Modulation of Cell Surface Heparan Sulfate Structure by Growth of Cells in the Presence of Chlorate†

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ABSTRACT: Swiss mouse 3T3 cells, when grown in the presence of 5 mM chlorate, an inhibitor of PAPS synthesis, produce heparan sulfate glycosaminoglycan chains containing only about 8% of the sulfate normally present and which have lost the ability to bind to fibronectin. These undersulfated chains are sensitive to nitrous acid at pH 4.5, indicating that many glucosaminyl residues have unsubstituted amino groups. The iduronic acid content of the heparan sulfate produced in the presence of chlorate is reduced to less than 7% as compared to the 36% in that from untreated cells. The chlorate-treated cells do not demonstrate any alterations in their growth control. However, the spreading behavior of these cells is altered to a flat rounded morphology compared to the more typical fibroblastic appearance of the untreated cell. The sulfation of chondroitin chains is also inhibited, but at a lower chlorate concentration which does not alter growth control or the spreading ability of the cells. These data indicate that (a) 3T3 cell surface heparan sulfate proteoglycan is not involved in growth control but may be involved in cell spreading, (b) the use of chlorate should be a valuable method for the study of the biosynthesis and structure/function relationships of sulfated glycosaminoglycans, and (c) the temporal sequence of the heparan sulfate chain modification reactions predicted from results of studies with cell-free extracts also operates in the cell.

Heparan sulfate proteoglycans (HSPGs)[1] are proteins which are glycosylated with HS glycosaminoglycan chains and are present on the surface of most vertebrate cells and in many extracellular matrices (Kraemer, 1979). Interest in the role of these glycoproteins in a variety of cell–cell and cell–matrix reactions has stemmed from the specific interactions of these chains with a variety of extracellular matrix proteins and the proven structural heterogeneity of the glycosaminoglycan chains which provide for modulation by cellular mechanisms (Gallagher et al., 1986). The sulfate residues, which may be present on at least four different positions of the polysaccharide backbone (Lindahl & Kjellén, 1987), are of particular interest since they have been shown to be major factors in the determination of specificity in protein–polysaccharide interactions (Lindahl et al., 1984). For example, the 3-*O*-sulfate on glucosaminyl residues in heparin (Lindahl et al., 1984) and in the HSs of endothelial cells (Marcum et al., 1986), Reichert's membrane (Pejler et al., 1987), and mouse mammary epithelial cells (Pejler & David, 1987) is required for interaction with antithrombin III.

The HS chains have been implicated in a variety of physiological processes including the regulation of glomerular basement membrane permeability to proteins (Kanwar et al., 1981), the assembly of basement membranes (Kleinman et al., 1986), the regulation of nuclear metabolism (Ishihara et al., 1986) and mammalian cell growth (Castellot et al., 1987), and the attachment and spreading of mammalian cells (Keller et al., 1982; Laterra et al., 1983; Cole et al., 1985; Izzard et al., 1986; McCarthy et al., 1986). The approaches which have been used to gain an understanding of the relationships between HS structure and biological function have included the modulation of its structure through the intervention of viruses (Underhill & Keller, 1977; Winterbourne & Mora, 1978), the isolation of mutants which make defective HS (Esko et al., 1985; Keller, K. M., et al., 1988), the use of degradative enzymes (Gill et al., 1986; Piepkorn et al., 1987), and the use of metabolic inhibitors (Johnston & Keller, 1979; Pratt et al., 1979). Previous results employing these techniques have shown that decreased sulfation is associated with changes in the organization and/or distribution of cytoskeletal and extracellular components (Keller, K. M., et al., 1988) and that decreases in O-sulfation specifically are associated with cell transformation by viral and mutagenic agents (Keller et al., 1980; Winterbourne & Mora, 1980) and with a diminished capacity to interact with fibronectin in an in vitro assay (Keller et al., 1982). HSPG has also been implicated in adhesive/spreading processes, possibly functioning as a transmembrane link between extracellular and cytoskeletal elements (Stamatoglou & Keller, 1981; Woods et al., 1986). However, such cell–matrix responses have been shown to vary between cell types (Singer et al., 1987).

Chlorate, an inhibitor of ATP sulfurylase (Farley et al., 1978) and, subsequently, the production of PAPS, the active sulfate donor for sulfotransferases (Lipmann, 1958), has been shown to inhibit sulfation of tyrosyl residues in various proteins and glycoproteins without inhibiting cell growth or protein synthesis (Baeuerle & Huttner, 1986). On the basis of this report, we initiated a detailed examination of the effects this sulfate analogue would have on the behavior of Swiss mouse 3T3 cells and the structure of the HS they produce. Our results add further support for a role of HSPG in the spreading mechanism of these cells through an interaction with fibronectin and indicate that the sulfation level of HSPG is not

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[1] Abbreviations: HSPG, heparan sulfate proteoglycan; HS, heparan sulfate; PAPS, phosphoadenosine phosphosulfate; DV, Dulbecco–Vogt; CS, chondroitin sulfate; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBSA, divalent cation free phosphate-buffered saline; CPC, cetylpyridinium chloride; HA, hyaluronic acid; UA, uronic acid; GlcA, D-glucuronic acid.

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directly involved with their contact regulation of cell growth. Our results also provide in vivo evidence for the temporal nature of the HS chain modifying reactions, which have previously been demonstrated with cell-free extracts (Lindahl & Kjellén, 1987). This result is of particular importance because of recent data which indicate that disruption of the membrane systems that contain the HS modification enzymes leads to alterations in the final structure of the HS produced by these cell-free systems (Riesenfeld et al., 1987). A preliminary account of this work has been presented (Keller & Keller, 1987b). Since completion of our work, several reports on effects of chlorate on the sulfation of glycosaminoglycans have been published (Humphries & Silbert, 1988; Greve et al., 1988). Our data fully confirm these reports and present extensive additional information which indicates the usefulness of this inhibitor in the study of HS biosynthesis and structure/function relationships of HS.

Experimental Procedures

Cell Culture. The murine cell line, 3T3-6A, used in these studies is a cloned line of the Swiss 3T3 cells (Keller, K. M., et al., 1988) and is identical with the parent in terms of growth behavior and PGs produced. The virally transformed Swiss 3T3 cell lines SV3T3 and SV40-1, the growth and maintenance of the cells, and the use of low-sulfate low-cysteine and/or low-glucose modifications of Dulbecco–Vogt (DV) medium for radioisotope labeling with [^{35}S]sulfate and/or [^{3}H]glucosamine have been previously described (Underhill & Keller, 1977; Keller & Keller, 1987a). The modified form of DV used for radioactive labeling and growth in chlorate contained low sulfate and/or low glucose, reduced cysteine (20 μM instead of 400 μM), and various concentrations of chlorate. The use of low-sulfate DV alone does not lead to the production of undersulfated glycosaminoglycans because the Swiss mouse 3T3 cells, including the 3T3-6A clone, are able to oxidize organic sulfides, e.g., cysteine, to sulfate in order to maintain an endogenous sulfate supply (Keller & Keller, 1987a), in marked contrast to other cells, such as those in the EHS sarcoma (Tyree et al., 1986) and bovine aortic endothelial cells (Humphries et al., 1986). However, the low-sulfate/cysteine DV medium potentiates the chlorate effect, presumably by lowering the cytosolic levels of sulfate. Previous studies have shown that >95% of macromolecular [^{35}S]sulfate is in the HS/CS pool (Underhill & Keller, 1977). Cell cloning efficiency, i.e., the number of colonies formed divided by the number of cells added times 100, was determined by a standard procedure (Freshney, 1987). Photomicrographs of cells examined by phase microscopy were taken with Kodak Panatomic-X film.

Cell Attachment Assay. Cells in the logarithmic phase of growth in low-sulfate/cysteine DV medium alone or in the presence of 5 or 10 mM chlorate were labeled with [*methyl*-^{3}H]thymidine overnight, then trypsinized, and washed 3 times in PBSA. After resuspension in serum-free 25 mM HEPES–low-sulfate/cysteine DV containing 4 mM sodium bicarbonate and 0, 5, or 10 mM chlorate, the cells were placed in a bacteriological-grade plastic dish, to which they do not attach, and incubated for 2 h at 37 °C. Although the cells do not attach and spread under these conditions, they do synthesize HSPG that is the same with respect to size and charge density as that from cells attached and spread on standard cell culture grade plastic dishes (Keller, K. M., et al., 1988). The cells were then removed by pipetting, washed once with PBSA, resuspended in HEPES–low-sulfate/cysteine DV, and seeded at 1×10^5 cells per 35-mm cell culture grade plastic dish. At various times, the medium was removed, and the attached cells were washed 3 times with PBSA. The cell sheet was then solubilized with 0.1 M NaOH containing 10% SDS, and radioactivity was determined by liquid scintillation spectroscopy.

Isolation of Proteoglycans. The isolation of trypsin-released cell surface PGs has been previously described (Underhill & Keller, 1975). Briefly, cells were washed in PBSA and digested with 1 mg/mL trypsin for 20 min. After removal of cells by centrifugation, the supernatant was further digested with Pronase (2 mg/mL) and dialyzed to reduce the ionic strength. The peptide-attached glycosaminoglycans were then isolated by ion-exchange chromatography on DEAE-cellulose employing a linear 0.01–1.0 M NaCl gradient or by successive precipitation with 10% CPC and ethanol in the presence of 0.5 mg/mL CS as carrier. Under these conditions, the only precipitated macromolecules labeled with [^{35}S]sulfate and [^{3}H]glucosamine were CS and HS or labeled with only [^{3}H]glucosamine, HA, and recovery was ≥89%. Medium PGs were isolated by either ion-exchange chromatography as described above or CPC precipitation after deproteination with 7% trichloroacetic acid, extensive dialysis, and volume reduction. The HS was subsequently purified by first digesting the PG sample with chondroitin ABC lyase (0.1 unit/mL) at both pH 8.5 and 5.5 and then hyaluronidase (20 TRU/mL) followed by gel filtration on Sephadex G-50 in 0.5 M ammonium bicarbonate. The material eluting at V_0 was pooled and shown to be sensitive to degradation with nitrous acid (Shively & Conrad, 1976) and to contain glucosamine as the only ^{3}H-labeled aminosugar. The latter observation indicated that all the unsulfated CS chains had been removed.

Analytical Procedures. Glycosaminoglycan chains were released from proteoglycans by alkaline borohydride treatment for 24 h at 45 °C and then analyzed by gel filtration on a dextran-calibrated 1 × 200 cm Sephacryl S-300 column using 1 M NaCl as eluant as previously described (Lowe-Krentz & Keller, 1983). Nitrous acid treatments were performed at both high (4.5) and low (1.5) pH values according to the methods of Lindahl et al. (1973) and Shively and Conrad (1976), respectively. The generated fragments were fractionated by gel filtration on 1 × 45 cm columns of Sephadex G-50 with 1 M NaCl. Acetylation of free amino groups was performed by a modification of a standard procedure (Danishevsky & Steiner, 1965). Samples were dissolved in 0.1 mL of 0.5 M potassium phosphate, pH 7.0, 10% (v/v) methanol and chilled to 3 °C. Three additions of 10 μL of acetic anhydride were made at 10-min intervals with constant shaking. At the end of 30 min, 1 mL of 0.5 M ammonium bicarbonate was added. The sample was concentrated, and salts were removed by repeated washing with water and centrifugation in a Centricon 10 microcentrator (Amicon). In some double-label experiments, two glycosaminoglycans, each with a single radioisotope label, were mixed in order to reduce large differences between levels of the two isotopes. Hexosamine and uronic acid analyses, based on the methods of Höök et al. (1974) and Fransson (1978), were performed on purified HSs metabolically labeled with D-[6-^{3}H(N)]glucosamine and D-[1-^{3}H]-galactose, respectively, as previously reported (Underhill & Keller, 1975; Keller et al., 1980). Protein was determined by the method of Lowry et al. (1951).

Computation of N- and O-Sulfate. The method used to calculate the relative *N*- and *O*-sulfate content has been described in detail (Keller et al., 1980). Briefly, HS labeled with [^{35}S]sulfate and [^{3}H]glucosamine was degraded with low-pH nitrous acid, and the resulting oligomers were fractionated on BioGel P2. Although the bulk of the [^{3}H]glucosamine-labeled

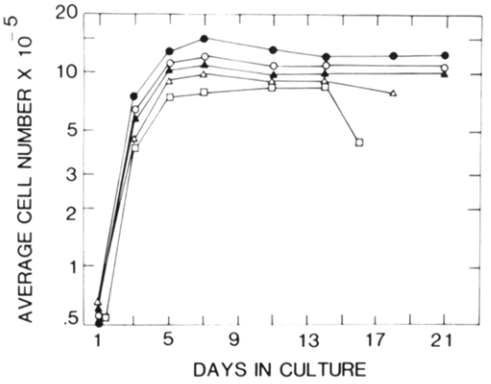

FIGURE 1: Effect of chlorate on the growth of Swiss mouse 3T3 cells. Control cells grown in DV (●); cells grown in low-sulfate/glucose/cysteine DV with no addition (○) and chlorate concentrations of 0.2 mM (▲), 1.0 mM (△), and 5.0 mM (□).

products are separated from the released [^{35}S]sulfate, a small fraction of sulfated disaccharides coelutes with the inorganic sulfate. This latter mixture is separated by ion-exchange chromatography on DEAE-Sephadex A-25. The ^{35}S label associated with the purified disaccharides is used to correct that associated with inorganic sulfate eluted from the BioGel P2 column. The latter figure, when multiplied by 100, is the percent *N*-sulfate. The percent *O*-sulfate is then calculated as 100 − (% *N*-sulfate).

Affinity Chromatography. The preparation and use of the human plasma fibronectin–Sepharose 4B matrix have been described (Stamatoglou & Keller, 1983). Briefly, the sample to be analyzed was applied to the column in a buffer composed of 10 mM Hepes, pH 6.5, and 0.15 M NaCl and washed with this buffer until no more radioactivity appeared in the effluent. The column was then washed with the same buffer containing 1.0 M NaCl. All steps were performed at room temperature, and the recovery of radioactivity was always >90%.

Materials. Trypsin (2× crystallized) was purchased from Cooper Biomedical. *Streptococcus hyalurolyticus* hyaluronidase (EC 3.2.1.35), chondroitin ABC lyase (EC 4.2.2.4), and pronase were obtained from ICN. The D-[6-^{3}H(N)]-glucosamine (30 Ci/mmol), $H_2{}^{35}SO_4$ (43 Ci/mg of S at 100% isotopic enrichment), D-[1-^{3}H(N)]galactose (25 Ci/mmol), and [*methyl*-^{3}H]thymidine (65 Ci/mmol) were obtained from ICN. Chromatographic column materials included DEAE-Sephadex A-25 and Sephadex G-50 from Pharmacia and BioGel P2 (−400 mesh), Dowex AG1-X8 (200–400 mesh), and Dowex 50-X8 (200–400 mesh) from Bio-Rad. Unless otherwise indicated, all reagents and chemicals were of the highest grade and purity available.

Results

Effect of Chlorate on the Attachment, Growth, and Morphology of Cells. When Swiss mouse 3T3 cells were grown in low-sulfate/cysteine DV medium, their growth rate resembled that of cells in unmodified medium (Figure 1). The addition of ≤5 mM chlorate caused no change in the rate of growth during the logarithmic phase. However, at 0.2, 1.0, and 5.0 mM chlorate, the final saturation densities were depressed 16, 24, and 36%, respectively. Although no change in the growth rate was observed within 2–4 days after the addition of chlorate, the cell number decreased after 14 days in culture in the presence of 5 mM chlorate. Morphological changes were noted 2–4 days after subconfluent cells were exposed to concentrations of ≥1 mM chlorate. In contrast to the control cells (Figure 2A), the chlorate-treated cells appeared flatter and spread more concentrically (Figure 2B). This altered morphology was retained even at confluence when growth was inhibited. In contrast to control cells which, with regular medium changes, can be held at confluence for many weeks, islands of chlorate-treated cells became vacuolated after 1 week and eventually detached. However, subconfluent growing cells could be passaged over at least a 2-month period with weekly transfers in low-sulfate/cysteine DV containing 5 mM chlorate. The cloning efficiency of chlorate-treated cells was reduced 7 times relative to the controls and the average colony size reduced by one-third. As measured over a 60-min period, cell attachment rates to standard cell culture grade plastic dishes remained unchanged even for cells passaged several times in 1 and 5 mM chlorate (data not shown). The transformed cells lines SV3T3 and SV40-1 showed no change in growth rate, but, in contrast to the 3T3 cells, their morphology was unaffected by chlorate concentrations up to 10 mM.

Table I: Effect of Chlorate on the Incorporation of [^{35}S]Sulfate into Glycosaminoglycans of Swiss Mouse 3T3 Cells

	cell surface		medium	
sample	cpm/μg of cell protein[a]	% control	cpm/μg of cell protein[a]	% control
control	349	100	21	100
0.2 mM chlorate	198	57	14	67
1.0 mM chlorate	152	44	8	38
5.0 mM chlorate	29	8	2	10

[a] Radioactivity measured on trypsin-released, Pronase-treated, and CPC- and ethanol-precipitated material (see Experimental Procedures).

Effect of Chlorate on the Biosynthesis of Proteoglycans. The effect of chlorate on the sulfation of proteoglycans in the Swiss mouse 3T3 cells was examined by growing the cells in the presence of chlorate for 2 days and then adding 5 μCi/mL [^{35}S]sulfate for an additional 2 days. The [^{35}S]sulfate incorporated into trypsin-released, Pronase-treated CPC-precipitable material was reduced to 57, 44, and 8% of the control value in the presence of 0.2, 1.0, and 5.0 mM chlorate, respectively (Table I). Further analysis of the trypsin-released

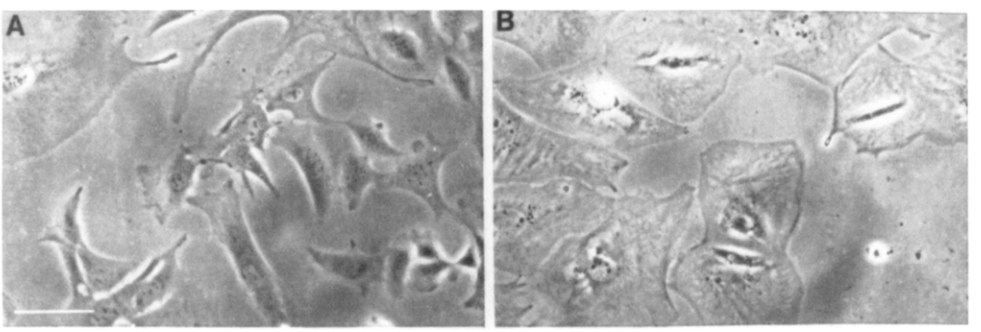

FIGURE 2: Effect of chlorate on the morphology of Swiss mouse 3T3 cells grown in low-sulfate/glucose/cysteine DV. (A) Control; (B) 5 mM chlorate. Bar = 100 μm.

Table II: [^{35}S]Sulfate Distribution in Glycosaminoglycans from Chlorate-Treated Swiss Mouse 3T3 Cells

	% ^{35}S in specified glycosaminoglycan			
	cell surface		medium	
sample	HS	CS	HS	CS
control	72	28	86	14
0.2 mM chlorate	95	5	94	6
1.0 mM chlorate	93	7	96	4
5.0 mM chlorate	96	4	95	5

Table III: Effect of Chlorate on the Incorporation of [^{35}S]Sulfate and [^{3}H]Glucosamine into the Trypsin-Releasable Glyosaminoglycans of Swiss Mouse 3T3 Cells

	cpm/μg of cell protein				
sample	^{3}H[a]	% control	^{35}S[a]	% control	^{35}S/^{3}H
control	67	100	121	100	1.8
1.0 mM chlorate	70	104	103	80	1.5
5.0 mM chlorate	84	121	27	21	0.3

[a] After Pronase digestion, the labeled macromolecules were fractionated on DEAE-cellulose, and the glycosaminoglycan fractions were pooled. The tritium is present in HA, CS, and HS, whereas the [^{35}S]sulfate is associated with only CS and HS (Underhill & Keller, 1975).

materials using chondroitin ABC lyase and degradation with low-pH nitrous acid revealed that only 4–7% of the cell surface material from chlorate-treated cells was CS as compared to 28% for the control cells (Table II). Incorporation of [^{35}S]sulfate into the CPC-precipitable material from the medium was reduced to 67, 38, and 10% of the control values when cells were grown in 0.2, 1.0, and 5.0 mM chlorate, respectively (Table I). As was observed in the case of the cell surface material, the relative amount of [^{35}S]sulfate-labeled CS compared to HS present in the medium decreased rapidly from 14% to approximately 5% of the total glycosaminoglycans which suggested that sulfation of CS was more sensitive to the effects of chlorate than was HS as has been observed in other systems [e.g., see Humphries et al. (1986)] (Table II).

In order to determine whether the decrease in sulfate incorporation was due to a general depression in proteoglycan synthesis or to a decrease in the sulfation of fully formed glycosaminoglycan chains, cells were grown in low-sulfate/glucose/cysteine DV medium containing 1.0 or 5.0 mM chlorate, and [^{3}H]glucosamine and [^{35}S]sulfate (5 μCi/mL each). The trypsin-released material showed a reduction in sulfate incorporation similar to that reported above (Table III). However, the amount of glucosamine incorporated into the backbone of the glycosaminoglycan was not reduced and, in fact, was increased 20% in the cells grown in 5.0 mM chlorate. It should be noted that the growth in chlorate had no effect on the protein content of cells (data not shown). Similar results were obtained with the transformed cells, SV3T3 and SV40-1 (data not shown), which indicated that the glycosaminoglycans in these cells were effected in the same way.

Effect of Chlorate on the Structure of Heparan Sulfate. Since the above data indicated that it was the chain sulfation reactions rather than formation of the polysaccharide backbone which were affected by chlorate, the HS chains produced in the presence of chlorate were examined by ion-exchange chromatography on DEAE-cellulose, a separation method which is sensitive to the number and distribution of the sulfate residues present (Underhill & Keller, 1977; Keller et al., 1982; Keller, K. M., et al., 1988). The [^{35}S]sulfate-labeled HSs prepared from cells grown in the absence or presence of 0.2, 1.0, or 5.0 mM chlorate were cochromatographed with

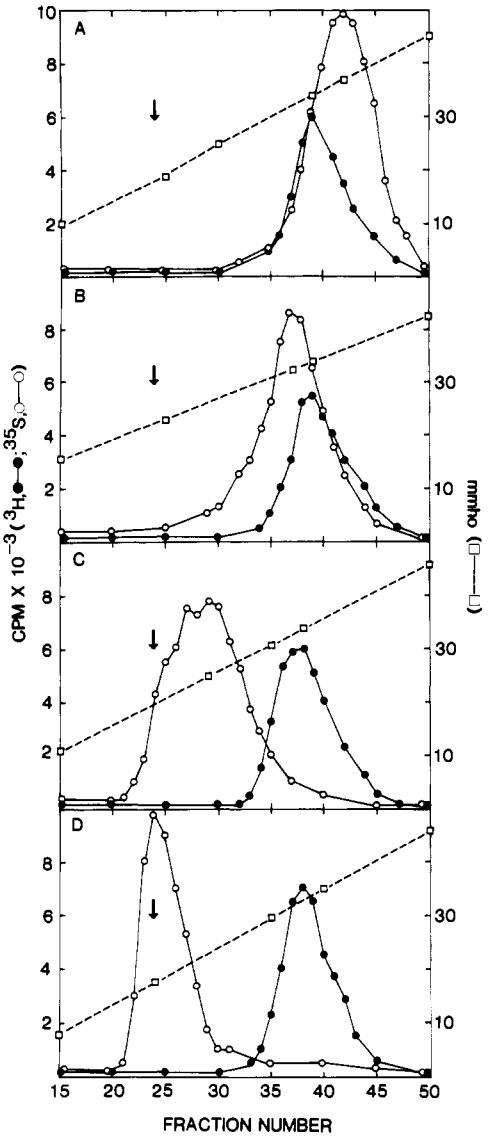

FIGURE 3: Effect of growth in chlorate on Swiss mouse 3T3 HS chromatographic behavior on DEAE-cellulose. [^{3}H]Glucosamine-labeled HS from SV3T3 cells (●); [^{35}S]sulfate-labeled HS from chlorate-treated cells (○). (A) Untreated; (B) 0.2 mM chlorate; (C) 1.0 mM chlorate; (D) 5.0 mM chlorate. The arrow indicates the elution position of HA.

[^{3}H]glucosamine-labeled HS from the virally transformed Swiss mouse cell line SV3T3 as an internal standard (Figure 3). The HS from the 3T3 cells (or clone 3T3-6A) required a higher salt concentration for elution from DEAE-cellulose than did the HS from the virally transformed cells SV3T3 (Underhill & Keller, 1975; Figure 3A). In contrast, HS isolated from cells treated with 0.2 mM chlorate was eluted before the SV3T3 HS (Figure 3B). As the concentration of chlorate was increased further, the elution position of the resultant HS was shifted even further to the left until the HS synthesized in the presence of $\geq$5.0 mM chlorate emerged at a position essentially identical with that of the unsulfated glycosaminoglycan, HA (Figure 3D).[2] When cells were simultaneously labeled with both [^{3}H]glucosamine and [^{35}S]-sulfate in varying concentrations of chlorate, similar results were obtained. Both the [^{3}H]glucosamine- and [^{35}S]sulfate-labeled HSs from cells grown in 5.0 mM chlorate coeluted at a position equivalent to HA. This change in elution behavior

[2] From the relative elution positions, the CS was most probably undersulfated, but no analysis of released disaccharides was performed.

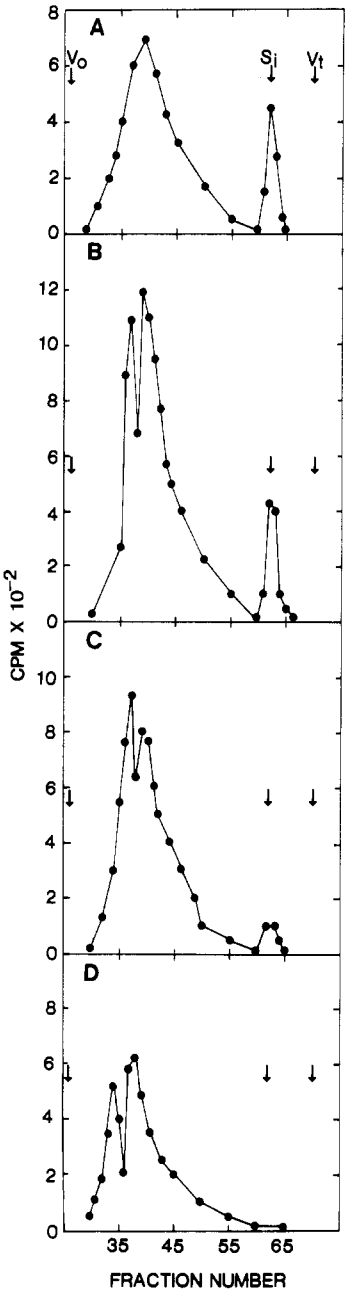

FIGURE 4: Molecular sieve chromatography on Sephacryl S-300 of alkaline borohydride released [^{35}S]sulfate-labeled HS chains isolated from control cells (A) and cells grown in chlorate concentrations of (B) 0.2, (C) 1.0, and (D) 5.0 mM.

reflected the change in the incorporation ratio of $^{35}S/^{3}H$ from 1.8 for control cells to 0.3 for cells grown in 5 mM chlorate (Table III). Similar results were obtained for the HSs isolated from SV3T3 and SV40-1 cells grown in ≥5 mM chlorate (data not shown).

Release of [^{35}S]sulfate-labeled carbohydrate chains from the core protein by β-elimination with alkaline borohydride and subsequent analysis by gel filtration on Sephacryl S-300 indicated that the chain sizes were not diminished (Figure 4). During the alkaline borohydride reaction, about 10% of the sulfate residues in the Swiss mouse 3T3 HS were released (Figure 4A). Increasingly less sulfate was released from the HSs produced in the presence of chlorate (Figure 4B–D), and no release was detected from HS synthesized in the presence of 5 mM chlorate (Figure 4D). Since studies on the alkaline borohydride release of sulfate from rat ovarian granulosa cell HSPGs have suggested that a particular class of *O*-sulfates

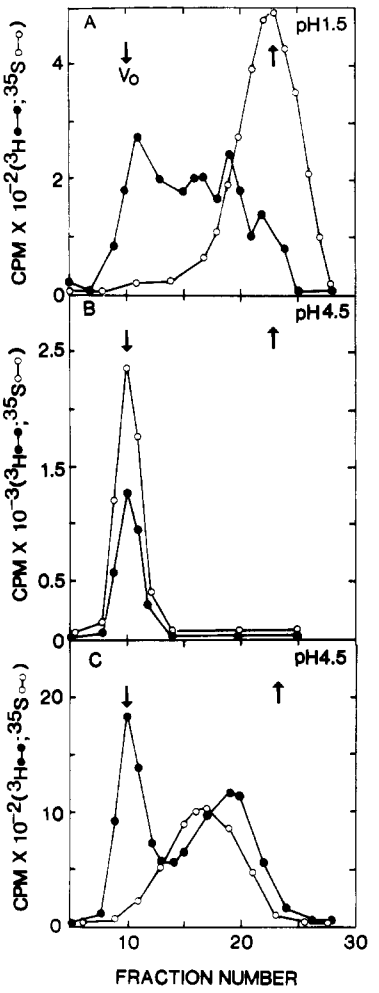

FIGURE 5: Molecular sieve chromatography on Sephadex-G-50 of high-pH (4.5) and low-pH (1.5) nitrous acid degradation products of HS purified from chlorate-treated Swiss mouse 3T3 cells labeled with [^{3}H]glucosamine (●) and [^{35}S]sulfate (○). HS from untreated cells degraded at pH 1.5 (A) and pH 4.5 (B). HS degraded at pH 4.5 from cells grown in 5.0 mM chlorate (C). The elution position of inorganic sulfate is indicated by the up arrow.

are released, i.e., 6-*O*-sulfates (Yanagishita & Hascall, 1983), the present data suggested that some of the charge density decrease in the HS from chlorate-treated cells resulted from the loss of a specific class of *O*-sulfate.

Degradation with nitrous acid at low pH (1.5) results in quantitative N-desulfation of HS and subsequent chain scission by β-elimination (Shively & Conrad, 1976). A typical low-pH degradation pattern resolved on Sephadex G-50 for $^{3}H/^{35}S$-labeled HS from the Swiss mouse 3T3 cells is shown in Figure 5A. Approximately 50% of the [^{35}S]sulfate label was released as inorganic sulfate which was the most retarded peak from the column. Also included in this peak was a proportion of nonsulfated and O-sulfated di- and tetrasaccharide fragments as previously shown (Keller et al., 1980). The earlier eluting larger oligomers have little associated sulfate in keeping with the known clustering of the sulfate substituents (Gallagher et al., 1986). During nitrous acid treatment at high pH (4.5), only glycosidic bonds adjacent to the glucosaminyl residues with unsubstituted amino groups are broken (Lindahl et al., 1973; Shively & Conrad, 1976). When the HS from control cells was subjected to high-pH degradation, no detectable change in the size of the molecule was observed (Figure 5B), indicating that few, if any, unsubstituted amino groups exist in the mature carbohydrate chain. In contrast, HS from cells metabolically labeled with [^{35}S]sulfate and [^{3}H]glucosamine

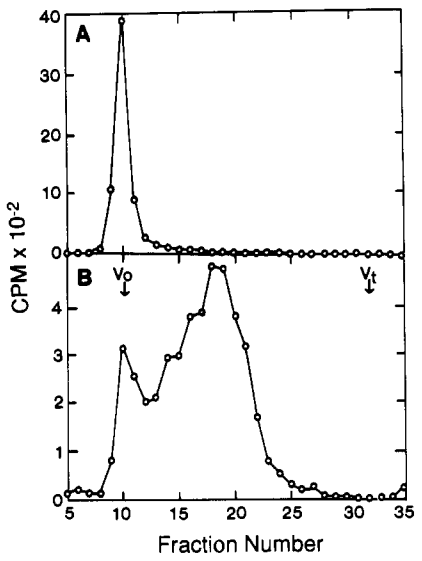

FIGURE 6: Effect of N-acetylation on the high-pH (4.5) nitrous acid degradation of [^{3}H]glucosamine-labeled HS purified from Swiss mouse 3T3 cells grown in the presence of 5 mM chlorate as demonstrated by molecular sieve chromatography on Sephadex G-50. V_0 and V_t are indicated. (A) N-Acetylated HS; (B) untreated HS.

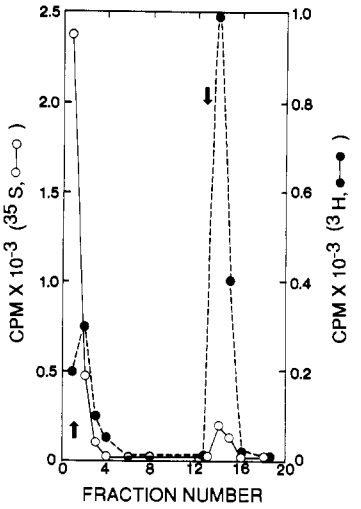

FIGURE 7: Affinity chromatography of HSs on fibronectin–Sepharose 4B. The mixed HS sample was applied to the column at fraction 1 (up arrow). The elution with high salt was initiated at fraction 13 (down arrow). [^{3}H]Glucosamine-labeled HS from control cells (●); [^{35}S]sulfate-labeled HS from cells grown in the presence of 5 mM chlorate (○).

in the presence of 5.0 mM chlorate showed significant degradation when treated with nitrous acid at pH 4.5 (Figure 5C), which indicated an increase in the amount of unsubstituted amino sugar. In the HS from cells grown in 5 mM chlorate, some of the [^{3}H]glucosamine label remained at V_0 (Figure 5C). This result suggested that contiguous nonsulfated disaccharides containing acetylated amino sugars also existed in the molecules. Together these data indicated that in the presence of chlorate there was an appearance of regions enriched in acetylated glucosaminyl groups as well as regions enriched in glucosaminyl residues with unsubstituted amino groups and a decrease in both *N*- and *O*-sulfates as was expected since the total sulfation of HS was depressed >89% as a result of synthesis in the presence of 5 mM chlorate. Analysis of the small amount of sulfate present in the HS produced in the presence of 5 mM chlorate (see Experimental Procedures) showed that 87% was N-linked and 13% was O-linked in contrast to the 49% and 51%, respectively, found in the HS from control cells. The sensitivity of the HS from cells grown in the presence of chlorate and [^{3}H]glucosamine to high-pH nitrous acid was tested after N-acetylation with acetic anhydride. As shown in Figure 6A, the chemically acetylated HS was totally resistant to nitrous acid at pH 4.5.

Since in vitro biosynthetic experiments have indicated that the conversion of D-glucuronosyl to L-iduronosyl residues occurs at the polymer level, is coupled to O-sulfation, and follows N-sulfation (Lindahl & Kjellén, 1987), the grossly undersulfated HS produced in the presence of chlorate would be expected to contain a greatly reduced level of iduronosyl residues. Uronic acid analyses of [^{3}H]galactose-labeled HS from control and chlorate-treated cells showed that for control cells the uronic acid composition was 64% glucuronic acid and 36% iduronic acid while that from the chlorate-treated cells contained ≥93% glucuronic acid and ≤7% iduronic acid.

Affinity Chromatography on Fibronectin–Sepharose. The facts that (a) the HS chains from cells grown in the presence of chlorate were grossly undersulfated, (b) the chlorate-treated cells demonstrated changes in their spreading behavior, and (c) the undersulfated HS chains have been shown to lack affinity for fibronectin (introduction) suggested that the chains produced by the chlorate-treated cells would not bind to fibronectin. As indicated in Figure 7, the HS chains produced by the treated cells were almost totally devoid of affinity for fibronectin, whereas those from the untreated cells demonstrated a high degree of binding under these physiological salt conditions as previously reported (Stamatoglou & Keller, 1983).

Discussion

The sulfated proteoglycans produced by Swiss mouse 3T3 are clearly altered when cells are grown in the presence of chlorate, a known inhibitor of PAPS biosynthesis (Baeuerle & Huttner, 1986). This effect is best manifested by the reduced charge density of the glycosaminoglycan chains from these cells as detected by ion-exchange chromatography on DEAE-cellulose. The chains produced in the presence of 5 mM chlorate behave as the nonsulfated glycosaminoglycan HA although they still contain a small amount of [^{35}S]sulfate, i.e., about 5% of the control value. The loss of sulfate is seen in both CS and HS, with the effect on CS occurring at a lower chlorate concentration. This result is consistent with the observation that the sulfotransferases involved in CS synthesis have higher K_m's for PAPS than those involved in HS synthesis (Humphries et al., 1988). Although we have not shown that the pool of PAPS in these cells is actually depleted in the presence of chlorate, the data which we have presented is fully consistent with this conclusion. That the effect of chlorate is not limited to Swiss mouse 3T3 cells is indicated by the fact that similar effects of chlorate are observed on the CSPGs produced by bovine aortic endothelial cells (Humphries & Silbert, 1988), human fibroblasts (Greve et al., 1988), and chick chondrocytes (Walters et al., 1988) and the HSPGs produced by human fibroblasts (Greve et al., 1988), bovine aortic endothelial cells (Humphries & Silbert, 1988), and a model basement membrane forming cell line (Brauer et al., 1988).

The effects of chlorate on the structure of the HS chains are in full accord with the temporal sequence of reactions which have been identified in the synthesis of this family of molecules with the use of cell-free preparations (Lindahl & Kjellén, 1987). In this pathway, the initial polymer of repeating GlcNAc-GlcA disaccharides is modified by the removal of *N*-acetyl groups, the addition of *N*-sulfate groups, the epimerization of D-glucuronosyl to L-iduronosyl, and the

addition of *O*-sulfate residues. The sensitivity of the HS chains from cells grown in the presence of chlorate to high-pH nitrous acid and the loss of this sensitivity after N-acetylation and our preliminary data on the products resulting from periodate oxidation of these chains (Keller, J. M., et al., 1988) demonstrate that the deacetylation reaction occurs without concomitant N-sulfation. As judged by the size range of the recovered glucosaminyl-containing oligomers, this deacetylation appears to occur to only a partial extent as would be predicted from the clustered regions of modifications characteristic of this family of molecules (Gallagher et al., 1986). These results are in agreement with those recently published by Greve et al. (1988) and those obtained from in vitro studies (above) but are in marked contrast to those obtained with the EHS sarcoma, in which no free amino groups in undersulfated HS produced by sulfate restriction were obtained (Tyree et al., 1986). The reason for this difference is not known but may reflect the well-characterized differences in cells to make use of organic sulfur as a source of sulfate (Keller & Keller, 1987a; Humphries et al., 1988). The HS formed in the presence of chlorate also has a greatly reduced content of iduronosyl residues and O-sulfation. Thus, those reactions which are expected to be dependent upon the N-sulfation reaction are essentially blocked. The fact that the deacetylation reaction that occurs in the presence of 5 mM chlorate appears to reproduce the high- and low-sulfated regions normally present in HSs indicates that, despite the greatly depressed N-sulfation reaction, the polymer appears to proceed through the biosynthetic machinery in a normal fashion. The fact that the size of the undersulfated HS chains is unchanged or slightly increased indicates that in this cell type there is no correlation between sulfation and chain elongation as has been reported for fibroblasts (Greve et al., 1988) and the EHS sarcoma (Tyree et al., 1986) in contrast to that proposed to occur in rabbit endothelial cells (Dietrich et al., 1988). These observations suggest that the inability to complete the chain modification reactions does not affect the movement of the HSPG through the Golgi and to the cell surface.

The behavior of the cells grown in the presence of chlorate has provided significant insight to the physiological function of their cell surface HS. However, the possible influence of an effect of chlorate on other cellular processes, e.g., it probably also inhibits the sulfation of other molecules, must temper some of these conclusions. Nevertheless, little if any sulfate is present in other proteins in these cells, and the morphological effects which we have observed are only seen at concentrations at which HS is affected. Despite considerable earlier correlative evidence suggesting that changes in the sulfation of these chains are somehow involved in the growth regulation of cells (Underhill & Keller, 1977; Winterbourne & Mora, 1978, 1980; Keller, K. M., et al., 1988), cells which carry the grossly undersulfated chains produced in the presence of chlorate still have full contact regulated growth control. Various transformed cells, which lack growth control and have slightly undersulfated surface HS chains, show no difference in their growth when induced in the presence of chlorate to produce grossly undersulfated cell surface HS. These data argue strongly against an involvement of the sulfate residues in the HS chains in the growth control demonstrated by the 3T3 cells. It will be of interest to see if this result holds true for hepatocytes in which growth control has been correlated with the presence of specific HS fragments in the nucleus (Fedarko & Conrad, 1986) or in skin fibroblasts where the addition of HS chains to the transferrin receptor has been correlated with their growth control (Cöster et al., 1986). The use of chlorate may provide a means to test these other correlations.

The role of HS in the attachment of cells has not been fully answered by our experiments. Our data demonstrate that cells with grossly undersulfated HS attach to cell culture grade plastic dishes with essentially normal kinetics, but the relationship between this plastic and a natural substrate is not apparent. The removal of cell surface HS with enzymes, either heparinase or heparitinase, has been reported to block the attachment of cells to plastic dishes (Gill et al., 1986), but this effect has not been reproducible with highly purified enzymes (Piepkorn et al., 1987). Although not quantitated, the behavior of the 3T3 cells in chlorate, as evidenced by their ability to cover a cell culture grade plastic dish surface without the formation of distinct islands of cells, indicates that the undersulfated HS chains do not affect the ability of the cells to migrate.

A most dramatic visible effect of chlorate on 3T3 cells is on the appearance of spread cells, which resemble flat disks in contrast to the fibroblastic shape of untreated control cells. Morphological changes have also been reported for cells which have been pretreated with heparinase prior to attachment and spreading (Laterra et al., 1983) and those which have been allowed to spread on fibronectin fragments that contain the cell binding domain but not a heparin binding domain (Izzard et al., 1986; Woods et al., 1986). In concert with the chlorate-induced morphological change, the formation of fibronectin fibrils appears to be delayed, and the arrangement of vinculin is somewhat altered (Keller, J. M., et al., 1988). These observations suggest that the cell surface HS may modulate the transmembrane signaling system in which an integrin mediates the interaction between extracellular fibronectin and the cytoskeleton. Studies with HS/CS-deficient mutants, which spread normally on fibronectin but do not form focal contacts or stress fibers, in contrast to the chlorate-treated 3T3 cells, have led to a similar conclusion regarding a relationship between HS structure and cell spreading (LeBaron et al., 1988). Our demonstration that the HS produced by cells grown in the presence of chlorate does not interact with fibronectin is in full accord with this idea. However, the generality of this observation is clearly limited since it has been shown that some cells appear to spread normally and form focal contacts on the cell binding domain of fibronectin (Singer et al., 1987) and endothelial cells grown in the presence of chlorate show no morphological changes (Humphries & Silbert, 1988). Whether these different responses are the result of multiple integrins (Buck & Horwitz, 1987), fibronectins (Hynes, 1985), or other extracellular matrix molecules (Buck & Horwitz, 1987) is not known. The fact that the viral transformed derivatives of 3T3 cells tested during our studies exhibited no changes in their spreading indicates that the mechanism used by these transformed cells to spread is altered at some point in addition to the previously reported changes in HS, which can result in the loss of binding to fibronectin (Keller et al., 1982). The nature of this (these) additional alteration(s) remains unknown but may be the phosphorylation of specific components of an integrin-mediated transmembrane signaling system (Kellie, 1988).

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