Table 2) were incubated with $^{35}SO_4$ and the β-xyloside, sulfation was restored to normal levels in four of the strains (Table 3). In contrast, strain S803 was defective in sulfate incorporation in both the presence and absence of the β-xyloside. These results suggested that mutants S719, S729, S745, and S761 were possibly defective in or upstream from the linking of xylose to the core proteins (Fig. 1), whereas mutant S803 was most likely defective in a step downstream from xylose addition.

Defective Xylosyltransferase Activity in Mutant S745. The first committed sugar transfer reaction in the assembly of glycosaminoglycans is the attachment of xylose to a serine residue of a core protein (Fig. 1; ref. 2). Recently, Campbell *et al.* (12) demonstrated that silk fibroin is a potent, artificial acceptor for the xylosyltransferase from cartilage. When cell extracts prepared from mutant and wild-type CHO cells were assayed for xylosyltransferase activity with silk fibroin (a generous gift from L. Rodén) and UDP-[1-^{3}H]xylose (Table 4), we observed that mutant S745 possessed less than 1/15th the enzyme specific activity (0.1 pmol/min per mg of cell protein) detected in the wild type (1.5 pmol/min per mg of cell protein). In contrast, the enzyme activity in the other mutants was comparable to that observed in the wild type. When equal amounts of protein from wild-type and mutant S745 cells were mixed and assayed for xylosyltransferase, the resultant specific activity was one-half that obtained when the wild type was assayed alone (1.0 vs. 2.0 pmol/min per mg of cell protein, respectively). This excluded the presence of soluble antagonists in mutant S745 and demonstrated that the defect in the synthesis of cellular glycosaminoglycans, judged by sulfate and glucosamine incorporation, was correlated with the loss of xylosyltransferase activity. Because CHO cells contain both chondroitin and heparan sulfate (*Experimental Procedures*), a common xylosyltransferase most likely participates in the formation of these polymers.

Table 4. Xylosyltransferase activity of mutant and wild-type cells assayed at 37°C

Strain	Enzyme specific activity, pmol/min per mg
Wild-type CHO-K1	1.5 ± 0.1
Mutant	
S719	1.2
S729	1.5
S745	0.1
S761	1.1

Approximately 25 μg of crude cell protein was mixed wth 50 μM UDP-[1-^{3}H]xylose (200–500 cpm/pmol), 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) buffer (pH 6.5), 50 mM potassium chloride, 10 mM magnesium chloride, 5 mM manganese chloride, 0.3% Triton X-100, and 2 mg of soluble silk fibroin per ml (12) in a total volume of 25 μl. Radioactive product generated after 1 hr of incubation at 37°C was precipitated with 10% trichloroacetic acid and collected on cellulose acetate membranes (Gelman 0.45-μm pore diameter) under reduced pressure. The discs were rinsed with 30 ml of 2% trichloroacetic acid and the radioactive product was quantitated by liquid scintillation spectrometry. Product formation in competent strains was proportional to time for 2 hr and to protein between 10 and 100 μg. The residual activity in mutant S745 was not.

DISCUSSION

Our genetic dissection of glycosaminoglycan assembly in CHO cells indicates that the incidence of mutants, like S745, is very high after mutagen treatment, suggesting that the entire biosynthetic pathway is potentially amenable to genetic dissection. The absence of growth characteristics in the mutants that might facilitate their direct selection or enrichment (22) demonstrates the utility of the polyester cloning technique (8, 9). This approach does not require any previous knowledge of glycosaminoglycan function and yields a spectrum of mutants with interesting properties. Indirect screening for animal cell mutants using polyester cloth and filter paper replicas has been used successfully to isolate mutants in lysosomal enzymes and cell-surface receptors (23, 24), lipid and membrane protein biosynthesis (25–29), amino acid transport (30), and DNA repair (31, 32). Reversion and complementation studies of the glycosaminoglycan mutants reported here are simplified since it should be possible to identify positive clones by $^{35}SO_4$ autoradiography without the use of drug-resistant strains and selections.

Studies of disproportionate dwarfisms in animals (33–35) have revealed the importance of core proteins and sulfation in the development of cartilage, but these systems are limited by the availability of rare strains bearing desirable mutations. In contrast, the screening technique described here permitted the identification of CHO mutants bearing mutations in glycosaminoglycan initiation (S719, S729, S745, S761), elongation (S803), and modification (S20, S733, S739, S757, S785, S813). Further studies of these strains should reveal whether the biosynthetic pathway (Fig. 1) inferred from enzyme activities detected in cell-free extracts is correct. Studies of revertants and complementation analysis may reveal alternate pathways and the presence of regulatory genes.

This report provides genetic evidence that the xylosyltransferase first described by Neufeld and co-workers (36) is responsible for the initiation of glycosaminoglycan assembly *in vivo* and that a common xylosyltransferase participates in the formation of chondroitin and heparan sulfate chains. Xylosyltransferase is thought to consist of two pairs of nonidentical subunits (37). Although enzymatic evidence demonstrates that the transfer of xylose is defective in strain S745, further studies are needed to determine whether the mutation lies in a regulatory gene as opposed to the structural gene encoding the catalytic subunit of the enzyme. The presence of normal xylosyltransferase activity in mutants S719, S729, and S761 is consistent with this possibility.

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