

# Developmental Changes in Heparan Sulfate Expression: In Situ Detection with mAbs

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**Abstract.** Two mAbs that are specific for heparan sulfate-related epitopes have been raised and used to analyze the cellular and tissular distribution of this glycosaminoglycan during development. mAb 10E4 reacts with an epitope that occurs in native heparan sulfate chains and that is destroyed by N-desulfation of the glycosaminoglycan. The antibody does not react with hyaluronate, chondroitin sulfate, or DNA, and reacts only poorly with heparin. The reactivity of proteoglycan extracts or tissue sections with the 10E4 antibody is completely abolished by heparitinase, but is only partially affected by heparinase. mAb 3G10, in contrast, reacts only with heparitinase-treated heparan sulfate chains, proteoglycans, or tissue sections. The 3G10 epitope is destroyed by treatment with mercuric acetate, which indicates that the desaturated uronate generated by the lyase is essential for the reactivity of the antibody. The 3G10 epitope is not generated by

treating heparan sulfate proteoglycans with heparinase or chondroitin sulfate proteoglycans with chondroitin sulfate lyases, which indicates that the 3G10 antibody recognizes desaturated uronates that occur in specific structural contexts. The antibody 10E4 and, after heparitinase treatment, the antibody 3G10 decorate the surfaces of many cell types and the extracellular matrix in proximity of the cells, in particular, the basement membranes. The analysis of embryonic and adult tissues reveals important temporal and regional differences in the abundance of the 10E4 and 3G10 epitopes at these sites. Moreover, the staining pattern of the two antibodies is not always superimposable, which is indicative of regional differences in the exposure or structure of the tissular heparan sulfates. As a whole the results suggest that heparan sulfate abounds at sites of active morphogenesis and that the expression of this glycosaminoglycan is developmentally regulated.

**T**HE heparan sulfate proteoglycans occur at the cell surface and in the extracellular matrix in the immediate vicinity of the cells (Gallagher, 1989). These localizations are assumed to be dictated, at least in part, by membrane- and matrix-anchoring structures in the protein moieties of these molecules. For human fetal fibroblastic cells, for example, this has been supported by molecular cloning experiments, which have identified complementary DNA structures coding for core proteins with extended sequences of hydrophobic amino acids that could function as membrane spanning domains (Marynen et al., 1989) and as signals for the attachment of a glycosyl phosphatidylinositol anchor (David et al., 1990), and by functional studies, which have documented a specific and high affinity binding interaction between fibronectin and the core protein of the matrix-bound proteoglycan (Heremans et al., 1990). The expression of at least some of these core proteins, e.g., that of the cell surface proteoglycan *syndecan* (Saunders et al., 1989), undergoes important changes during embryonic morphogenesis, suggesting that HSPGs actively participate in and may even modulate developmental processes (Bernfield and Sander-son, 1990).

Many of the functions of these proteoglycans, however, depend on their polysaccharide moieties, whereby the number, length, charge, and sequence complexity of the heparan sulfate chains play an important role (Ruoslahti, 1989). These chains appear to vary, not only between different proteoglycans, but also for different representatives of a single proteoglycan species. To a certain extent this variability appears intrinsic to the molecular mechanisms that underlie the post-translational modification of proteins in general, and the biosynthesis of these complex carbohydrates in particular (Lindahl et al., 1986). Yet, other variations seem less fortuitous. Studies of syndecan, for example, have revealed changes in the type, number, and the length of the glycosaminoglycan chains, depending on the source of the proteoglycan (Sander-son and Bernfield, 1988; Salmivirta et al., 1991) and the physiological environment of the cells (Rapraeger, 1989). An even more extreme example is provided by serglycin, which is substituted with heparin chains in mastocytoma cells (Kjellén et al., 1989), but primarily with chondroitin sulfate chains in basophilic leukemia cells (Seldin et al., 1985), indicating that the carbohydrate moiety of the proteoglycan varies with the differentiation and functional status

of the cells. Growth factors seem to be important in this context (Rapraeger, 1989), but the variables that influence these structural aspects of the proteoglycans remain largely unknown. Since the heparan sulfate chains determine the matrix, growth factor, and proteinase inhibitor-binding activities of the proteoglycans, these structural differences may not be trivial and influence the adhesiveness, growth, and migration of the cells, and the outcome of the developmental processes that depend on these cellular properties.

The establishment of structure-function relationships in the proteoglycans and the evaluation of their expression will therefore necessarily include the characterization of their carbohydrate moieties. For a long time chemical and enzymatic susceptibility tests have played an important and exclusive role in this characterization. More recently, for chondroitin sulfate (Couchman et al., 1984; Yamagata et al., 1987), keratan sulfate (Caterson et al., 1983), heparan sulfate (Kure and Yoshie, 1986), and heparin (Pejler et al., 1988), these approaches have been supplemented with immunological assays using mAbs specific for epitopes that are unique to these glycosaminoglycans or their degradation products. In the present report we describe two mAbs that extend this approach to the detection and characterization of the heparan sulfate proteoglycans. One detects intact heparan sulfate chains, the other the desaturated uronates produced during the cleavage of the heparan sulfate chains with heparitinase, allowing the mapping of heparan sulfate and of the heparitinase-resistant proteoglycan cores in tissues and tissue extracts. These antibodies reveal that the expression of heparan sulfate undergoes important changes during embryonic development.

## Materials and Methods

### Biochemicals

Heparin from porcine mucosa was obtained from Calbiochem-Behring Corp. (San Diego, CA). Chondroitin sulfate A from whale cartilage, chondroitin sulfate B from pork skin, chondroitin sulfate C from shark cartilage, heparan sulfate from bovine kidney, and hyaluronic acid from human umbilical cord were obtained from Sigma Chemical Co. (St. Louis, MO). Dextran and dextran sulfate were obtained from Pharmacia LKB (Uppsala, Sweden). Salmon sperm DNA was from Sigma Chemical Co. Heparinase (heparin lyase, EC 4.2.2.7), heparitinase (heparan sulfate lyase, EC 4.2.2.8), chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4), and chondroitinase AC II (chondroitin AC lyase, EC 4.2.2.5) were from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Proteinase K was from Merck (Darmstadt, Germany), and thermolysin from Calbiochem-Behring Corp. mAb IB5 was a kind gift of Dr. Bruce Caterson (University of North Carolina, Chapel Hill, NC).

### Cell Culture

Normal human fetal lung fibroblasts were cultured in DME (Gibco Europe, Paisley, Scotland, UK) supplemented with 10% (vol/vol) FCS. Labeling of confluent monolayers with carrier-free  $^{35}\text{S}$  sulfuric acid (New England Nuclear, Boston, MA) in culture medium with reduced sulfate content was as described before (David and Van den Berghe, 1983).

### Heparan Sulfate Proteoglycan Isolation

Heparan sulfate proteoglycans were isolated from the detergent extract, extracellular matrix, and the conditioned media of cultured human fetal lung fibroblasts as described before (Lories et al., 1986). Hydrophobic membrane-associated heparan sulfate proteoglycans were purified by liposome incorporation, followed by gel filtration on Sepharose CL4B in 4 M guanidinium chloride, and by ion exchange chromatography on Mono Q in

0.5% Triton X-100, 6 M urea, 50 mM Tris-HCl, pH 8.0 (Lories et al., 1987). Secreted heparan sulfate proteoglycans from the matrix and media fractions were purified by cesium chloride density gradient centrifugation, ion-exchange chromatography on Mono Q, and gel filtration on Sepharose CL4B in 4 M guanidinium chloride (Heremans et al., 1988).

### Enzymatic and Chemical Treatments

**Heparinase, Heparitinase, and Chondroitinase ABC Digestions.** Purified samples of the heparan sulfate proteoglycans or 200- $\mu\text{g}$  aliquots of the different commercial glycosaminoglycans were digested for 3 h at 37°C with 1 mU of heparinase, 1 mU of heparitinase, or 5 mU of chondroitinase ABC in 0.1 ml of 100 mM sodium chloride, 1 mM calcium chloride, 50 mM sodium hepes, pH 7.0, containing 5  $\mu\text{g}$  of BSA. The proteoglycan samples were digested in the presence of proteinase inhibitors, as described before (David and Van den Berghe, 1985).

**Proteinase Digestion.** Proteoglycan samples were digested for 2 h at 65°C with 100  $\mu\text{g}/\text{ml}$  of proteinase K in 50 mM Tris-HCl buffer, pH 8.0, or with 100  $\mu\text{g}/\text{ml}$  of thermolysin in 5 mM calcium chloride, 50 mM Tris-HCl, pH 8.0.

**Desulfation, Acetylation, and Sulfation Reactions.** Heparan sulfate proteoglycans or heparin were dissolved in 1 M pyridinium chloride, 1 M guanidinium chloride, pH 5.2, and N-desulfated by treatment with 19 vol DMSO for 4 h at 50°C as described before (David and Van den Berghe, 1985). The N-desulfated samples were dissolved in 0.125 M sodium bicine, pH 9.0, and acetylated as described before (David and Van den Berghe, 1985) or resulfated by treatment with 250 mM sulfurtrioxide-trimethylamine complex (Inoue and Nagasawa, 1976). Trinitrobenzene sulfonic acid was used to monitor the degree of substitution of the modified N-desulfated samples (Glazer et al., 1975).

**Nitrous Acid Degradation.** 1 vol of purified heparan sulfate proteoglycans dissolved in water was mixed with 4 vol of ice-cold 0.5 M nitrous acid, pH 1.5, and left at room temperature (Shively and Conrad, 1976). After 10 min the sample was neutralized with sodium hydroxide and dialyzed against 20 mM Tris-HCl, pH 7.4.

**Treatment with Mercuric Acetate.** Proteoglycan samples eluted from DEAE with 0.8 M sodium chloride, 0.2 M sodium acetate, pH 5.0, and heparitinase-digested proteoglycan samples in heparitinase buffer were mixed with 3 vol of 0.1 M sodium acetate, 25 mM mercuric acetate, pH 5.0, and left for 10 min at room temperature (Ludwigs et al., 1987).

**Reduction.** Heparitinase-digested heparan sulfate proteoglycans were reduced with 1 M potassium borohydride for 2 h at room temperature.

**Radioiodination.** The proteoglycans were labeled using sodium  $^{125}\text{I}$  and chloramine-T as described before (Lories et al., 1987).

### Isolation of the Hybridomas

BALB/c mice were immunized with liposome-incorporated membrane heparan sulfate proteoglycans (F58) or with heparitinase-digested medium heparan sulfate proteoglycans (F69) from human fetal lung fibroblasts. The immunization schemes and the protocols for cell fusion and hybridoma selection were as described before (De Boeck et al., 1987). The supernatants of the hybridoma cultures were screened by immunodot blotting on Zeta probe membranes and by reverse immunodot blotting using rabbit anti-mouse Ig-coated nitrocellulose sheets and [ $^{35}\text{S}$ ]sulfate-labeled or  $^{125}\text{I}$ -labeled heparan sulfate proteoglycan, as described (De Boeck et al., 1987). Hybridomas F58-10E4 and F69-3G10 were subcloned twice by limiting dilution. The corresponding mAbs were purified from ascites fluid by ammonium sulfate precipitation and gel filtration. mAb 10E4 was identified as an IgM and mAb 3G10 as an IgG2b, using Mouse Typer (Bio-Rad Laboratories, Richmond, CA). Other antibodies used were the antiglypican mAb S1 (De Boeck et al., 1987), the antiperlecan mAb 9C9 (Heremans et al., 1989), and mAb IB5, which is directed against unsaturated uronate linked to N-acetylgalactosamine (Couchman et al., 1984).

### Proteoglycan Binding Assays

mAb 10E4 was coupled to CNBr-activated Sepharose (2 mg/ml gel) following the instructions of the supplier (Pharmacia LKB). Aliquots (40,000 cpm) of  $^{125}\text{I}$ -iodinated or [ $^{35}\text{S}$ ]sulfate-labeled proteoglycan or glycosaminoglycan were incubated with 20  $\mu\text{g}$  immobilized antibody in the presence of 0.5% Triton X-100 and of various buffers, salts, and competitors (see Results). After overnight incubation at 4°C with mixing, the immunobeads were pelleted by centrifugation, and the radioactivity was determined in the supernatant and pellet fractions.

## Immunocytochemistry

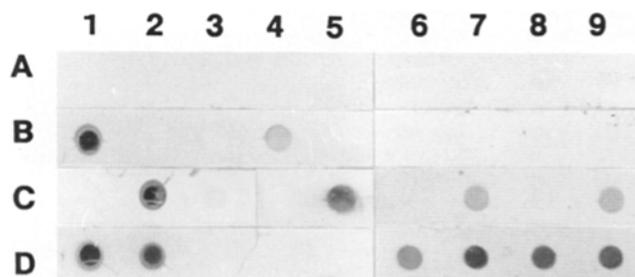
Cryosections and deparaffinated sections of tissues fixed in 2% paraformaldehyde were overlaid with 2.5 mU of heparitinase in 0.5 ml of buffer or with buffer only, for 2 h at 37°C. After rinsing with 0.5% casein (wt/vol) in PBS, the sections were incubated for 1 h (cryosections) to 3 h (paraffin sections) with mAb 10E4 (5 µg/ml) or with mAb 3G10 (2 µg/ml) in 0.5% casein-PBS, rinsed, and further incubated for 1 h with horseradish peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark). After rinsing, the sections were stained using 0.05% 3,3'-diaminobenzidine (wt/vol) and 0.01% hydrogen peroxide (wt/vol). In some experiments the tissue sections were treated for 10 min at room temperature with 14 mM mercuric acetate at pH 5.0 (Ludwigs et al., 1987) before or after the enzyme treatment, and then further processed for immunocytochemistry.

## Results

### mAb 10E4 Reacts with Heparan Sulfate

Several hybridomas were obtained after immunization with intact heparan sulfate proteoglycan. During the screening assays the culture supernatant from hybridoma 10E4 was found to react with intact proteoglycan, but not with heparitinase-treated proteoglycan, which implied that the corresponding epitope might reside in the heparan sulfate chains. This hybridoma was subcloned twice and its antibody was further characterized.

In dot-blot assays, mAb 10E4 reacted strongly with heparan sulfate proteoglycans from the culture medium of human fetal lung fibroblasts (Fig. 1, B1). It also reacted with heparan sulfate proteoglycans that were isolated from the detergent extracts (Lories et al., 1987) or from the detergent-insoluble matrices (Heremans et al., 1988) of these cells (not shown). Heparitinase treatment of the proteoglycans abolished their reactivity with mAb 10E4 (Fig. 1, B2), without interfering with the binding of the proteoglycans to the membrane, as revealed by the persistence of the signal obtained with mAb S1 (Fig. 1, D1 and D2), a mAb that reacts with the core pro-



**Figure 1.** Enzyme and chemical susceptibilities of the epitopes. Heparan sulfate proteoglycans from the culture medium of human lung fibroblasts were spotted on Zeta probe membranes either without any pretreatment (lane 1), or after one of the following regimens: a treatment with heparitinase only (lane 2); with heparitinase first and then with borohydride (lane 3); with borohydride only (lane 4); with borohydride first and then with heparitinase (lane 5); with nitrous acid only (lane 6); with nitrous acid followed by heparitinase (lane 7); with heparitinase followed by mercuric acetate (lane 8); or inversely with mercuric acetate followed by heparitinase (lane 9). The strips were exposed to either no first antibody (row A), mAb 10E4 (row B), mAb 3G10 (row C), or mAb S1 (row D), and stained using a peroxidase-conjugated rabbit anti-mouse second antibody.

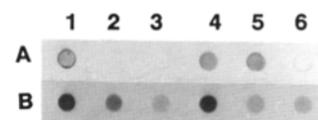
tein of glypican, a cell surface heparan sulfate proteoglycan that is shed to the culture medium (David et al., 1990). mAb 10E4 also reacted with heparan sulfate chains that were isolated from these proteoglycans by extensive digestion with proteinase K and precipitation with ethanol (Fig. 2, A1). Digestion with chondroitinase ABC did not affect the reactivity of the fractions (not shown). Treating the proteoglycans (not shown) or the protein-free heparan sulfate chains with DMSO under conditions that selectively remove N-sulfate, in contrast, completely abolished the reactivity (Fig. 2, A2). Autoradiography to detect the residual O-linked [<sup>35</sup>S]sulfate (~50% of the total [<sup>35</sup>S]sulfate initially present) confirmed that the N-desulfated chains remained bound to the Zeta-probe membrane during the assay (Fig. 2 B). The reactivity of the DMSO-treated samples was restored by resulfation (Fig. 2, A5), but not by acetylation (Fig. 2, A3). Moreover, acetylation of the DMSO-treated samples prevented restoration of the immunoreactivity by treatment with trimethylamine sulfurtrioxide complex (Fig. 2, A6). Treatment with DMSO, in contrast, did not affect the reactivity of the proteoglycans with, for example, mAb S1, as reported previously (De Boeck et al., 1987). As a whole, the effects of these different modifications confirmed that mAb 10E4 was reacting with the glycosaminoglycan moiety of the heparan sulfate proteoglycans, and suggested that N-sulfate formed part of the epitope.

### Variables That Affect the Reactivity of mAb 10E4

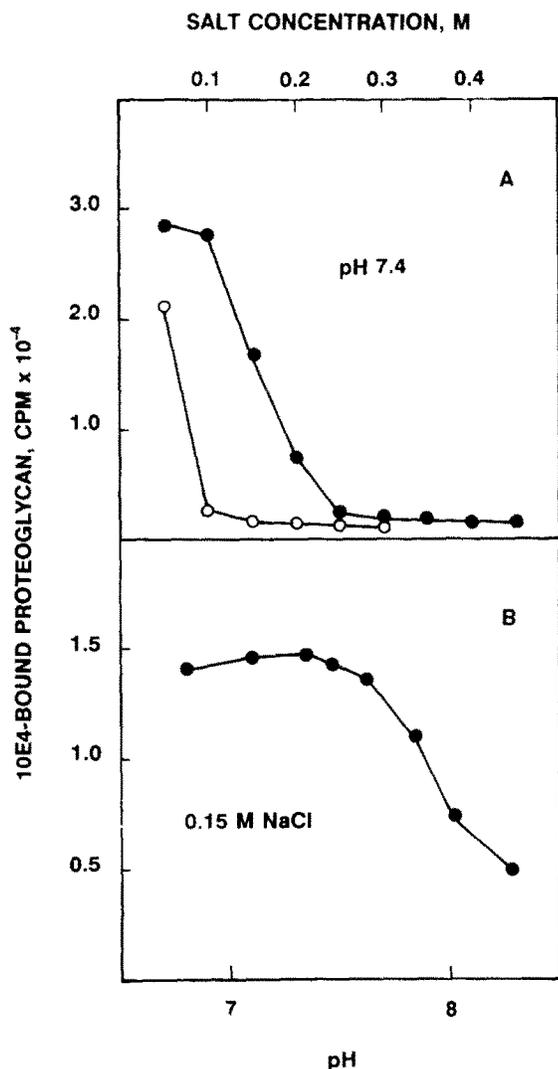
The reactivity of mAb 10E4 with heparan sulfate or with heparan sulfate proteoglycan was salt and pH sensitive. At physiological pH the binding of soluble labeled glycosaminoglycan or proteoglycan to immobilized 10E4 antibody was markedly reduced by concentrations of NaCl >0.15 M, and virtually inexistent at NaCl concentrations >0.25 M (Fig. 3 A). At similar molar concentrations multivalent salts were more effective inhibitors than monovalent salts. The binding was also pH sensitive. At physiological ionic strength (0.15 M NaCl), it was significantly reduced at pH values >7.6 (Fig. 3 B). On the basis of these findings further binding experiments were conducted at pH 7.4 in the presence of 50 mM NaCl.

### Specificity of mAb 10E4

To assess the specificity of mAb 10E4, the Sepharose-bound antibody was used to fractionate <sup>35</sup>S-proteoglycans pro-



**Figure 2.** Reactivity of mAb 10E4 with chemically modified heparan sulfate chains. Protein-free heparan sulfate chains from metabolically labeled proteoglycans were spotted on Zeta probe membrane strips without any pretreatment (lane 1), or after one of the following treatments: N-desulfation (lane 2); N-desulfation followed by acetylation of the free aminogroups (lane 3); exposure to sulfurtrioxide-trimethylamine complex; (lane 4); N-desulfation followed by resulfation with sulfurtrioxide (lane 5); or N-desulfation followed by acetylation and then exposure to sulfurtrioxide (lane 6). The strips were incubated with antibody 10E4 and stained using peroxidase-conjugated second antibody (row A) and then exposed for autoradiography (row B).



**Figure 3.** Salt and pH sensitivity of the heparan sulfate proteoglycan-mAb 10E4 binding interaction. The membrane proteoglycan fraction from cultured human lung fibroblasts was labeled with <sup>125</sup>I, and aliquots (40,000 cpm) were allowed to bind to Sepharose CL4B beads substituted with 10E4 antibody (6 μg/assay) at different concentrations of either sodium chloride (●) or sodium sulfate (○) (A), and at different pH values (B). The inhibition curves obtained with sodium phosphate and ammonium sulfate were superimposable on that obtained with sodium sulfate (not shown).

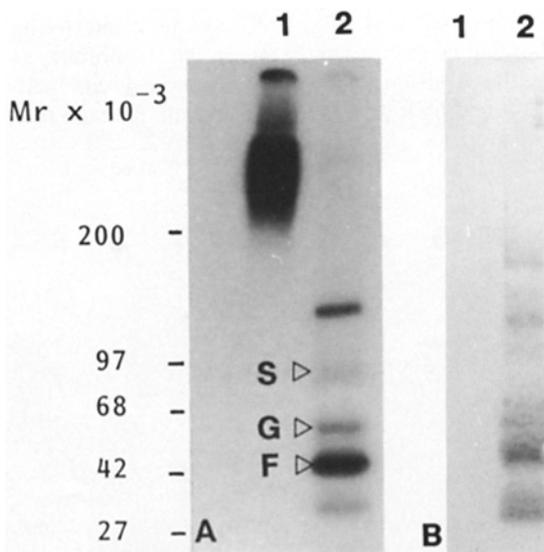
duced by cultured human fetal lung fibroblasts (Table I). Compared with the composition of the cell extracts and media fractions that were originally applied, the immunopurified materials were substantially enriched in <sup>35</sup>S-heparan sulfate, whereas the fall-through fractions were partially depleted of <sup>35</sup>S-heparan sulfate. When this fall through was reapplied to a fresh column, again part of the heparan sulfate was bound (not shown). This indicated that mAb 10E4 preferentially retained heparan sulfate and heparan sulfate proteoglycan. On the other hand, the fractionation of <sup>125</sup>I-labeled lipophilic proteoglycans yielded 10E4-bound label, which was retained near the origin during electrophoresis in SDS-polyacrylamide gradient gels, but which migrated as multiple sharp bands after heparitinase digestion (Fig. 4 A).

**Table I. Proteoglycan and Glycosaminoglycan Fractionation on mAb 10E4**

Source	HS composition		
	Applied	Bound	Fall through
		(%)	
PG			
C	54.3	94.7	44.5
M	31.6	83.9	21.5
GAG			
C	51.6	96.0	40.4
M	25.9	89.1	16.4

The proteoglycans (PG) from <sup>35</sup>SO<sub>4</sub>-labeled human lung fibroblast cell layers (C) and from the conditioned culture media of these cells (M) and the glycosaminoglycans (GAG) prepared from these proteoglycans by exhaustive protease treatment were mixed with Sepharose CL4B-coupled 10E4 antibody. After an overnight incubation the beads were collected, washed, and eluted with 1 M sodium chloride. The heparan sulfate content of the applied, bound, and nonbound fractions was measured by cetylpyridinium chloride precipitation of control and nitrous acid-treated samples (David and Van den Berghe, 1983), and expressed as a percentage of the total <sup>35</sup>S-GAG in each of these fractions.

This result was consistent with the evidence that this proteoglycan fraction is composed of multiple distinct proteoglycans (Lories et al., 1989), and indicated that the 10E4-reactive heparan sulfate chains were carried by all core proteins present in this fraction. The specificity of mAb 10E4 for heparan sulfate was also apparent from competition experiments in which the 10E4 immunobeads were incubated with <sup>125</sup>I-iodinated heparan sulfate proteoglycan or with <sup>35</sup>S-labeled



**Figure 4.** Occurrence of the 10E4 and 3G10 epitopes in individual proteoglycans. (A) The <sup>125</sup>I-labeled cell surface proteoglycans from human lung fibroblasts, a mixture of heparan sulfate and chondroitin sulfate proteoglycans, were incubated with solid phase-immobilized mAb 10E4. Bound proteoglycans were eluted with salt and analyzed by SDS-PAGE and autoradiography either without (lane 1) or after digestion with heparitinase (lane 2). (B) Nondigested (lane 1) and heparitinase-digested (lane 2) nonlabeled cell surface proteoglycans were submitted to SDS-PAGE, blotted onto Zeta probe, and stained with mAb 3G10. The positions of fibroglycan (F), glypican (G), and syndecan (S) have been indicated.

heparan sulfate in the presence of various anionic polymers (Table II). In these binding experiments, chondroitin sulfate chains and casein at concentrations of 100 µg/ml had no effect. Heparan sulfate, in contrast, at this and even lower concentrations, was an effective inhibitor. Some commercial preparations of hyaluronate and dermatan sulfate were inhibitory (Table II), but this activity appeared due to contamination with heparan sulfate (Table III). The inhibitions were completely abolished by pretreating the glycosaminoglycan samples with heparitinase or with nitrous acid, while a pretreatment with heparinase had only a moderate effect. A chondroitinase ABC treatment, in contrast, had no influence on the inhibitory effect of these samples, further ruling out any reactivity of hyaluronate or dermatan sulfate itself. On a weight basis heparin was less inhibitory than heparan sulfate (Table II). Its inhibitory effect was abolished by N-desulfation (Table II) and by heparinase (Table III), but also by heparitinase, albeit less efficiently (Table III). Various dextran sulfates were strong inhibitors, but dextran was not. Treating these dextran sulfates with heparitinase, heparinase, chondroitinase, or nitrous acid had no effect on their inhibitory activity (not shown).

#### ***mAb 3G10 Reacts with Heparitinase-generated Eliminate Reaction Products***

Several hybridomas were also obtained after immunization with heparitinase-digested heparan sulfate proteoglycan. The culture medium of hybridoma 3G10 was found to immunoprecipitate heparitinase-digested <sup>125</sup>I-iodinated proteoglycans, but not the intact <sup>125</sup>I-iodinated or metabolically <sup>35</sup>S-<sub>4</sub>-labeled proteoglycans. This finding implied that the

**Table II. <sup>125</sup>I-HSPG and <sup>35</sup>S-HS Binding to mAb 10E4: Effect of Anionic Polymers**

Compound added	Bound label	
	<sup>125</sup> I	<sup>35</sup> S
	(%)	
None	69.5	65.3
Heparin	34.6	46.5
N-desulfated heparin	62.2	60.6
Acetylated N-desulfated heparin	63.3	61.4
Hyaluronate	8.8	23.3
Chondroitin sulfate		
A	-	64.9
C	63.2	64.7
B	44.8	-
Dextran sulfate	0.1	10.8
Dextran	67.7	-
Heparan sulfate		
50	11.1	-
20	33.0	32.7
10	41.5	-
Casein	-	60.9
Salmon sperm DNA	63.5	-

Radioiodinated heparan sulfate proteoglycans (<sup>125</sup>I-HSPG) and metabolically labeled heparan sulfate chains (<sup>35</sup>S-HS) were incubated with Sepharose CL4B-coupled 10E4 antibody in the presence of various compounds. All compounds were added at a concentration of 100 µg/ml, except heparan sulfate, which was added at concentrations of 50, 20, and 10 µg/ml. The amount of label bound is expressed as a percentage of the total label added to each incubation mixture.

**Table III. <sup>125</sup>I-HSPG Binding to mAb 10E4: Effect of GAGs and GAG Oligosaccharides**

GAG treatment	GAG added				
	None	HA	DS	HP	HS
None	60.4	8.1	37.5	32.0	33.0
Chondroitinase ABC	-	5.7	37.1	31.8	33.0
Heparitinase	-	57.2	58.4	41.4	56.7
Heparinase	-	23.3	43.3	54.0	43.4
Nitrous acid	-	56.3	-	52.8	-

Commercial preparations of hyaluronate (HA), dermatan sulfate (DS), heparin (HP) (100 µg/assay), and heparan sulfate (HS) (20 µg/assay) were added to <sup>125</sup>I-HSPG-mAb 10E4 mixtures as intact polymers or after preincubation with either chondroitinase ABC, heparitinase, heparinase, or nitrous acid. The enzyme-treated GAGs were boiled and added as such. The nitrous acid treated GAGs were ethanol precipitated twice and redissolved in H<sub>2</sub>O before addition to the mixtures. The amount of mAb 10E4-bound label is expressed as a percent of the total label added to each assay.

mAb was reacting with epitopes resulting from or exposed by the heparitinase digestion.

This reactivity of mAb 3G10 was confirmed in indirect immunoperoxidase dot-blot assays. Untreated proteoglycan fractions that were stained by mAbs 10E4 and S1 (see above) went undetected by mAb 3G10 (Fig. 1, C1). A heparitinase treatment that abolished the reactivity of the proteoglycan fraction with mAb 10E4 (see above) induced a strong reaction with mAb 3G10 (Fig. 1, C2). Reduction of the heparitinase-treated proteoglycans with borohydride abolished the reactivity with mAb 3G10 (Fig. 1, C3). This treatment also abolished the reactivity with mAb S1 (Fig. 1, D3). mAb S1, which was known not to react with dithiothreitol-treated proteoglycans or core proteins (De Boeck et al., 1987), also failed to stain proteoglycans that were treated with borohydride before being treated with heparitinase (Fig. 1, D5). This sequence of treatments, however, did not prevent the proteoglycans from reacting with mAb 3G10 (Fig. 1, C5), which suggested that the reduction affected an epitope created by the heparitinase treatment itself. Treating the proteoglycan fractions with nitrous acid at pH 1.5 did not affect their reactivity with mAb 3G10. No reactivity was generated by nitrous acid treatment only (Fig. 1, C6), nor did nitrous acid treatment prevent the generation of the 3G10 epitope by heparitinase (Fig. 1, C7). The reactivity of mAb 10E4 with these samples, in contrast, was abolished by nitrous acid, with or without heparitinase (Fig. 1, B6 and B7), whereas neither treatment affected the reactivity with mAb S1 (Fig. 1, D6 and D7). Finally, exposing the heparitinase-treated proteoglycans to mercuric acetate at pH 5.0 abolished their reactivity with mAb 3G10 (Fig. 1, C8). This treatment, which has been shown to eliminate the unsaturated uronic acids generated by eliminate reactions (Ludwigs et al., 1987), did not affect the epitope of mAb S1 (Fig. 1, D8 and D9) and did not prevent the generation of the 3G10 epitope by the heparitinase treatment (Fig. 1, C9). Additional experiments indicated that the 3G10 epitope could not be generated in heparan sulfate proteoglycans by treating the proteoglycans with heparinase, nor in chondroitin sulfate proteoglycans by treating them with chondroitinase AC or ABC (not shown). mAb 3G10 reacted with heparitinase-digested heparan sulfate proteoglycans isolated from the detergent extracts, the extracellular matrices, or the culture media of cells of

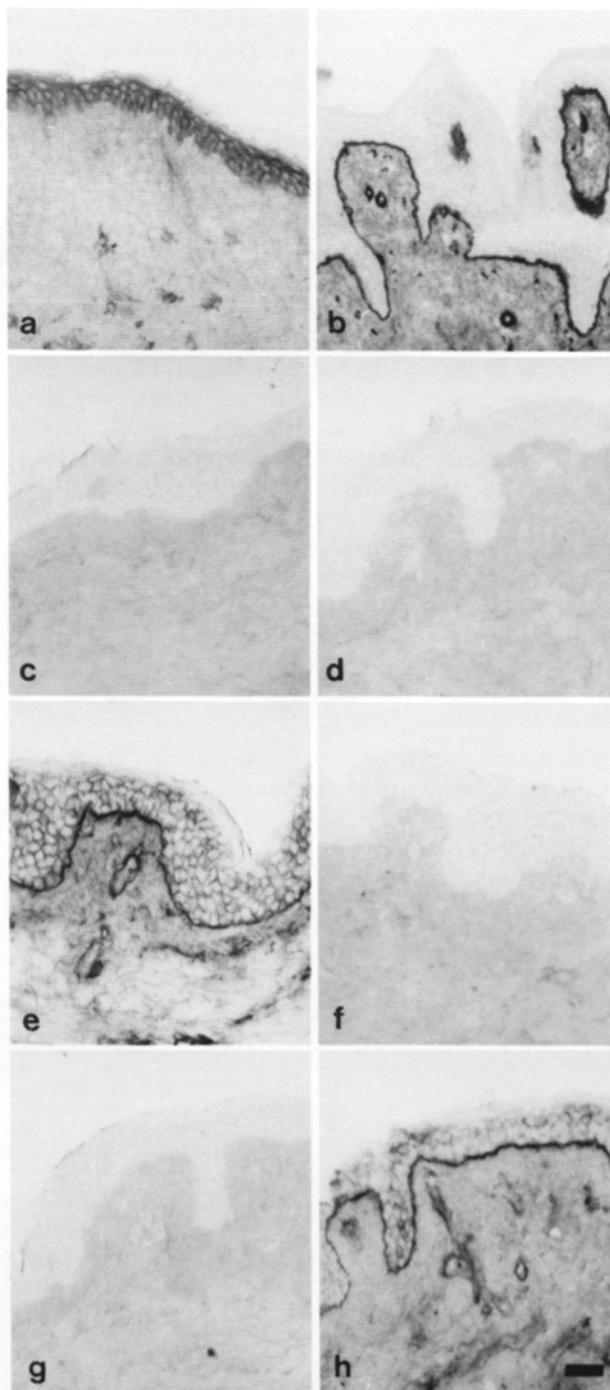
various origins and species (not shown). In Western blots of heparitinase-digested membrane proteoglycans, mAb 3G10 was staining the different core protein forms (Fig. 4 B). Finally, the reactivity of heparitinase-digested proteoglycans with mAb 3G10 was inhibited by supplementing the incubation with heparitinase-digested heparan sulfate chains, but not by heparan sulfate or heparitinase added separately, nor by chondroitinase ABC-digested chondroitin sulfate chains (Fig. 5). These results suggested that the epitope of mAb 3G10 was formed, at least in part, by the desaturated uronic acid residues generated during the cleavage of the heparan sulfate chains by heparan sulfate lyase.

#### mAbs 10E4 and 3G10 as Histochemical Probes

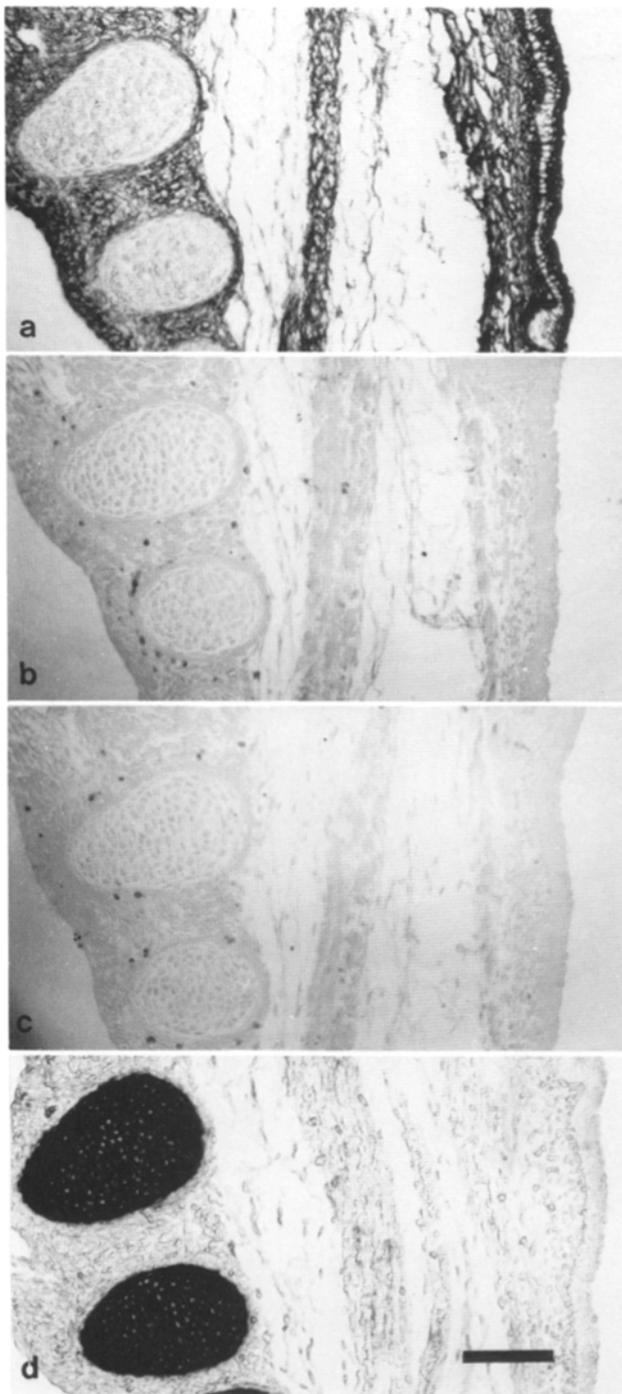
Since the above results indicated that mAb 10E4 and, indirectly, mAb 3G10 were specific probes for heparan sulfate, we investigated whether both antibodies could be used to map this glycosaminoglycan in tissue sections. In cryosections of adult human skin, for example, mAb 10E4 strongly demarcated the dermo-epidermal junction (Fig. 6 e). This was consistent with the occurrence of heparan sulfate-rich basement membrane proteoglycan at this site, as revealed with a mAb specific for the core protein of this proteoglycan (Fig. 6 b). mAb 10E4 also stained the contours of the epidermal keratinocytes (Fig. 6 e), consistent with the occurrence of cell surface-associated heparan sulfate proteoglycans with similar localizations in these cells, as revealed with the glypican core protein-specific mAb S1 (Fig. 6 a). Pretreatment of the sections with heparitinase abolished the 10E4 staining, consistent with the specificity of the antibody (Fig. 6 f).

	1	2	3	4	5	6
F69-3G10	+	+	+	+	+	+
Heparitinase	-	+	+	-	+	-
Heparan S	10	10	0	100	100	0
Heparitinase-digested HSPG	<b>A</b>	●	●	●	●	●
	<b>B</b>	●	●	●	●	●
Chondroitin S	10	10	0	100	100	0
Chondroitinase ABC	-	+	+	-	+	-
F69-3G10	+	+	+	+	+	-

**Figure 5.** Inhibition of mAb 3G10 binding by heparan sulfate oligosaccharides. Heparan sulfate proteoglycans from the culture medium of human lung fibroblasts were digested with heparitinase and blotted onto Zeta probe strips. The strips were incubated with antibody 3G10 in the presence of either 10 (lanes 1 and 2) or 100 (lanes 4 and 5)  $\mu$ g of heparan sulfate (row A) or chondroitin sulfate (row B), that had been left untreated (lanes 1 and 4) or that had been mixed and preincubated (lanes 2 and 5) with, respectively, heparitinase (row A) or chondroitinase ABC (row B). Control strips were incubated with mAb 3G10 in the absence of additives (A6) or in the presence of the enzymes only (A3 and B3), or without mAb 3G10 and any additives (B6). After washing away the incubation mixtures, all strips were incubated with a peroxidase-conjugated second antibody.



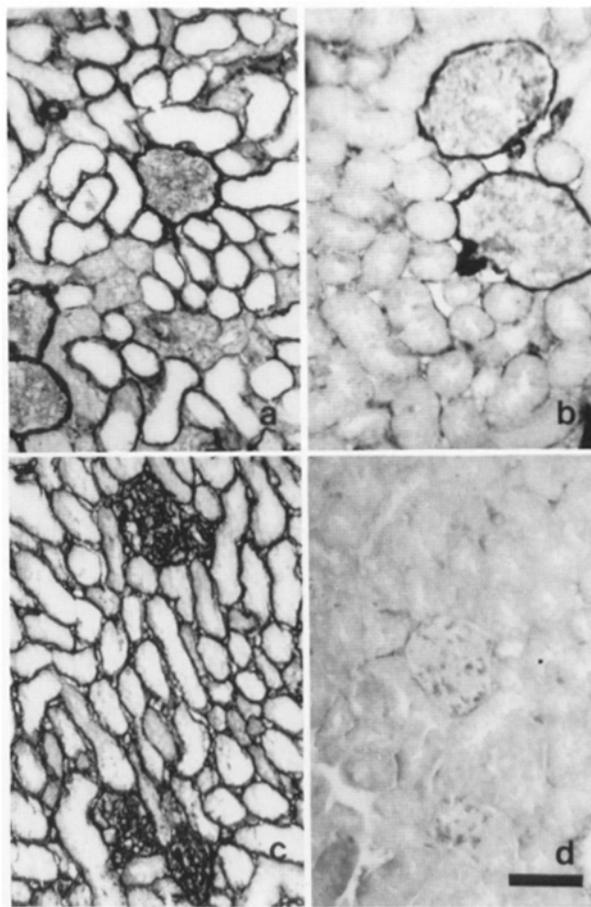
**Figure 6.** mAbs 10E4 and 3G10 as histochemical probes. Unfixed cryosections of human skin were incubated with the core protein-specific antibodies S1 (a) and 9C9 (b) to reveal the distribution of glypican (a cell surface proteoglycan) and that of a matrix-associated heparan sulfate proteoglycan, respectively. Other cryosections were briefly fixed in 2% paraformaldehyde and incubated for 1 h in heparitinase buffer with or without enzyme. The mock-treated (c, e, and g) and heparitinase-treated (d, f, and h) sections were further incubated either without first antibody (c and d), with mAb 10E4 (e and f), or with mAb 3G10 (g and h). All sections were stained using peroxidase-conjugated anti-mouse antibodies and diaminobenzidine. Bar, 10  $\mu$ m.



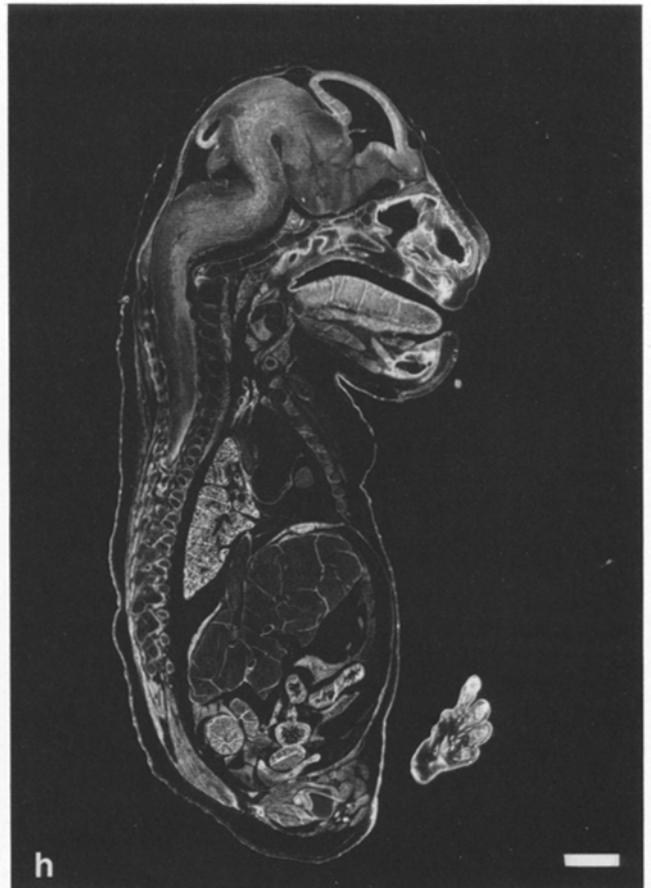
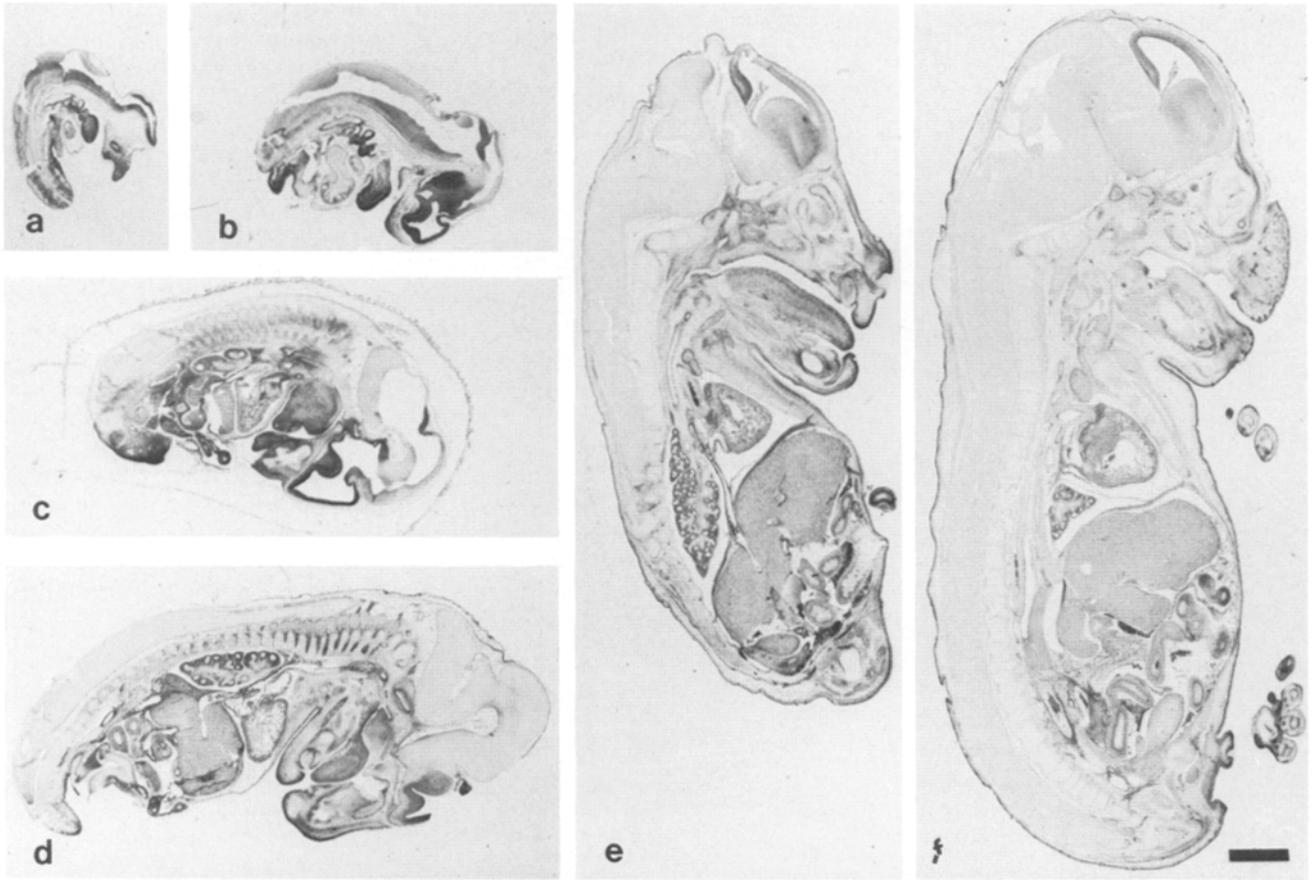
**Figure 7.** Specificity of the 3G10 antibody. Deparaffinated sections of 14-d-old hamster embryos were incubated for 2 h with heparitinase (*a* and *b*) or with chondroitinase AC (*c* and *d*). One of the heparitinase-treated sections was posttreated with 15 mM mercuric acetate (*b*). After further incubation with mAb 3G10 (*a-c*) or mAb 1B5 (*d*), the sections were stained using peroxidase-conjugated rabbit anti-mouse antibodies. Treating the sections with mercuric acetate before the heparitinase treatment had no effect on the stainings of control antibody or mAb 3G10-treated sections (not shown). Bar, 100  $\mu$ m.

A complementary image was obtained with mAb 3G10. This antibody left nonenzyme-treated sections unstained (Fig. 6 *g*). In contrast, it decorated the cell contours and matrix structures in heparitinase-treated sections, staining the keratinocytes, the dermo-epidermal junction, and the microvascular basement membranes of the dermis (Fig. 6 *h*). Some control experiments confirmed the histochemical specificity of mAb 3G10. Strong epithelial and mesenchymal stainings obtained with mAb 3G10 on heparitinase-treated sections of hamster embryos (Fig. 7 *a*) were completely prevented by exposure to mercuric acetate (Fig. 7 *b*). Chondroitinase AC-treated embryonic cartilage nodules, in contrast, were not stained by mAb 3G10 (Fig. 7 *c*), but strongly stained by mAb 1B5 (Fig. 7 *d*), an antibody that has been shown to be directed against unsaturated uronate linked to N-acetylgalactosamine (Couchman et al., 1984).

The patterns and intensities of the stainings obtained with mAbs 10E4 and 3G10 were, however, not always superimposable. In adult hamster kidney sections, for example, mAb 10E4 strongly stained the Bowman's capsule and the basement membranes of only part of the tubules, whereas the



**Figure 8.** Heterogeneity of the heparan sulfate chains. Cryosections of adult hamster kidneys were briefly fixed with paraformaldehyde and treated with buffer only (*a*), with heparitinase (*b* and *d*), or with heparitinase (*c*). The mock-treated and enzyme-treated sections were incubated with mAb 10E4 (*a* and *b*) or mAb 3G10 (*c* and *d*), and stained with peroxidase-conjugated rabbit anti-mouse antibodies. Bar, 100  $\mu$ m.



glomeruli and the basement membranes of some tubuli were only weakly stained (Fig. 8 *a*). mAb 3G10, in contrast, strongly stained the glomeruli and stained all tubular basement membranes uniformly (Fig. 8 *c*). This suggested that at these specific sites the heparan sulfate chains contained less 10E4 epitope, or that these chains, although accessible for heparitinase, did have 10E4 epitopes that were less accessible for the antibody. Interestingly, a heparinase treatment strongly reduced the reactivity of the basement membranes with mAb 10E4, except that of the Bowman's capsule (Fig. 8 *b*). mAbs 3G10 and 10E4 appeared, therefore, to be useful to probe the tissular distribution of heparan sulfate and to map regional differences in the structure or exposure of this glycosaminoglycan.

### **Developmental Changes in Heparan Sulfate Expression**

Both antibodies were then used to investigate the expression of heparan sulfate during embryonic development, where the programmed changes in cell growth, cell adhesion, and matrix assembly that are instrumental in the shaping of the body plan are thought to depend, at least in part, on heparan sulfate-catalyzed processes.

**Expression of the 10E4 Epitope.** Staining tissue sections of 8–13-d hamster embryos with mAb 10E4 revealed outspoken temporal and regional differences in the abundance of this heparan sulfate epitope (Fig. 9, *a–g*). The strongest stainings were obtained during early developmental stages, with particularly intense reactions in certain areas of the brain and in the mesenchymal tissues of the head, limbs, lungs, gut, and kidneys. In these mesenchymes the staining was most intense in the juxtaepithelial areas. Staining also occurred on basement membranes, but the epithelial cells themselves were only moderately stained and, except for some areas of the ectoderm, in later stages even mostly negative. Some of these localizations and changing patterns were investigated in greater detail and illustrated at higher magnifications (Figs. 10–12).

**Nervous Tissues.** In the 8-d embryo, the contours of virtually all cells of the neural tube were distinctly outlined by the antibody (Fig. 10 *a*), but in later stages, most cells of the central nervous system had lost their stain (Fig. 9). A striking exception was formed by the telencephalic areas, where the 10E4 epitope persisted up to day 13 on the cells that formed the walls of the lateral ventricles (Fig. 10 *b*). In contrast to the central nervous tissues, the peripheral nerves and the ganglia were strongly positive for 10E4 (to a certain extent this can be seen in Fig. 9 *d*).

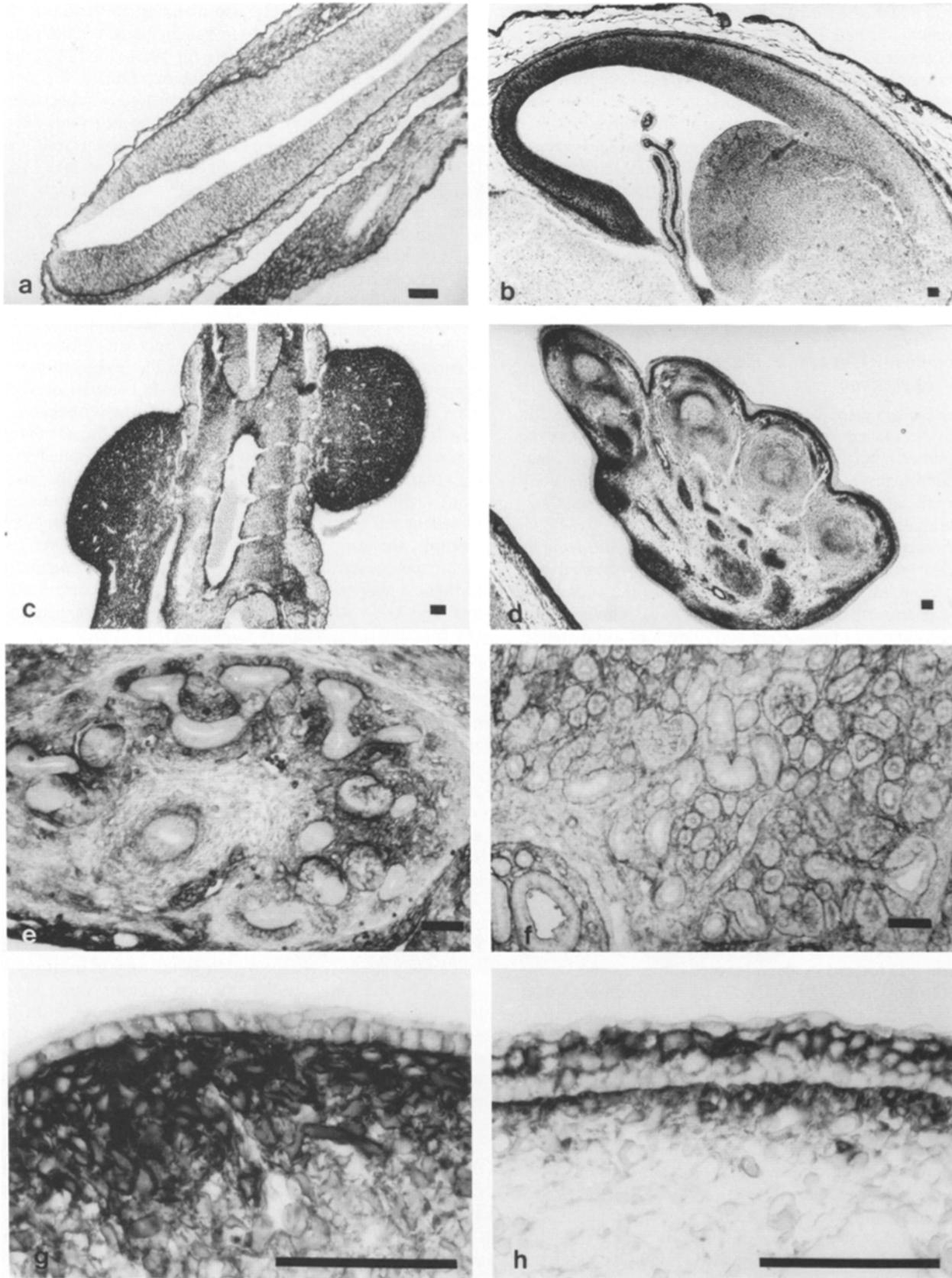
**Mesenchymal Tissues.** The 10E4 epitope was also abundant on the mesenchymal tissues of the maxillar and mandibular processes of the 8–13-d embryo, where it ultimately became concentrated on the subectodermal cells, particularly those underlying the vibrissal rudiments (Fig. 11, *a* and *c*), on the dental mesenchyme, and on the cells of the tongue that condensed around the papillae and those that differentiated into muscle cells (Fig. 9). Strong and changing pat-

terns of 10E4 staining were also observed in the limbs. In the 9–10-d embryo the whole mesenchyme of the limb bud was strongly and diffusely positive for 10E4 (Fig. 10 *c*), but in later stages and at the level of the extremities, the stain was mainly concentrated over the immediately subectodermal mesoderm and over the myotendinous structures (Fig. 10 *d*). For the mesenchymes of the visceral organs, particularly strong reactions were obtained in the lungs (see Figs. 9 and 12). At day 10 the lung mesenchyme was strongly and nearly homogeneously decorated by the antibody (Fig. 12 *a*). With further development strong 10E4 stain persisted on the mesenchymal cells that were in the immediate vicinity of the secondary and tertiary branches and growing tips of the lung epithelium, but the mesenchymal cells that surrounded the major branches were only weakly stained (Fig. 12, *b* and *c*). In the terminal stages a light and diffuse stain occurred in the alveolar septae and in the basement membranes of the airway ducts (Fig. 12 *d*). In comparison with lung, the mesenchymes of the kidneys and other viscera were only moderately stained by the 10E4 antibody, but as in lung this stain was mostly concentrated over the mesenchymal cells that were in contact with the epithelia and over the basement membranes that formed the interfaces between the mesenchymal and epithelial tissues. In the 11-d kidney, for example, the 10E4 stain was mostly concentrated over the pretubular mesenchymal cell aggregates that subsequently form the S-shaped bodies and fuse with the branching ureter tree and over still undifferentiated peri-ureteral mesenchymal cells (Fig. 10 *e*). In later stages of kidney development, the stain was virtually limited to the basement membranes (Fig. 10 *f*).

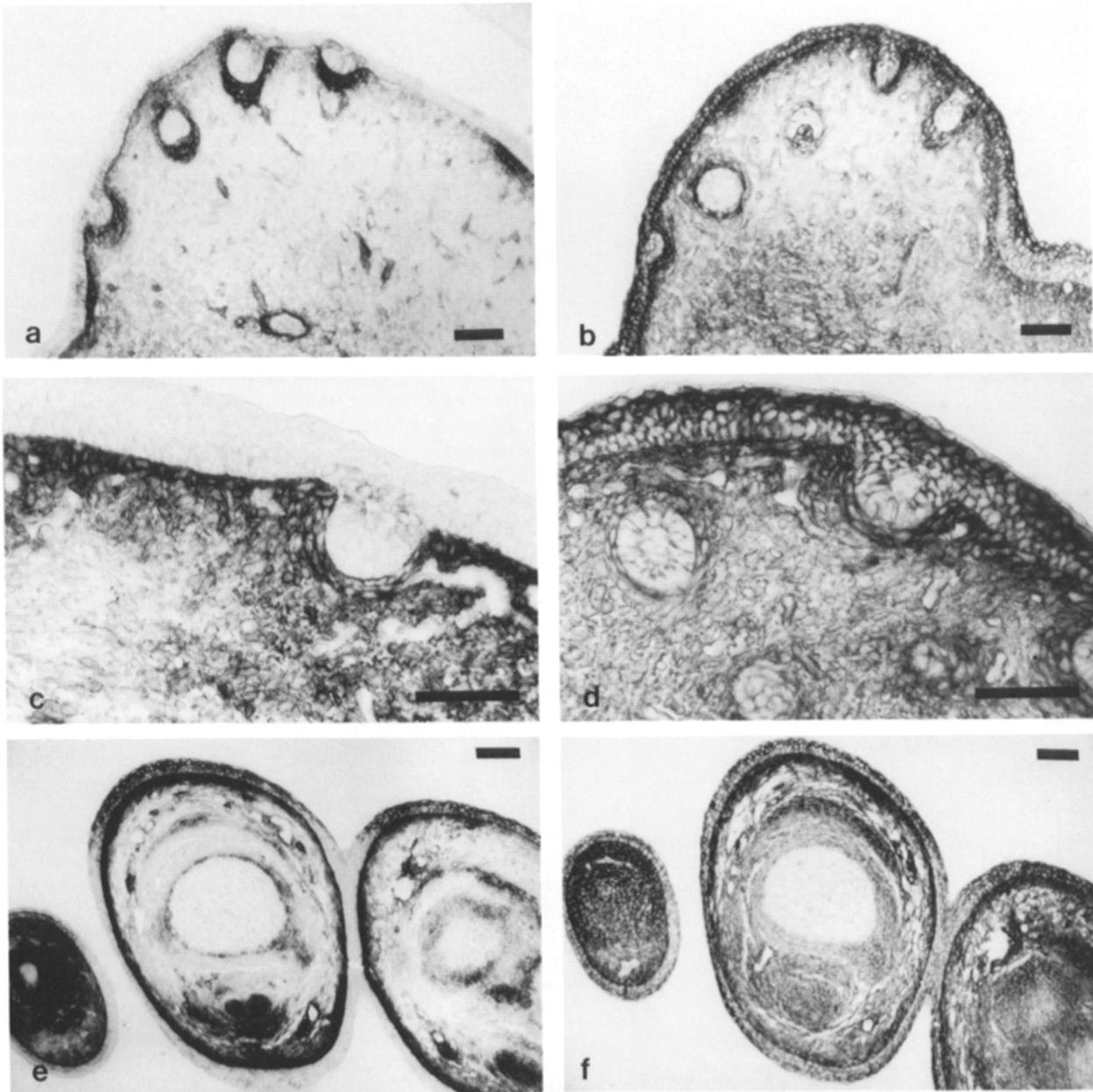
**Epithelial Tissues.** In general, and in comparison with these mesenchymal stainings, the epithelial cell surfaces of endodermal and mesodermal origin were only weakly stained by the 10E4 antibody (see, for example, the kidney and lung epithelium in Figs. 10 and 12), and this staining was mostly limited to the earliest stages examined. The ectodermal staining patterns, in contrast, were more heterogeneous and complex. The very early flattened ectoderm was virtually unstained by mAb 10E4. Later, when these cells assumed a cuboidal shape they became positive for the 10E4 epitope, but the overlying periderm was negative (Fig. 10 *g*). Finally, in the multilayered epiderm the basal cells were moderately stained, and the middle layers were even strongly stained by the antibody, but the upper layers were negative (Fig. 10 *h*). In some areas, however, the entire thickness of the epithelium was only weakly stained or even negative for 10E4. These striking exceptions occurred at the level of the epiderm of the face (Fig. 11, *a* and *c*), and at the level of the limb extremities, where the epiderm of the dorsal side of the digits was strongly stained, but the lateral and volar sides were virtually negative (Fig. 11 *e*). For all these localizations a treatment of the sections with heparitinase completely abolished the staining reaction for the 10E4 epitope (not shown).

**Expression of the 3G10 Epitope.** To a certain extent, the staining pattern obtained by mAb 3G10 on heparitinase-

**Figure 9.** Developmental changes in heparan sulfate expression. Sagittal sections of paraffin-embedded 9- (*a*), 9.5 (*b*), 10- (*c*), 11- (*d*), 12- (*e*), and 13-d (*f–h*) hamster embryos were stained with the anti-“chain” mAb 10E4 (*a–g*), or treated with heparitinase and stained with the anti-“stub” mAb 3G10 (*h*). Indirect immunoperoxidase staining. *g* and *h* are negative prints. Bars, 100  $\mu$ m.



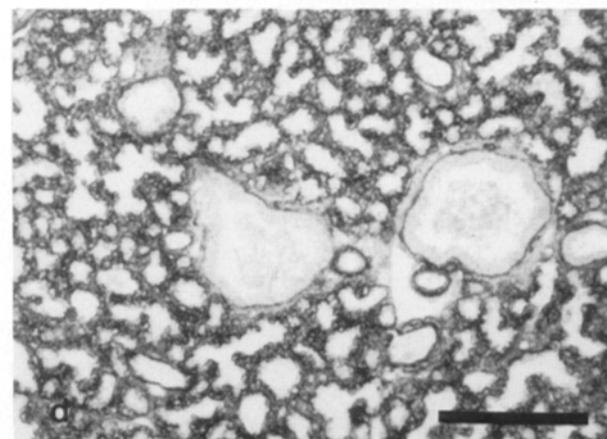
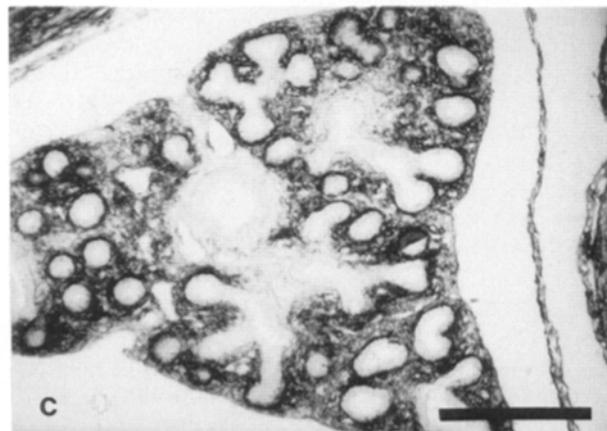
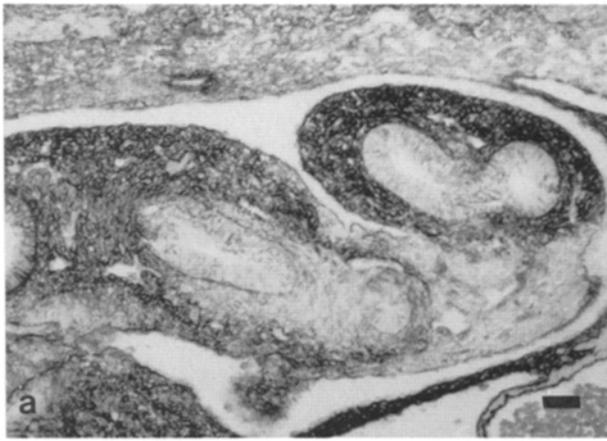
**Figure 10.** Temporal and regional differences in heparan sulfate expression. Paraffin sections of hamster embryonic tissues stained with the anti-“chain” mAb 10E4, showing the general neural tube staining at 8 d (a) and the localized staining of the lateral ventricle at 13 d (b), the strong diffuse staining of the hind limb bud at 9.5 d (c) and the localized foot stainings at 12 d (d), the staining of the kidney mesenchyme and basement membranes at 11 (e) and 14 d (f), and the staining of the surface ectoderm of the chest area at days 11 (g) and 13 (h) of development. Bars, 100  $\mu$ m.



**Figure 11.** Regional differences in embryonic heparan sulfate chain composition or exposure. Paraffin sections through the lips and vibrissae (*a-d*), and through the digits (*e* and *f*) of a 13-d hamster embryo, left untreated and stained with the anti-“chain” mAb 10E4 (*a*, *c*, and *e*), or treated with heparitinase and stained with the anti-“stub” mAb 3G10 (*b*, *d*, and *f*). Bars, 100  $\mu$ m.

treated sections mimicked the distribution of the 10E4 epitope in nonenzyme-treated embryonic tissues, with accentuated stainings of the walls of the lateral ventricles, the lungs, and, for example, the foot process (compare Fig. 9, *g* and *h*). However, in general, the patterns were less contrasted, the 3G10 epitope being more widely and evenly distributed over the cell surface and matrix structures than the 10E4 epitope. In the central nervous tissue the 3G10 antibody also slightly stained the mesencephalic, cerebellar, and spinal structures. In the mesenchymes the 3G10 stain was less confined to the periepithelial condensations, and several epithelia were moderately to strongly positive for 3G10, where they had been virtually negative for the 10E4 epitope. This was exem-

plified in the tissues of the face and digits, where virtually all ectodermal and mesenchymal cells, except those of cartilage, were nearly homogeneously decorated by mAb 3G10 (Fig. 11, *b*, *d*, and *f*), not or imperfectly reproducing the mesenchymal concentrations and the dorso-volar ectodermal polarization noted with the 10E4 antibody (Fig. 11, *a*, *c*, and *e*). These discrepancies suggested that some of the patterns obtained with the 10E4 antibody were to be explained by the local accumulation of heparan sulfate chains with a distinctive structure rather than by an increased density of heparan sulfate chains at these sites. As a whole, these data provided clear evidence for a differential and regulated expression of heparan sulfate during development.



## Discussion

In this report we describe the properties of two anti-heparan sulfate mAbs that, especially when used in conjunction with each other and with core-specific probes, provide unique tools for investigating the structure and expression of the heparan sulfate proteoglycans. The study of the distribution of the corresponding epitopes in embryonic tissues suggests that the expression of heparan sulfate is highly regulated.

### *An Antibody against Native Heparan Sulfate Chains*

One of these antibodies (mAb 10E4) reacts with an epitope that occurs in native heparan sulfate, and that seems to be determined, at least in part, by the N-sulfate residues that are characteristic for this type of glycosaminoglycan. This conclusion is based on the observations that the reaction of the proteoglycans with this antibody is abolished by nitrous acid or heparitinase; that protein-free heparan sulfate chains bind to the antibody; and that the reactivity of the proteoglycans or chains is abolished by selective N-desulfation and restored by resulfation. The salt and pH sensitivity of the binding suggests that the interaction of the glycosaminoglycan with the antibodies is largely electrostatic in nature. This is consistent with the notion that the epitope comprises N-sulfate, and suggests that binding may involve a critical histidine residue (the only amino acid with a pK near 7.0) on the antibody. Yet, despite involving ionized structures, the interaction is endowed with remarkable specificity. The antibody does not react with DNA or hyaluronate, or with sulfated glycosaminoglycans such as chondroitin sulfate and dermatan sulfate, which have similar average charge densities as heparan sulfate. The binding properties of this antibody are therefore more discriminating than those of the "natural" anti-heparan sulfate antibodies frequently encountered in patients with systemic lupus erythematosus, which most often recognize heparan sulfate and double-stranded DNA (Faaber et al., 1986; Aotsuka et al., 1988). The precise nature of the epitope, however, remains undefined. The occurrence of N-sulfate is not sufficient and may even not be an absolute requirement for interaction with mAb 10E4. Indeed, heparin, which compared with heparan sulfate is much more enriched in N-sulfate, is a less effective inhibitor than heparan sulfate in the binding assays. On the other hand, dextran sulfate, which has no N-sulfate, is one of the most effective inhibitors. Since there are no known modifications of the heparosan polymer that are present in heparan sulfates but not in heparin, this observation suggests that recognition by the antibody requires both the presence of N-sulfate, and the absence of further modifications that may occur in the glucosamine and vicinal uronate residues after the initial N-deacetylation and N-sulfation reaction. Then, the heparitinase and nitrous acid-sensitive contaminants in some hyaluronate preparations that are inhibitory for the binding of the antibody could possibly represent the other end of the spectrum and be lowly sulfated heparosan chains with intermediate degrees of chain modifications and a

**Figure 12.** Changing patterns of heparan sulfate expression during lung development. Paraffin sections through the lungs of 10- (a), 11- (b), 13- (c), and 14-d (d) hamster embryos, stained with the anti-"chain" mAb 10E4. Bars, 100  $\mu$ m.

higher incidence of the epitope than characteristic heparan sulfate chains, but this was not further investigated. In a sense, the specificity of the antibody and its preference for heparan sulfate over heparin mimics that of the enzyme heparitinase. This enzyme cleaves the  $\alpha$ -glycosidic bonds between N-sulfated or N-acetylated glucosamines and glucuronic acid, and therefore depolymerizes heparan sulfate extensively while leaving heparin mostly undegraded (Hovingh and Linker, 1970). It is significant, in this context, that heparitinase is actually destroying the 10E4 epitope (see Table III) and not just removing it from the preparation by cleaving the chains between the epitope and the protein linkage region. However, heparinase, which cleaves  $\alpha$ 1,4-glycosidic bonds between N-sulfated glucosamines and 2-O-sulfated iduronates, also destroys some 10E4 epitopes in the heparan sulfate preparations and abolishes the inhibiting structures in heparin more effectively than heparitinase (Table III). This may be an indication that in the build up of the epitope there is some tolerance as to the nature of the uronates to which the N-sulfated glucosamines are linked, but that further O-sulfation of the N-sulfated glucosamines (which happens more extensively in heparin than in heparan sulfate) abolishes their reaction with mAb 10E4. On the other hand, the strong inhibitory activity of dextran sulfate implies that, to a certain extent, the recognition sequence may also be mimicked in this polymer. To propose that the natural recognition sequence must therefore also comprise O-sulfate would seem contradictory to the conclusions drawn from the results obtained with heparin. Possibly the epitope is mimicked in dextran sulfate because it includes sulfated glucose derivatives that are engaged in an  $\alpha$ -glycosidic bond. Such sequences are present in the heparinoids and dextran sulfates but in none of the other natural glycosaminoglycans tested. The  $\alpha$ -glycosidic linkages by themselves, however, do not suffice to form the 10E4 epitope, since N-desulfated heparan sulfates and unsulfated dextran are not recognized by the antibody. On the other hand, both the dextran sulfates and heparan sulfates are made up of the alternation of regions of high sulfation with bloc regions of low or no sulfation (Gallagher and Walker, 1985; Turnbull and Gallagher, 1990). If these alternations were important for the reactivity with the 10E4 antibody, it would account for the observation that heparin, wherein these sulfate-free regions occur less frequently, is less well recognized. Clearly, further characterizations are needed. As a whole, the specificity of the 10E4 antibody may mimic that of mAb HK-249, a mAb raised against the proteoglycan produced by the Engelbreth-Holm-Swarm tumor, believed to recognize a glucosamine sulfate  $\alpha$ 1,4-glucuronic acid-containing determinant in the intact chain of the proteoglycans (Snow et al., 1990). It would seem different, however, from that of mAb Hep SS-1, which is thought to recognize an O-sulfated, N-acetylated glucosamine linked to uronic acid (Kure and Yoshie, 1986).

The repetitive but nonuniform structure of the heparan sulfate polymers then implies that individual heparan sulfate chains are likely to contain multiple but variable numbers of the 10E4 epitope, and that different segments of the polymer may react differently with the antibody. The segment adjacent to the protein linkage region, for example, consists of an extended N-acetylated sequence (Lyon et al., 1987), and is not recognized by the antibody (Fig. 1). Also, the residual staining of some basement membranes after heparinase

treatment, where the reactivity of the mesenchymal matrix and that of the cell surfaces is abolished (Fig. 8), indicates that the relative positions of the 10E4 epitopes and heparinase cleavage sites with respect to the linkage region must be different at these different sites. On average, however, the 10E4 antibody is expected to trace the mass of heparan sulfate expressed on individual proteoglycans and at individual sites.

### *An Antibody That Traces Residual Heparan Sulfate Stubs*

mAb 3G10, in contrast, does not react with the intact heparan sulfate chains. It appears to be directed, but only in part, against the desaturated uronate residues generated by a heparitinase treatment. The effect of the mercuric salt indicates that the uronate is an essential component of the epitope. The absence of reactivity of the antibody with heparinase-treated heparan sulfate proteoglycans, or chondroitinase ABC- and AC-treated chondroitin sulfate proteoglycans, however, suggests that this  $\Delta$ -uronate is recognized in a specific context. The specificity of heparitinase, which cleaves the chains at the level of the 2-acetamido and 2-sulfamido-2-deoxy-D-glucosyl  $\alpha$ (1-4) D-glucuronate linkages, and the structure of the heparan sulfate chains, which is rich in extended N-acetylated sequences in the region adjacent to the protein linkage, suggest that this context is an N-acetylated glucosamine. This context would indeed explain why nitrous acid degradation of the chains before the enzyme digestion (leaving only N-acetylated sequences attached to the cores) has no effect on the amount of 3G10 epitope generated in the core protein preparation. The failure to detect any cross-reactivity of 3G10 with chondroitin sulfate proteoglycans that have been extensively digested with chondroitinase AC, an enzyme that can depolymerize the chondroitin sulfate chains down to the initial glucuronate, which is linked to the gal-gal-xylose linkage sequence (thought to be common to chondroitin sulfate and heparan sulfate chains), is also significant in this context. It means either that the heparitinase preparation cannot depolymerize the heparan sulfate chains down to this initial glucuronate, leaving a desaturated heparosan disaccharide to react with 3G10, or that the antibody is recognizing a difference between the desaturated uronate on the linkage region of heparan sulfate and that of chondroitin sulfate chains. The first possibility would be reminiscent of the activity of chondroitinase ABC on chondroitin sulfate chains, as this enzyme, unlike chondroitinase AC, always leaves a desaturated chondroitin (sulfate) disaccharide attached to the glucuronic acid-gal-gal-xylose linkage region (Christner et al., 1980). The second possibility would be of potential interest as it is not known what signals direct a cell to extend the linkage region by either heparosan or chondroitin sequences. Yet the 3G10 antibody also reacts with heparan sulfate oligosaccharides liberated from Sepharose CL4B-coupled proteoglycan by heparitinase, indicating that the linkage sequence itself is not required for the epitope (not shown).

Irrespective of the precise context in which the desaturated uronates are recognized by 3G10, only one desaturated uronate residue per chain will remain linked to the core protein. The extent to which 3G10 reacts with heparitinase-treated core proteins or tissue sections will therefore trace the num-

ber of heparan sulfate chains carried by these cores or expressed in a particular tissue rather than the mass of heparan sulfate that was originally present.

### Differential Expression of Heparan Sulfate

Not unexpectedly, the surfaces and extracellular matrices of virtually all adherent cells in adult and embryonic animals are stained by either one or a combination of both these antibodies, revealing the occurrence of heparan sulfate at these sites. However, the intensity of the staining reactions shows marked tissue and stage-dependent variations, suggesting that the expression of heparan sulfate is highly regulated and subject to both temporal and spatial controls. In general, heparan sulfate seems to abound in the early embryo, especially in the mesenchymal tissues of those areas that undergo active morphogenesis, and is less conspicuous in the tissues of the healthy adult. This evidence for a developmentally controlled expression of heparan sulfate seems consistent with the data on the differential and regulated expression of the core proteins of the cell surface proteoglycans syndecan (Bernfield and Sanderson, 1990) and fibroglycan (David et al., unpublished results) in embryonic tissues and in healing wounds (Elenius et al., 1991). However, the distribution of these two cell surface proteins in the embryo overlaps only partially with that of the 10E4 and 3G10 epitopes, suggesting that additional proteoglycans may be involved and under developmental control, or that the substitution of these proteoglycans with heparan sulfate is not uniform, or both. A striking example is provided in the developing lung, where syndecan and fibroglycan are mainly expressed in, respectively, the lung epithelium (Brauker et al., 1991) and the mesenchymal cells that surround the epithelial stalks and branches, but where the 10E4 epitope, in contrast, abounds on the mesenchymal cells that confront the tips of the expanding epithelium (Fig. 12). The involvement of additional proteoglycan forms might be expected from the complex composition of the cell surface proteoglycan fractions from most cell types, including fetal lung fibroblasts (Fig. 4), and from the recent identification of additional members of the syndecan-fibroglycan family (Carey et al., 1992; David et al., 1992; Gould et al., 1992; Kojima et al., 1992), whereas direct evidence has been obtained for the variability of the glycanation of syndecan during lung development. From day 12 to 18 of development, the relative mass of syndecan in the mouse embryonic lung was noted to decrease progressively, due to a reduction in the size of its glycosaminoglycan chains (Brauker et al., 1991), which is not inconsistent with the progressive reduction in 10E4 staining of the respiratory epithelium noted here over a similar time frame (Fig. 12). Moreover, the results obtained in the embryos (Fig. 11) and in the adult kidney sections (Fig. 8) indicate that the distributions of the 3G10 and 10E4 epitopes do not always coincide. This finding could be interpreted as discrepancies in epitope exposure, but may also reflect regional differences in the structure of the proteoglycans, for example, with respect to the length or the sulfation (heparin likeness) of the chains that are implanted on the cores. Such regional differences may be physiologically meaningful, for example, for the local activity and stability of growth factors in the embryo (Klagsbrun and Baird, 1991), or for the permeability of the matrix in kidney tissues specialized in transport phenomena (Kanwar et al., 1980). In that context it may be interesting

to note that the distribution of the 10E4 rather than the 3G10 epitope in the 13-d hamster embryo resembled that reported for basic fibroblast growth factor in the rat embryo at a similar stage of development (Gonzales et al., 1990). The heparan sulfate sequences involved in growth factor binding and activation are unknown but the sequence requirements for the high affinity binding interaction between heparin and antithrombin indicate how important also the fine structure of these complex polysaccharides can be for proteoglycan function (Lindahl et al., 1986). Antibody probes that would be able to trace a variety of defined heparan sulfate sequences would therefore be important additions to the present antibody panel, and find wide applications in the in situ mapping and probing of heparan sulfate during developmental processes.

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### References

- Aotsuka, S., M. Okawa-Takatsuji, M. Kinoshita, and R. Yokohari. 1988. Analysis of negatively charged dye-binding antibodies reactive with double-stranded DNA and heparan sulfate in serum from patients with rheumatic diseases. *Clin. Exp. Immunol.* 73:436-442.
- Bernfield, M., and R. D. Sanderson. 1990. Syndecan, a morphogenetically regulated cell surface proteoglycan that binds extracellular matrix and growth factors. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 327:171-186.
- Brauker, J. H., M. S. Trautman, and M. Bernfield. 1991. Syndecan, a cell surface proteoglycan, exhibits a molecular polymorphism during lung development. *Dev. Biol.* 147:285-292.
- Carey, D. J., D. M. Evans, R. C. Stahl, V. K. Asundi, K. J. Conner, P. Garbes, and G. Cizmeci-Smith. 1992. Molecular cloning and characterization of N-syndecan, a novel transmembrane heparan sulfate proteoglycan. *J. Cell Biol.* 117:191-201.
- Caterson, B., J. E. Christner, and J. R. Baker. 1983. Identification of a monoclonal antibody that specifically recognizes corneal and skeletal keratan sulfate. *J. Biol. Chem.* 258:8848-8854.
- Christner, J. E., B. Caterson, and J. R. Baker. 1980. Immunological determinants of proteoglycans. *J. Biol. Chem.* 255:7102-7105.
- Couchman, J. R., B. Caterson, J. E. Christner, and J. R. Baker. 1984. Mapping by monoclonal antibody detection of glycosaminoglycans in connective tissues. *Nature (Lond.)* 307:650-652.
- David, G., and H. Van den Berghe. 1983. Transformed mouse mammary epithelial cells undersulfate basement membrane proteoglycan. *J. Biol. Chem.* 258:7338-7344.
- David, G., and H. Van den Berghe. 1985. Heparan sulfate-chondroitin sulfate hybrid proteoglycan of the cell surface and basement membrane of mouse mammary epithelial cells. *J. Biol. Chem.* 260:11067-11074.
- David, G., V. Lories, B. Decock, P. Marynen, J. J. Cassiman, and H. Van den Berghe. 1990. Molecular cloning of a phosphatidylinositol-anchored membrane heparan sulfate proteoglycan from human lung fibroblasts. *J. Cell Biol.* 111:3165-3176.
- David, G., B. Van der Schueren, P. Marynen, J. J. Cassiman, and H. Van den Berghe. 1992. Molecular cloning of amphiglycan, a novel integral membrane heparan sulfate proteoglycan expressed by epithelial and fibroblastic cells. *J. Cell Biol.* 118:961-969.
- De Boeck, H., V. Lories, G. David, J. J. Cassiman, and H. Van den Berghe. 1987. Identification of a 64 kDa heparan sulphate proteoglycan core protein from human lung fibroblast plasma membranes with a monoclonal antibody. *Biochem. J.* 247:765-771.
- Elenius, K., S. Vainio, M. Laato, M. Salmivirta, I. Thesleff, and M. Jalkanen. 1991. Induced expression of syndecan in healing wounds. *J. Cell Biol.* 114:585-595.
- Faaber, P., T. P. M. Rijke, L. B. A. van de Putte, P. J. A. Capel, and J. H. M. Berden. 1986. Cross-reactivity of human and murine anti-DNA antibodies with heparan sulfate. *J. Clin. Invest.* 77:1824-1830.
- Gallagher, J. T., and A. Walker. 1985. Molecular distinctions between heparan sulphate and heparin. *Biochem. J.* 230:665-674.

- Gallagher, J. T. 1989. The extended family of proteoglycans: social residents of the pericellular zone. *Curr. Opin. Cell Biol.* 1:1201-1218.
- Giazer, A. N., R. J. DeLange, and D. S. Sigman. 1975. Chemical modification of proteins. In *Laboratory Techniques in Biochemistry and Molecular Biology*. T. S. Work and E. Work, editors. North-Holland, Amsterdam-Oxford. 76-78.
- Gonzalez, A.-M., M. Buscaglia, M. Ong, and A. Baird. 1990. Distribution of basic fibroblast growth factor in the 18-day rat fetus: localization in the basement membranes of diverse tissues. *J. Cell Biol.* 110:753-765.
- Gould, S. E., W. B. Upholt, and R. A. Kosher. 1992. Syndecan 3: a member of the syndecan family of membrane-intercalated proteoglycans that is expressed in high amounts at the onset of chicken limb cartilage differentiation. *Proc. Natl. Acad. Sci. USA.* 89:3271-3275.
- Heremans, A., J. J. Cassiman, H. Van den Berghe, and G. David. 1988. Heparan sulfate proteoglycan from the extracellular matrix of human lung fibroblasts. Isolation, purification, and core protein characterization. *J. Biol. Chem.* 263:4731-4739.
- Heremans, A., B. Van der Schueren, B. Decock, M. Paulsson, J. J. Cassiman, H. Van den Berghe, and G. David. 1989. Matrix-associated heparan sulfate proteoglycan: core protein-specific monoclonal antibodies decorate the pericellular matrix of connective tissue cells and the stromal side of basement membranes. *J. Cell Biol.* 109:3199-3211.
- Heremans, A., B. Decock, J. J. Cassiman, H. Van den Berghe, and G. David. 1990. The core protein of the matrix-associated heparan sulfate proteoglycan binds to fibronectin. *J. Biol. Chem.* 265:8716-8724.
- Hovingh, P., and A. Linker. 1970. The enzymatic degradation of heparin and heparitin sulfate. *J. Biol. Chem.* 245:6170-6175.
- Inoue, Y., and K. Nagasawa. 1976. Selective N-desulfation of heparin with dimethyl sulfoxide containing water or methanol. *Carbohydr. Res.* 46:87-95.
- Kanwar, Y. S., A. Linker, and M. G. Farquhar. 1980. Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. *J. Cell Biol.* 86:688-693.
- Kjellén, L., I. Pettersson, P. Lillhager, M.-L. Steen, U. Pettersson, P. Lehtonen, T. Karlsson, E. Ruoslahti, and L. Hellman. 1989. Primary structure of a mouse mastocytoma proteoglycan core protein. *Biochem. J.* 263:105-113.
- Klagsbrun, M., and A. Baird. 1991. A dual receptor system is required for basic fibroblast growth factor activity. *Cell.* 67:229-231.
- Kojima, T., N. W. Shworak, and R. D. Rosenberg. 1992. Molecular cloning and expression of two distinct cDNA-encoding heparan sulfate proteoglycan core proteins from a rat endothelial cell line. *J. Biol. Chem.* 267:4870-4877.
- Kure, S., and O. Yoshie. 1986. A syngeneic monoclonal antibody to murine meth-A sarcoma (HepSS-1) recognizes heparan sulfate glycosaminoglycan (HS-GAG): cell density and transformation dependent alteration in cell surface HS-GAG defined by HepSS-1. *J. Immunol.* 137:3900-3908.
- Lindahl, U., D. S. Feingold, and L. Rodén. 1986. Biosynthesis of heparin. *TIBS* 11:221-225.
- Lories, V., G. David, J. J. Cassiman, and H. Van den Berghe. 1986. Heparan sulfate proteoglycans of human lung fibroblasts. Occurrence of distinct membrane, matrix and secreted forms. *Eur. J. Biochem.* 158:351-360.
- Lories, V., H. De Boeck, G. David, J. J. Cassiman, and H. Van den Berghe. 1987. Heparan sulfate proteoglycans of human lung fibroblasts. Structural heterogeneity of the core proteins of the hydrophobic cell-associated forms. *J. Biol. Chem.* 262:854-859.
- Lories, V., J. J. Cassiman, H. Van den Berghe, and G. David. 1989. Multiple distinct membrane heparan sulfate proteoglycans in human lung fibroblasts. *J. Biol. Chem.* 264:7009-7016.
- Ludwigs, U., A. Elgavish, J. D. Esko, E. Meezan, and L. Rodén. 1987. Reaction of unsaturated uronic acid residues with mercuric salts. *Biochem. J.* 245:795-804.
- Lyon, M., W. P. Steward, I. N. Hampson, and J. T. Gallagher. 1987. Identification of an extended N-acetylated sequence adjacent to the protein-linkage region of fibroblast heparan sulphate. *Biochem. J.* 242:493-498.
- Marynen, P., J. Zhang, J. J. Cassiman, H. Van den Berghe, and G. David. 1989. Partial primary structure of the 48- and 90-kilodalton core proteins of cell surface-associated heparan sulfate proteoglycans of lung fibroblasts. *J. Biol. Chem.* 264:7017-7024.
- Pejler, G., U. Lindahl, O. Larm, E. Scholander, E. Sandgren, and A. Lundblad. 1988. Monoclonal antibodies specific for oligosaccharides prepared by partial nitrous acid deamination of heparin. *J. Biol. Chem.* 263:5197-5201.
- Rapraeger, A. 1989. Transforming growth factor (type  $\beta$ ) promotes the addition of chondroitin sulfate chains to the cell surface proteoglycan (syndecan) of mouse mammary epithelia. *J. Cell Biol.* 109:2509-2518.
- Ruoslahti, E. 1989. Proteoglycans in cell regulation. *J. Biol. Chem.* 264:13369-13372.
- Salmivirta, M., K. Elenius, S. Vainio, U. Hofer, R. Chiquet-Ehrismann, I. Thesleff, and M. Jalkanen. 1991. Syndecan from embryonic tooth mesenchyme binds Tenascin. *J. Biol. Chem.* 266:7733-7739.
- Sanderson, R. D., and M. Bernfield. 1988. Molecular polymorphism of a cell surface proteoglycan: distinct structures on simple and stratified epithelia. *Proc. Natl. Acad. Sci. USA.* 85:9562-9566.
- Saunders, S., M. Jalkanen, S. O'Farrell, and M. Bernfield. 1989. Molecular cloning of syndecan, an integral membrane proteoglycan. *J. Cell Biol.* 108:1547-1556.
- Seldin, D. C., K. F. Austen, and R. L. Stevens. 1985. Purification and characterization of protease-resistant secretory granule proteoglycans containing chondroitin sulfate Di-B and heparin-like glycosaminoglycans from rat basophilic leukemia cells. *J. Biol. Chem.* 260:11131-11139.
- Shively, J. E., and H. E. Conrad. 1976. Formation of anhydrosugars in the chemical depolymerization of heparin. *Biochemistry.* 15:3932-3942.
- Snow, A. D., T. N. Wight, D. Noehlin, Y. Koike, K. Kimata, S. J. Dearmond, and S. B. Prusiner. 1990. Immunolocalization of heparan sulfate proteoglycans to the prion protein amyloid plaques of Gerstmann-Strausler syndrome, Creutzfeldt-Jakob disease and scrapie. *Lab. Invest.* 63:601-611.
- Turnbull, J. E., and J. T. Gallagher. 1990. Molecular organization of heparan sulphate from human skin fibroblasts. *Biochem. J.* 265:715-724.
- Yamagata, M., K. Kimata, Y. Oike, K. Tani, N. Maeda, K. Yoshida, Y. Shimomura, M. Yoneda, and S. Suzuki. 1987. A monoclonal antibody that specifically recognizes a glucuronic acid 2-sulfate-containing determinant in intact chondroitin sulfate chain. *J. Biol. Chem.* 262:4146-4152.