

Cells Selected for High Tumorigenicity or Transformed by Simian Virus 40 Synthesize Heparan Sulfate with Reduced Degree of Sulfation*

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Cell lines, selected from two independent clones of an established mouse embryo cell line by their ability to grow as solid tumors in immunocompetent syngeneic hosts, were found to have the same alteration in anion exchange properties as was previously reported for simian virus 40 (SV40)-transformed subclones. One tumor cell line (219CT) and one SV40-transformed subclone (215CSC) were selected for further detailed comparison with their common parent clone (210C). Cellulose acetate electrophoresis at pH 1.0 showed that 215CSC heparan sulfate had a slight overall decrease in sulfation compared with heparan sulfate from 210C; however, no gross difference in sulfation could be detected between heparan sulfate from 219CT and 210C. Analysis of the products of deaminative cleavage of heparan sulfate by nitrous acid under conditions where cleavage occurs quantitatively at *N*-sulfated glucosamine residues showed that, although heparan sulfate from the three cell lines gave similar yields of *O*-sulfated disaccharides, both 215CSC and 219CT had only about half as many *O*-sulfate residues in higher molecular weight oligosaccharides compared to heparan sulfate from 210C. Enzymatic degradation of heparan sulfate with a mixture of enzymes from *Flavobacterium heparinum* showed that this common alteration in heparan sulfate from both 215CSC and 219CT resulted from a 30% decrease in glucosamine residues bearing 6-*O*-sulfate groups. As this decrease in 6-*O*-sulfated glucosamine residues occurs in regions of the chain containing relatively few sulfate groups, it is clear that certain sequences of charged groups present in heparan sulfate from 210C will be found only rarely in heparan sulfate from 215CSC and 219CT. It is suggested that this will result in alterations of the interaction of heparan sulfate with other molecules in the microenvironment at the cell surface which may be important in the control of such phenomena as cell growth and adhesion.

In attempting to elucidate the alterations of cellular metabolism which define the neoplastic state, the most frequently used model has been the *in vitro* transformation of cells by viruses, such as SV40.¹ It is well known that, upon infection of

cells by SV40, the transformation, *i.e.* permanent insertion of SV40 DNA into the cellular genome and heritable expression of the "early" or A half of the SV40 gene as detected by the synthesis of its products the T-antigens, is a very rare event (1). We have previously shown that, after infection of a "parent" cloned mouse cell (210C) by SV40, the metabolism of heparan sulfate is altered only in the subclones which possess SV40 DNA and express the SV40 T-antigens, but not in an untransformed "sister" clone (2). Such changes in heparan sulfate were first reported by others in SV40-transformed 3T3 cells selected for their ability to form foci in cell culture (3, 4). Thus, the altered metabolism can be detected consistently and only in the cells which express the SV40 T-antigens, the proteins thought to be responsible in transformed cells for stimulation of DNA synthesis and for loss of growth control (1).

As an alternative to the virus transformation model, we have isolated highly tumorigenic tumor cell lines by *in vivo* selection from two independent clones (210C and 216C) of AL/N mouse fibroblasts which possess very low tumorigenicity. Because these tumor cell lines were isolated from tumors induced in the syngeneic mouse by injecting 10⁷ or 10⁶ cloned cells and because the tumor lines possess heritably very high tumorigenicity (median tumorigenic dose < 10² cells/mouse), the simplest explanation is that they represent variants (possibly mutants) which arose spontaneously at a very low frequency, and which must have been selected out from the clones on the basis of their high tumorigenicity. We reasoned that, if there is a specific biochemical difference which is common between at least two different tumor cell lines and their respective parent clones, which is also the same between the parent and the SV40-transformed daughter clones, this is unlikely to be just a coincidence as both models of transformation select rare transformed cells by very different mechanisms, but probably reflects basic and common biochemical alterations in both types of transformation events.

In this paper we demonstrate that tumor cell lines, like SV40 transformants, have alterations in the anion exchange elution characteristics of heparan sulfate when compared to the parent clones. A detailed structural analysis of the substitution patterns of sulfate groups in heparan sulfate from one tumor and one SV40-transformed cell line and the parent clone from which they were derived, is also presented.

MATERIALS AND METHODS

D-[6-³H(*N*)]glucosamine hydrochloride (21 Ci/mmol), D-[1-¹⁴C]glucosamine hydrochloride (55 mCi/mmol), sodium [³⁵S]sulfate (866

lactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-diS, disulfated unsaturated disaccharide.

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¹ The abbreviations used are: SV40, simian virus 40; ΔDi-OS, 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-ga-

mCi/mmol), and Aquasol were obtained from New England Nuclear. Chondroitinase ABC, Δ Di-6S, Δ Di-4S, Δ Di-OS, chondroitin sulfate A, hyaluronidase from bovine testes, and hyaluronic acid from pig skin were from Miles Laboratories. Heparin (170 units/mg) was obtained from Sigma. A crude preparation of heparinase from *Flavobacterium* was a generous gift from A. Linker, Veterans Administration Hospital, Salt Lake City, UT. Other materials were as previously described (2).

Cell Culture—Conditions of growth of cells in tissue culture and the isolation of two clones (210C and 216C) by microscopic examination of 1- μ l droplets of a cell suspension, was previously described (2), as was the infection of 210C by SV40 and the isolation of one T-antigen-negative (213CSC) and two T-antigen-positive subclones (214CSC and 215CSC) (2).

Tumorigenicity was determined by inoculation of 10-fold dilutions of a cell suspension into the hind legs of batches of 8 to 10 syngeneic AL/N mice. Tumor cell lines 219CT and 220CT were established from tumors obtained by inoculation of 10^7 210C cells and 10^6 216C cells into separate young adult (8-week-old) AL/N mice. A nonnecrotic portion of tumors approximately 2 cm in diameter was minced, washed twice in phosphate-buffered saline, and trypsinized. The resulting cell suspension was plated in plastic culture flasks and re-established as a tumor line, as described previously (5). The relationship between the various cell lines is shown in Fig. 1.

None of the cell lines were contaminated by mycoplasma as determined by culture methods (including sensitive fluorescent antibody and fluorescent DNA staining tests for cell dependent strains of mycoplasma) performed by Flow Laboratories, Inc.

Labeling of Cells and Isolation of Glycoconjugates—Cell lines were labeled for 24 h at an early and similar number of cumulative subcultures at similar cell densities during exponential growth. For the initial experiment five replicate 25-cm² flasks of cells were labeled as previously described (2) but using 1.25 μ Ci/ml of [³H]glucosamine for the parent clones and 0.125 Ci/ml [¹⁴C]glucosamine for the tumor cells. In both cases the final concentration of glucosamine in the medium was 2.5 μ M. For the structural analysis of heparan sulfate, cells were labeled in single 75-cm² flasks either using 50 μ Ci/ml of [³H]glucosamine for the parent clone and 10 μ Ci/ml of [¹⁴C]glucosamine for the daughter at a final glucosamine concentration of 0.18 mM in Dulbecco-Vogt modification of Eagle's medium with one-tenth the normal glucose concentration or using 50 μ Ci/ml of [³H]glucosamine and 200 μ Ci/ml of [³⁵S]sulfate in medium with one-tenth the normal glucose concentration and with the magnesium sulfate replaced by magnesium chloride. The labeled cultures were prepared for and analyzed by ion exchange as previously described (2). Scintillation counting in Aquasol was carried out in a Beckman LS 9000 counter calibrated with a series of standards of [³H]- and [¹⁴C]sucrose (LKB Instruments Inc.) and dioctyl [³⁵S]sulfide (Radiochemical Centre, Amersham) in Aquasol. The results were plotted by computer as previously described.

Analytical Procedures—Heparan sulphate isolated by anion exchange was purified by digestion for 5 h at 37°C with 0.33 units of chondroitinase ABC in enriched Tris buffer (6) in a final volume of 1 ml. Under these conditions 200 μ g of standard hyaluronic acid or chondroitin sulfate A or C were completely degraded to the expected unsaturated disaccharide. Chondroitin sulfate synthesized by the cells was digested under identical conditions, but in a final volume of 0.1 ml and the reactions products were analyzed by descending paper chromatography as described previously (2).

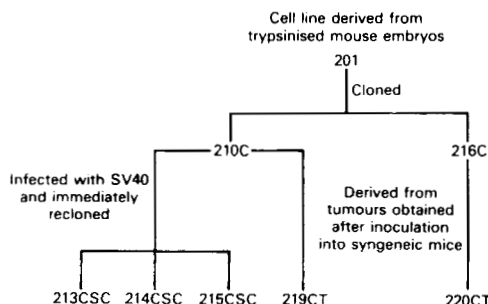


FIG. 1. Relationship between the AL/N mouse embryo cell lines. Each cell line was given a specific number and a letter code describing its origin (C, clone; S, SV40-infected; T, isolated from a tumor). Cell lines 214CSC and 215CSC were SV40 T-antigen-positive by nuclear immunofluorescence, whereas 213CSC was T-antigen-negative.

Nitrous acid degradation of heparan sulfate was performed by the low pH procedure, which has been demonstrated to cleave heparin at *N*-sulfated glucosamine residues generating oligosaccharides terminating in anhydromannose, with quantitative release of the sulfate group (7). Reactions were initiated by the addition of 0.4 ml of the nitrous acid reagent (7) at -5°C to 0.1 ml of heparan sulfate in distilled water. The mixture was allowed to warm to room temperature and after 10 min the reaction was stopped by the addition of 50 μ l of 5 M ammonium sulfamate followed by neutralization with 1 M NaOH to a phenol red end point.

To estimate the amount of [³⁵S]sulfate released by the nitrous acid reaction, an aliquot was spotted onto a sheet of Whatman No. 3MM chromatography paper and subjected to high voltage electrophoresis at 20 V/cm for 40 min in 1.6 M formic acid, pH 1.7 (8). Radioactivity was detected by scintillation counting of 0.5-cm wide strips after elution with 1 ml of distilled water in the scintillation vial before addition of 10 ml of Aquasol.

The frequency and distribution of *N*-sulfated glucosamine residues in the heparan sulfate chain was determined by exclusion gel chromatography of the nitrous acid degradation products on a column (1 \times 50 cm) of Bio-Gel P10-400 eluted at 8 ml h⁻¹ cm⁻² with 0.5 M ammonium bicarbonate at room temperature. Such a column was found to have far higher resolution than an identical column of Sephadex G-50 superfine. Resolution of the Bio-Gel column was not significantly improved by increasing the length to 200 cm. The column was calibrated with oligosaccharides produced by digestion of hyaluronic acid with testicular hyaluronidase (1600 IU/ml) in 0.15 M sodium chloride, 0.1 M sodium acetate, pH 5.0, for 3 h at 37°C. Bovine serum albumin was a marker for the void volume (*V*₀) and sodium azide or sodium [³⁵S]sulfate (both of which eluted in the same volume) for the totally included volume (*V*_i).

The percentage of glucosamine residues in the degradation products present as anhydromannose (and, hence, the percentage of residues which were *N*-sulfated in the intact molecule) can be calculated from the data in the elution profiles by considering the number of glucosamine residues/oligosaccharide (determined from its elution position) and the fact that each oligosaccharide terminates in an anhydromannose residue. Thus, the amount of activity (*a*_{*n*}) in an oligosaccharide composed of "*n*" disaccharide repeat units that is present as anhydromannose is given by *a*_{*n*}/*n*, and the percentage of anhydromannose residues in the degradation product is

$$\frac{\sum_{n=1}^{\infty} a_n/n}{\sum_{n=1}^{\infty} a_n}$$

The material degraded by nitrous acid was also examined by ion exchange on columns (1 \times 4 cm) of Whatman DE52 equilibrated with 10 mM Tris-HCl, pH 8.4, and eluted at room temperature with a linear gradient of 0 to 0.25 M NaCl at 50 ml h⁻¹ cm⁻². Fractions of 1.0 ml were collected. The column was calibrated with di-, tetra-, and hexasaccharides isolated from testicular hyaluronidase-digested hyaluronic acid, as described above, and with monosulfated disaccharides from a chondroitinase ABC digestion of chondroitin sulfate produced by 210C cells.

In some experiments heparan sulfate was degraded by a nitrous acid reaction (Reaction B (9)) which cleaves the chain only at unsubstituted amino groups, the reaction products were analyzed by exclusion gel chromatography.

The overall degree of sulfation was estimated by cellulose acetate electrophoresis in hydrochloric acid at pH 1.0 with the strips suspended and cooled in Varsol, conditions under which the mobility of glycosaminoglycans is directly proportional to the degree of sulfation (10). Standards of hyaluronic acid, chondroitin sulfate A, and heparin were run on the same strip and detected by staining with Alcian blue.

Crude heparinase was used to degrade heparan sulfate to the constituent monosaccharides (11) by digesting an aliquot of the radiolabeled heparan sulfate in a final volume of 20 μ l of 0.1 M sodium acetate buffer, pH 7.0, containing 20 μ g of crude heparinase and 100 μ g of carrier heparin. After 20 h at 30°C the reaction was terminated by boiling for 3 min and the whole sample was spotted onto a sheet of Whatman No. 1 chromatography paper and subjected to descending chromatography in butan-1-ol:acetic acid:water (10:3.5, v/v) for 40 h. Standards of *N*-acetylglucosamine and heparinase-degraded heparin were detected on the chromatogram by the silver nitrate-sodium hydroxide method (12).

RESULTS

Cell Growth Properties—When a sufficiently large number of cells from the two clones, *i.e.* 10^7 210 cells and 10^6 216C cells, were inoculated into immunocompetent syngeneic AL/N mice, tumors appeared after a long latency period (Table I). When cells from such tumors were re-established in tissue culture and subsequently the resulting tumor cell lines, 219CT and 220CT, were tested for their ability to grow as tumors in the syngeneic mouse, they were found to have a dramatically increased tumorigenicity. This was detectable both in the reduction (by 1000-fold or more) in the number of cells required to induce tumors and in the increased speed with which tumors could be detected when a dose sufficient to produce tumors in all of the mice was injected (Table I).

In cell culture on plastic substratum, when half the medium was changed each day, the doubling time of the cell lines was similar at about 14 h (data not shown, but presented in part in ref. 2). The maximum number of cells attainable (saturation density) under these conditions was slightly higher for the tumor cell lines than for their parent, although one of the parent clones itself (216C) had a high saturation density (Table I). Colony formation in suspension in viscous medium was low for the various cell lines of Table I and, therefore, in these cells did not correlate with tumorigenicity.

Comparison of Glycoconjugates Produced by Tumor Cell Lines and Their Parent Clones—Glucosamine-labeled glycoconjugates from the three culture compartments (medium, trypsin, and trypsinized cells) were analyzed by anion exchange chromatography using two isotopes to allow simultaneous analysis of paired cell lines eliminating the introduction of artifacts by the procedures involved. The peaks from the column have been characterized previously (2) as follows: hyaluronic acid, heparan sulfate, and chondroitin sulfate eluted at approximately fraction 45, 60, and 70 respectively, and glycopeptides eluted before fraction 40.

Analysis of the glycoconjugates produced by the tumor cell lines showed that, like the two SV40-transformed daughter clones (2), they had similar alterations in the elution properties of heparan sulfate. In both the trypsin and the medium heparan sulfate from 219CT eluted from the column at lower salt concentrations than that from the parent clone 210C (Fig. 2, *b* and *c*). Very little, if any material eluting at the position of heparan sulfate was found in the cell compartment of 219CT (Fig. 2*a*). The same altered elution profiles were found for the independently obtained tumor cell line 220CT when it was compared to 216C, the clone from which it was derived;

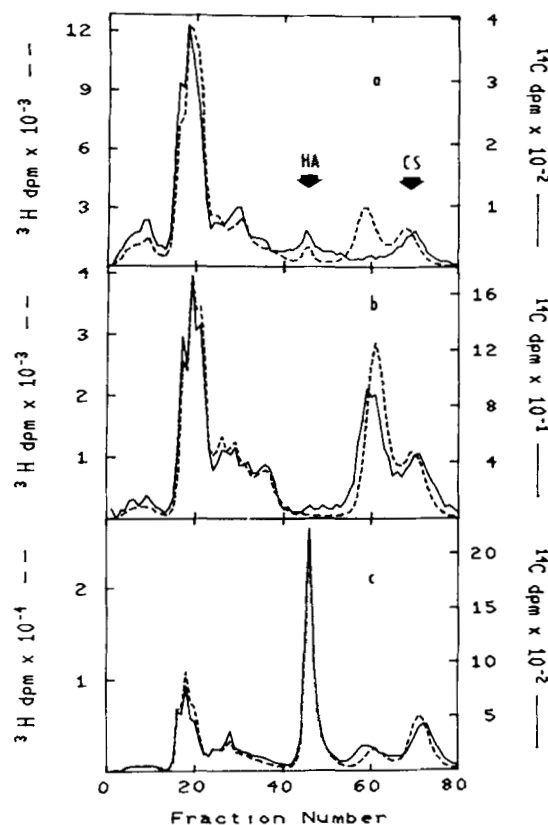


FIG. 2. Anion exchange profiles of glycoconjugates produced by 210C and 219CT cell lines. Nondialyzable papain-digested glycoconjugates were applied to a DEAE-cellulose column (4×1 cm) in 10 mM Tris-HCl, pH 8.4. A 300-ml linear gradient of 0–0.6 M NaCl was superimposed, starting at fraction 14. Aliquots of 1 ml were taken from the 4-ml fractions for scintillation counting. Compartments from 210C labeled with [3 H]glucosamine (---) had been mixed at the time of harvest with corresponding compartments from 219CT labeled with [14 C]glucosamine (—). The three graphs show elution profiles of: *a*, the cell; *b*, the trypsin; *c*, the medium compartments. Elution positions of standards of hyaluronic acid (HA) and chondroitin sulfate A (CS) run under identical conditions on a separate column are shown. Heparan sulfate was the peak eluting between these two markers at about fraction 60.

however, neither of these cell lines had much material eluting at the position of heparan sulfate in the cell compartments. The different elution properties of heparan sulfates for this pair was clearly seen when material isolated from the papain-digested medium by ion exchange was digested by chondroitinase ABC to remove any contaminating hyaluronic acid or chondroitin sulfate and rerun on an anion exchange column using a shallower salt gradient (Fig. 3).

Chondroitin sulfate from both tumor cell lines consistently eluted from the columns at slightly higher salt concentrations than the material from the parent clones, as illustrated in Fig. 2 for 219CT compared with 210C. This was in contrast to the results with two SV40-transformed cell lines where a tendency for chondroitin sulfate to elute earlier was observed (2). Glycoconjugates produced by a reclone of the parent clone were unaltered (2), showing that such changes as described above are not always obtained when randomly selecting subpopulations from the parent clones.

For a detailed study of the structure of the heparan sulfate chains, one SV40-transformed subclone (215CSC) and one tumor cell line (219CT) were chosen for comparison with their common parent 210C. For the results described below five doubly labeled samples of heparan sulfate from the papain-digested trypsinates were isolated by anion exchange and

TABLE I
Growth characteristics of the cell lines

The maximum densities (saturation densities) observed in cell culture were obtained under conditions in which half the medium was changed daily. Plating efficiency in Methocel is expressed as the percentage of inoculated cells able to grow into colonies of 0.1 mm or greater. Tumorigenicity was expressed as the TD_{50} , the cell number at which 50% of the animals had tumors (15). The latency periods, shown here, are the times required for tumors to appear in 50% of the animals receiving an inoculum of cells one log above the TD_{50} . All tumors were progressively growing lethal fibrosarcomas.

Cell line	Saturation density	Methocel plating efficiency	Tumorigenicity	Latency period
	10^5 cells/cm ²	%	TD_{50}	days
210C ^a	1.9	0.2	10^6	78
215CSC ^a	3.5	1.4	10^6	50
219CT	4.8	<0.001	< 10^2	10
216C	4.0	0.2	10^5	73
220CT	5.2	<0.001	10^2	33

^a Data has been presented already (2); it is included here for comparative purposes.

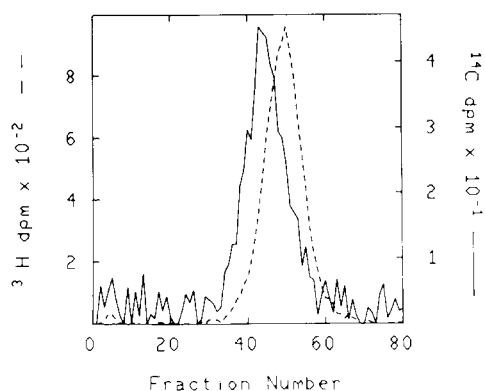


FIG. 3. Anion exchange profile of heparan sulfate purified from the papain-digested medium of cell lines 216C (---) and 220CT (—). Heparan sulfate isolated from the medium compartment by anion exchange chromatography was digested with chondroitinase ABC to remove contaminating glycosaminoglycans, dialyzed against 0.25 M NaCl, 10 mM Tris-HCl, pH 8.4, and reappplied to a column of DEAE-cellulose equilibrated with the same buffer. A 150-ml linear gradient of 0.25–0.5 M NaCl was applied from fraction 20. Aliquots of 1 ml were taken from the 2-ml fractions for scintillation counting.

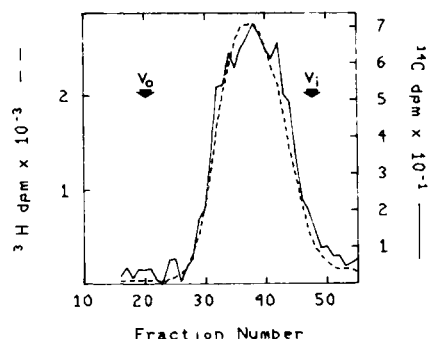


FIG. 4. Exclusion gel chromatography of heparan sulfate from the cell surface of 210C and 219CT. Heparan sulfate released from the surface of glucosamine-labeled cells was isolated after papain digestion by anion exchange chromatography and further purified by chondroitinase ABC digestion. The sample, to which was added blue dextran and sodium azide as markers for the void volume (V_0) and the totally included volume (V_t), respectively, was analyzed by chromatography on a column (100×1.1 cm) of Ultrogel AcA 22 in 0.5 M sodium acetate, pH 7, eluted at $10 \text{ ml h}^{-1} \text{ cm}^{-2}$. Aliquots of 1 ml were counted from 2-ml fractions. ---, heparan sulfate from 210C; —, heparan sulfate from 219CT.

purified by chondroitinase ABC digestion. In two of the samples, [^3H]glucosamine-labeled material from 210C was being compared with [^{14}C]glucosamine-labeled material from 215CSC and 219CT, respectively. The other three samples were obtained from each cell line doubly labeled with [^3H]glucosamine and [^{35}S]sulfate.

Molecular Size and Degree of Sulfation of Heparan Sulfate—The possibility that these altered DEAE-cellulose binding properties were due to reduced size of heparan sulfate chains was investigated by exclusion gel chromatography. No difference could be detected between the heparan sulfates from 210C, 215CSC, or 219CT. All three had K_{av} values of 0.6 on an Ultrogel AA-22 column. The elution profile for heparan sulfate from the cell surface of 210C and 219CT is shown in Fig. 4; identical results were obtained with heparan sulfate from 215CSC. This confirms the lack of size difference previously reported for 210C and 215CSC in SV40 transformation (2) and extends it to the spontaneously transformed 219CT tumor cell line.

The degree of sulfation of glycosaminoglycans can be esti-

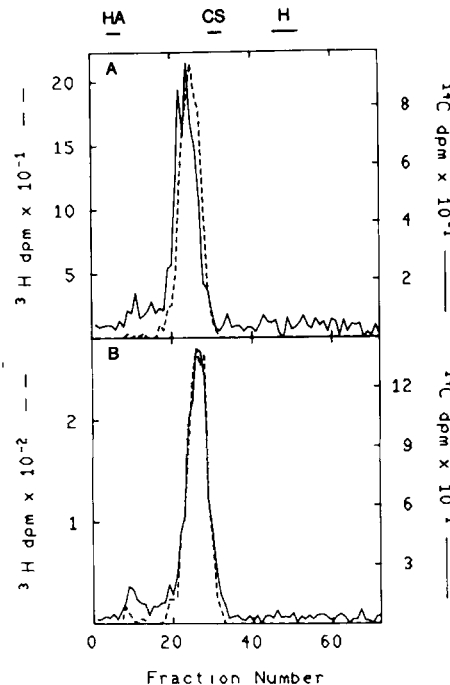


FIG. 5. Cellulose acetate electrophoresis of cell surface heparan sulfate at pH 1.0. Heparan sulfate released from the surface of glucosamine-labeled cells by trypsin was isolated by anion exchange chromatography and purified by chondroitinase ABC digestion. Samples were subjected to electrophoresis at 2.5 V/cm in 0.1 M HCl on cooled strips of cellulose acetate. After electrophoresis, the dried strips were cut into 1-mm fractions with the origin at fraction 10, and radioactivity was eluted with 1 ml of distilled water for 1 h in scintillation vials before addition of scintillation fluid and counting. Standards of hyaluronic acid (HA), chondroitin 4-sulfate (CS), and heparin (H) were run on the same cellulose acetate strip, and detected by Alcian blue staining. A, heparan sulfate from 210C (---) and 215CSC (—). B, heparan sulfate from 210C (---) and 219CT (—).

mated by cellulose acetate electrophoresis at pH 1.0 when only the sulfate groups are ionized (10). Heparan sulfate from the surface of the three cell lines was found to migrate less rapidly than chondroitin 4-sulfate, showing that it had on average under one sulfate group/disaccharide (Fig. 5).

Heparan sulfate from 215CSC migrated slightly less rapidly than from the parent clone 210C, indicating that after SV40 transformation there was a small overall reduction in the degree of sulfation (Fig. 5A). In contrast, material from the tumor cell line (219CT) had the same electrophoretic mobility as that from the parent, indicating that the overall degree of sulfation was unaltered (Fig. 5B). This result suggests that the altered anion exchange properties of heparan sulfate from the tumor cell line are due to alterations in the distribution of sulfate groups between the three possible positions: *N*-sulfate and 6-*O*-sulfate on the glucosamine residue and 2-*O*-sulfate on iduronic acid. That such alterations are detected by anion exchange and not by electrophoresis is presumably because the fractionation obtained by DEAE-cellulose chromatography at high pH depends on the position of the charged groups as well as the overall charge of the glycosaminoglycans. This is supported by the finding that a heparin standard with more than two sulfate groups/hexosamine residue elutes at approximately the same salt concentration as chondroitin sulfate under the conditions of ion exchange chromatography employed.² It is possible, therefore, that investigations involving ion exchange under different conditions would not detect such changes as reported here.

² Unpublished observations.

Number and Distribution of *N*-Sulfate Groups—*N*-sulfate groups are sensitive to attack by nitrous acid, and recently a detailed investigation of the reaction demonstrated conditions which resulted in quantitative release of the sulfate group with deaminative chain cleavage (7). Under these conditions a side reaction resulting in deaminative ring contraction without chain cleavage was found to occur at only about 7% of the *N*-sulfate groups of heparin (13).

High resolution exclusion gel chromatography of the nitrous acid degradation products of cell surface heparan sulfate gave information concerning the frequency and number of *N*-sulfate residues (Fig. 6). The slightly larger amount of radioactivity which was consistently found in heparan sulfate from 215CSC compared to that from 210C demonstrated a greater number of adjacent *N*-acetylated glucosamine residues (which are resistant to nitrous acid cleavage) in the 215CSC heparan sulfate and, therefore, a reduction in either the distribution frequency or number of *N*-sulfate residues (Fig. 6A). Data for the tumor cell line (219CT) indicated either no difference or a very slight increase in frequency or number of *N*-sulfate residues (Fig. 6B).

As described under "Materials and Methods," the percentage of glucosamine residues in the undegraded heparan sulfate which were *N*-sulfated can be calculated from the elution profile, provided the number of disaccharide units in the oligosaccharides of each peak is known. The five or six smallest sized oligosaccharides were clearly separated on the Bio-Gel column and their size was determined by their elution

relative to oligosaccharides obtained by testicular hyaluronidase degradation of hyaluronic acid. The remaining radioactivity which was not so well resolved appeared to correspond to oligosaccharides containing 7 to 9 or 10 disaccharide units. The exact designation of oligosaccharide size for the higher molecular weight oligosaccharide is not critical as the formula used to calculate the number of *N*-sulfate residues is heavily weighted for radioactivity in the lower molecular weight fractions. Results of these calculations are shown in Table II. Approximately one-half of the glucosamine residues were *N*-sulfated and only small differences between the various cell lines were found.

These heparan sulfates were not significantly cleaved by nitrous acid under conditions which only cleave at unsubstituted glucosamine residues (9) (results not shown) and, therefore, it is probable that the residues which are not *N*-sulfated are mostly *N*-acetylated, ruling out the possibility that the altered anion exchange properties in tumor or SV40-transformed cells was due to increased amounts of free amino groups.

Number and Distribution of *O*-Sulfate Groups—When heparan sulfate from a single cell line doubly labeled with [^3H]-glucosamine and [^{35}S]-sulfate was degraded with nitrous acid, it was evident that very little [^{35}S]-sulfate was associated with high molecular weight oligosaccharides (Fig. 7; Table III). Free sulfate released from *N*-sulfate residues by nitrous acid migrated in the totally included volume of the column together with the *O*-sulfated disaccharides. The amount of free [^{35}S]-sulfate was determined by high voltage paper electrophoresis and this value was used to determine the percentage of

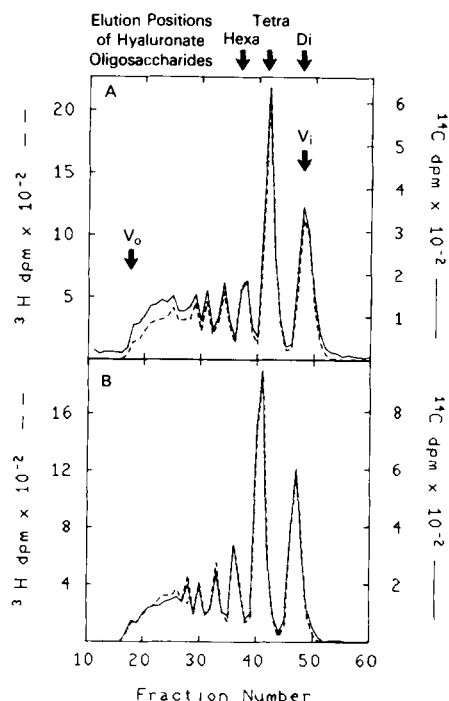


FIG. 6. Exclusion gel chromatography of nitrous acid-degraded glucosamine-labeled heparan sulfate. Purified cell surface heparan sulfate was degraded by nitrous acid and the resulting oligosaccharides were analyzed by elution through a column (50×1 cm) of Bio-Gel P10-400 in 0.5 M ammonium bicarbonate at a flow rate of $8 \text{ ml h}^{-1} \text{ cm}^{-2}$. Fractions of 0.75 ml were collected in scintillation vials and analyzed for radioactivity. The void volume (V_0) was measured with bovine serum albumin and the totally included volume (V_i) was measured with sodium azide or sodium [^{35}S]-sulfate. The column was calibrated with oligosaccharides produced by testicular hyaluronidase degradation of hyaluronic acid. A, nitrous acid-degraded heparan sulfate from 210C (---) and 215CSC (—); B, nitrous acid-degraded heparan sulfate from 210C (---) and 219CT (—).

TABLE II

*Degree of *N*-sulfation of cell surface heparan sulfate*

Heparan sulfate released from the surface of glucosamine-labeled cells by trypsin was isolated by anion exchange chromatography and purified by digestion with chondroitinase ABC before degrading the material with nitrous acid and fractionating the oligosaccharides so produced by exclusion gel chromatography on a column of Bio-Gel P10-400. The percentage of glucosamine residues that were converted to anhydromannose at the reducing end of the oligosaccharides—and, hence, were *N*-sulfated in the intact heparan sulfate—were calculated as described under "Materials and Methods." Three preparations of heparan sulfate from 210C and two preparations for 215CSC and 219CT were analyzed.

Cell line	<i>N</i> -sulfated glucosamine residues %
210C	47.3 ± 0.7 (6) ^a
215CSC	46.5 ± 0.8 (4)
219CT	49.9 ± 1.0 (4)

^a Mean \pm standard deviation (number of experiments shown in parentheses).

TABLE III

Distribution of [^{35}S]-sulfate radioactivity after nitrous acid degradation of heparan sulfate

Purified heparan sulfate released by trypsin from the surface of cells labeled with [^3H]-glucosamine and [^{35}S]-sulfate was degraded by nitrous acid. ^{35}S radioactivity released from *N*-sulfated glucosamine residues as inorganic sulfate was determined by high voltage paper electrophoresis and ester sulfate in various oligosaccharides by Bio-Gel P10 chromatography, as shown in Fig. 7. The percentage of [^{35}S]-sulfated disaccharides was determined by subtracting the contribution of inorganic [^{35}S]-sulfate from the total ^{35}S radioactivity present in the totally included peak.

Cell line	N-sulfate	O-sulfated oligosaccharides			
		Di-	Tetra-	Hexa-	Higher oligo-
% total ³⁵ S radioactivity					
210C	54	32	10	1.0	3
215CSC	59	32	6	0.6	2
219CT	59	32	6	0.6	1

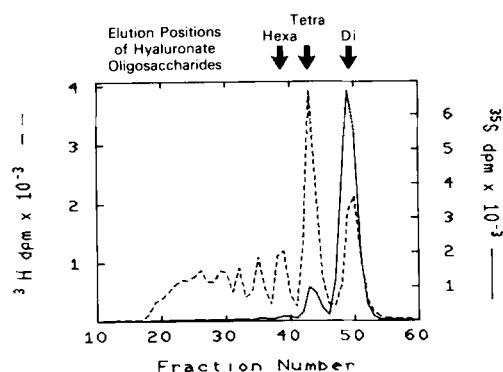


FIG. 7. Exclusion gel chromatography of nitrous acid-degraded heparan sulfate from 210C cells labeled with [^3H]glucosamine and [^{35}S]sulfate. Heparan sulfate released by trypsin from the surface of 210C labeled simultaneously with [^3H]glucosamine and [^{35}S]sulfate was isolated by anion exchange chromatography and purified by chondroitinase ABC digestion before nitrous acid degradation. The resulting oligosaccharides were analyzed by exclusion gel chromatography as described in Fig. 6.

^{35}S radioactivity present as *O*-sulfated disaccharides in the various oligosaccharides (Table III). From these results it is apparent that both the virus-transformed subclone and the tumor cell line have fewer sulfate groups *O*-substituted on oligosaccharides higher than disaccharides, compared to the parent clone. As the proportion of [^{35}S]sulfate and [^3H]glucosamine present as *N*-substituted residues is known, the distribution and frequency of *O*- and *N*-sulfate groups in the heparan sulfate chain can be determined (Table IV). As anhydromannose is at the end of each oligosaccharide, the number of oligosaccharides produced by nitrous acid cleavage of 100 disaccharide units of heparan sulfate is equal to the number of anhydromannose residues (Table IV). On average just over one *O*-sulfate residue/molecule was present in the disaccharide peak of the nitrous acid degradation products, indicating that in this region of the heparan sulfate chain all of the disaccharide units may have contained two sulfate groups and a few may have been maximally substituted with three sulfate groups. In contrast oligosaccharides larger than disaccharides produced by nitrous acid degradation had insufficient ^{35}S radioactivity to allow for an average of even one *O*-sulfate group/oligosaccharide molecule, resulting in an average density of less than one sulfate residue/disaccharide unit in this region of the heparan sulfate. It was in this region of the chain that the greatest difference between the heparan sulfate of the tumor- and virus-transformed cells on the one hand, and the parent clone on the other, was detected (Table IV).

The results presented in Table IV indicated that individual oligosaccharide peaks obtained by fractionation according to size after nitrous acid degradation were composed of a variety of molecules differing in the presence or absence of *O*-sulfate residues. It is also to be expected that the molecules bearing *O*-sulfate groups may differ in the substitution position, being either on the C-2 of iduronic acid or C-6 of glucosamine.

Confirmation of these conclusions was obtained by chromatography of individual oligosaccharide peaks obtained by exclusion chromatography of nitrous acid-degraded heparan sulfate on a calibrated ion exchange column. Forty-two per cent of the glucosamine label from tetrasaccharides isolated from nitrous acid-degraded heparan sulfate from 210C eluted in two peaks with ^{35}S : ^3H ratios³ equivalent to monosulfated

³ The ^{35}S : ^3H ratio of the tetrasaccharide peak was approximately half the ratio found in both $\Delta\text{Di-4S}$ produced by chondroitinase ABC digestion of chondroitin sulfate from the same preparation of cell

TABLE IV

Frequency and distribution of various residues in oligosaccharides obtained by nitrous acid degradation of heparan sulfate

Purified cell surface heparan sulfate labeled with [^3H]glucosamine and [^{35}S]sulfate was degraded with nitrous acid and analyzed by a combination of exclusion gel chromatography and high voltage paper electrophoresis. The distribution of various residues among the different sized oligosaccharides (i.e. as a function of the frequency of *N*-sulfate groups) was calculated as follows, all values being normalized to an original chain length of 100 disaccharide units. The number of anhydromannose (AHM) residues (derived from *N*-sulfated glucosamine residues) was determined as the percentage of ^3H radioactivity in the oligosaccharide peak (equivalent to the percentage of disaccharide units from the undegraded heparan sulfate in that size of oligosaccharide) divided by the number of disaccharide units in the oligosaccharide; the remaining ^3H radioactivity gave the number of glucosamine residues. The total number of sulfate residues/100 disaccharide units was determined from the percentage of ^3H activity present as anhydromannose and the percentage of ^{35}S radioactivity as inorganic sulfate after nitrous acid treatment, from this value the number of *O*-sulfate residues was calculated from the data in Table III. For example, the calculation of the distribution of sulfate residues in 210C heparan sulfate was as follows: there were 47 AHM residues/100 disaccharides, and therefore 47 *N*-sulfate residues. As 54% of the ^{35}S radioactivity was originally present as *N*-sulfate, the total number of sulfate residues/100 disaccharides was $47 \div 0.54 = 87$. Therefore, the number of *O*-sulfate residues in the disaccharide peak, which contained 32% of the ^{35}S radioactivity, was $87 \times 0.32 = 28$. Similar calculations were applied to other peaks.

Oligosaccharide fraction	Residue	Residue frequency ^a		
		210C	215CSC	219CT
Disaccharide	GlcNH ₂	0	0	0
	AHM	22	23	25
	<i>O</i> -SO ₄	28	25	27
Tetrasaccharide	GlcNH ₂	14	14	16
	AHM	15	13	16
	<i>O</i> -SO ₄	9	5	5
Hexasaccharide	GlcNH ₂	7	6	7
	AHM	3	3	3
	<i>O</i> -SO ₄	1.0	0.5	0.5
Higher oligosaccharides	GlcNH ₂	30	33	27
	AHM	7	7	6
	<i>O</i> -SO ₄	3	1.6	1.0

^a Measured in numbers of residues/100 disaccharide units of undegraded heparan sulfate.

tetrasaccharides, 32% of the glucosamine label eluted at a similar position to the hyaluronate tetrasaccharide; this heparan sulfate tetrasaccharide was not labeled by [^{35}S]sulfate. The remainder of the radioactivity eluted at a position corresponding to unsulfated hexasaccharide and as material which was more strongly bound to the column than the monosulfated tetrasaccharides and also had a higher ^{35}S : ^3H ratio. For 215CSC the percentage of glucosamine label as putative monosulfated tetrasaccharides and unsulfated tetrasaccharide was 24 and 46%, respectively, and for 219CT it was 29 and 40%, respectively. These results on the distribution of glucosamine label (a measure of the carbohydrate chain) confirm those inferred from the distribution of [^{35}S]sulfate groups (Tables III and IV).

As the tetrasaccharide peak is the major product of nitrous acid-degraded heparan sulfate it should be possible to detect the differences outlined above by ion exchange chromatography of an unfractionated nitrous acid-degraded heparan sulfate mixture from the paired cell lines. The results in Fig. 8 where peaks 5 and 7 (the two putative monosulfated tetrasaccharide peaks) were reduced in both 215CSC (Fig. 8A) and surface material and the monosulfated monosaccharides released by heparinase degradation of the heparan sulfate (Fig. 8).

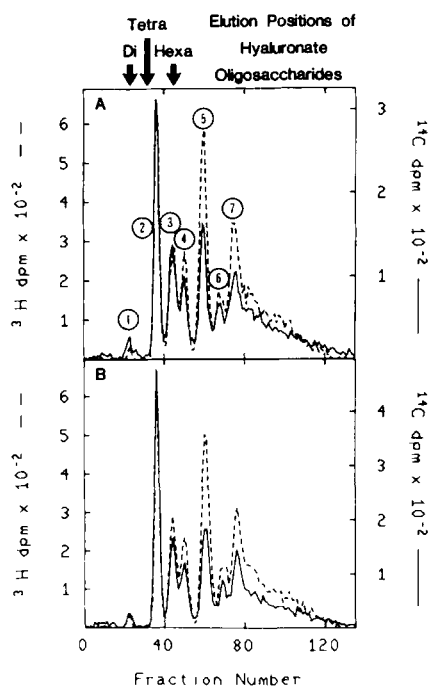


FIG. 8. Anion exchange chromatography of nitrous acid-degraded heparan sulfate. Purified heparan sulfate released from the surfaces of glucosamine-labeled cells by trypsin was degraded by nitrous acid and the resulting oligosaccharides were analyzed by anion exchange chromatography on a column (4×1 cm) of DEAE-cellulose equilibrated with 10 mM Tris-HCl, pH 8.4. A 125-ml gradient of 0–0.25 M NaCl was superimposed on this buffer starting at fraction 11. The elution positions of di-, tetra-, and hexasaccharides isolated from testicular hyaluronidase-degraded hyaluronic acid by exclusion gel chromatography and run on DEAE-cellulose columns under identical conditions is shown; these three oligosaccharides were clearly separated from each other. Monosulfated disaccharides from chondroitin 4-sulfate eluted at fraction 43. By reference to the elution positions of these standards and the ^{35}S : ^3H ratio of oligosaccharides produced by nitrous acid degradation of heparan sulfate labeled with [^{35}S]sulfate and [^3H]glucosamine and separated according to size on a Bio-Gel P10 column before anion exchange chromatography, the numbered peaks are thought to correspond with: 1, 2, 4, and 6, unsulfated di-, tetra-, hexa-, and octasaccharides; 3, monosulfated disaccharides; 5 and 7 (in part), in monosulfated tetrasaccharides. The elution profiles were normalized on the unsulfated tetrasaccharide peak (2). A, nitrous acid-degraded heparan sulfate from 210C (---) and 215CSC (—). B, nitrous acid-degraded heparan sulfate from 210C (---) and 219CT (—).

219CT (Fig. 8B) as compared to the parent clone 210C confirmed this. Putative designations of the other peaks in the ion exchange profile are given in the figure legend; however, more work is required to define these peaks conclusively. In agreement with the data in Table IV only small amounts of the unsulfated disaccharide (peak 1) were detected in contrast to the significant quantities of unsulfated tetra-, hexa- and octasaccharides (Fig. 7, peaks 2, 4, and 6, respectively). Similar low levels of unsulfated disaccharides were found by other workers (14) and presumably are characteristic of the biosynthesis of these molecules.

Distribution of 6- and 2-O-Sulfate Groups—The principal difference between the heparan sulfate from the tumor- and the virus-transformed cells and the parent clone was in the O-sulfate groups. It has been shown that a crude preparation from *Flavobacterium heparinum* induced to grow on heparin is capable of degrading heparin and heparan sulfate to monosaccharides (11). Under the conditions used here this preparation demonstrated only low activities of sulfatases capable of releasing N- and 6-O-sulfates from glucosamine residues²;

and it degraded heparin to yield three spots by paper chromatography with mobilities which corresponded with those previously characterized as 6-O-sulfo-2-sulfoamino-2-deoxy-D-glucose (the major spot), 2-sulfoamino-2-deoxy-D-glucose, and an α -keto acid (11). The crude heparinase was used to digest heparan sulfates doubly labeled with [^3H]glucosamine and [^{35}S]sulfate in the presence of carrier heparin. Under these conditions complete depolymerization was obtained as more than 90% of the glucosamine label migrated as monosaccharides on a Bio-Gel P2 column (results not shown). The degradation products were analyzed by paper chromatography to determine the amount of 6-O-sulfation of the glucosamine residues and the results in Fig. 9 show four glucosamine-labeled peaks in addition to a small amount of ^3H label at the origin. Peaks 2 and 3 had the same R_f values as spots released from heparin and corresponding to 6-O-sulfo-2-sulfoamino-2-deoxy-D-glucose and 2-sulfoamino-2-deoxy-D-glucose, respectively (11). Peak 5 had the same R_f value as N-acetylglucosamine which was shown to be liberated from heparan sulfate (11). The ^{35}S : ^3H ratio of peaks 2, 3, and 5 were consistent with these designations. The fourth glucosamine-labeled peak had the same ^{35}S : ^3H ratio as 2-sulfoamino-2-deoxy-D-glucose and

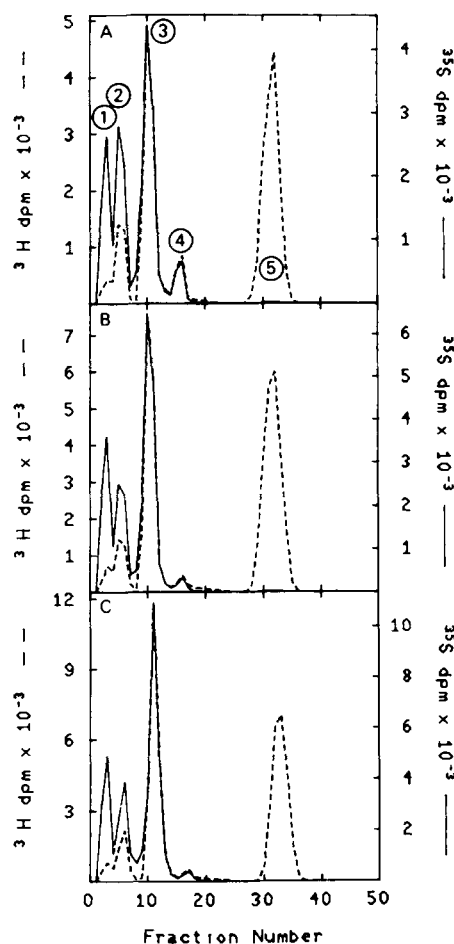


FIG. 9. Paper chromatography of heparinase-degraded heparan sulfate. Purified heparan sulfate from the surface of cells labeled with [^3H]glucosamine and [^{35}S]sulfate was degraded with crude heparinase in the presence of carrier heparin. The products were analyzed by descending paper chromatography in butan-1-ol:acetic acid:water (10:3:5). In this system inorganic sulfate remained mostly at the origin (peak 1), the other peaks were characterized as: peak 2, 6-O-sulfo-2-sulfoamino-2-deoxy-D-glucose; peak 3, 2-sulfoamino-2-deoxy-D-glucose; peak 4, 6-O-sulfo-2-acetamido-2-deoxy-D-glucose; and peak 5, N-acetylglucosamine. A, heparan sulfate from 210C. B, heparan sulfate from 215CSC. C, heparan sulfate from 219CT.

one-half that of 6-*O*-sulfo-2-sulfoamino-2-deoxy-D-glucose and was, therefore, a monosulfated glucosamine derivative most likely 6-*O*-sulfo-2-acetamido-2-deoxy-D-glucose. In this experiment derivatives of the uronic acid residues were not detected as they were not labeled; however, the [³⁵S]sulfate originally present at position 2 of iduronic acid was released as free sulfate the majority of which remains at the origin in this solvent system and is seen as peak 1 in Fig. 9.

Assuming these designations are correct it is possible to calculate directly the number of *N*-sulfate and 6-*O*-sulfate residues/glucosamine residue and, by difference, the number of 2-*O*-sulfate groups on iduronic acid residues. The major difference between the heparan sulfate from the tumor and SV40-transformed cell lines as compared with the parent clone was a reduction by about one-third in the number of 6-*O*-sulfate groups/chain (Table V). The number of 2-*O*-sulfate groups on iduronic acid residues were similar for all three cell lines (Table V). Results for the number of *N*-sulfate residues determined by this procedure (Table V) agreed well with values obtained by nitrous acid degradation followed by exclusion chromatography (Table II) and support evidence for a slight increase in *N*-sulfation in the heparan sulfate for 219CT which might compensate for the reduced 6-*O*-sulfation, explaining the similarity in overall degree of sulfation of heparan sulfate from 219CT and 210C (Fig. 5).

Position of Sulfate Groups in Chondroitin Sulfate—It was repeatedly found that chondroitin sulfate from both tumor cell lines eluted at a slightly higher salt concentration than

that from the parent clones; in contrast the chondroitin sulfate from SV40-transformed cell eluted at nearly the same or a slightly lower salt concentration than the parent clone (2). To investigate these differences, the structure of chondroitin sulfate peaks was determined by chondroitinase ABC digestion followed by paper chromatography. Chondroitin sulfate from both the tumor and the SV40-transformed cell line had relatively less chondroitin-6-sulfate (Table VI). In the tumor cell line there also appeared to be a slight increase in the amount of the disulfated disaccharide units, this result being clearly seen in the chromatographic profile of a doubly labeled sample of chondroitinase ABC digested sulfate from 210C and 219CT (results not shown). Similar chromatographic profiles of 210C and 215CSC showed no difference in the relative amount of radioactivity in the disulfated disaccharide peaks.

DISCUSSION

Sulfated glycosaminoglycans, particularly heparan sulfate which is thought to be enriched at the cell surface, have been suggested to play an important role in phenomena such as cell-cell and cell-substrate adhesion (16–19), growth control (20–22), masking of cell surface receptors (23), and modulation of cation levels, *e.g.* Ca²⁺ (24). Many of these phenomena are thought to be altered after neoplastic transformation, and it is relevant therefore that changes in glycosaminoglycan metabolism in control and transformed cells have been reported (reviewed in Ref. 25). The majority of these reports concern the amounts and proportions of various glycosaminoglycan classes labeled with radioactive precursors. Few details of the structure of heparan sulfate have been reported, despite evidence that it is the sequence of residues in such molecules which determines biological activity (26, 27).

Alterations in the anion exchange elution profile of heparan sulfate have been found consistently after SV40 transformation (2–4) and we have found similar changes after *in vivo* selection of highly tumorigenic variants from cloned cell lines (communicated in preliminary form in Ref. 28). The detailed structural analysis of cell surface heparan sulfate reported in this paper indicated that these alterations in ion exchange profiles were due to a decrease by about one-third in the degree of 6-*O*-sulfation of the glucosamine residues in the glycosaminoglycan in both types of transformed cells. The structures of heparan sulfate from the SV40-transformed derivative cell line (215CSC) and from the highly tumorigenic variant cell line (219CT) obtained from the same parent clone (210C) were not identical, however, as, although they had similar quantitative reductions in 6-*O*-sulfation, this was partially compensated for by an increase in *N*-sulfation in heparan sulfate from 219CT.

In the structural analysis of heparin and heparan sulfate, the most useful tool has been the specific degradation of *N*-sulfated glycosaminoglycans by nitrous acid (7, 9, 13, 14, 26, 27, 29). This reaction is important because, as well as allowing determination of the relative amounts of *N*- and *O*-sulfate groups and the percentage of glucosamine residues which were *N*-sulfated, it also provides information concerning the relative location of various residues in the heparan sulfate chain. Such sequence information is obtained from detailed analysis of the oligosaccharides produced after nitrous acid treatment and depends on quantitative chain cleavage at the *N*-sulfated glucosamine residues. Recently, a detailed study of the effects of pH on this reaction has shown that some commonly used conditions are inadequate for such an analysis because a substantial proportion of the *N*-sulfated glucosamine residues of carboxyl-reduced heparin undergo deaminative ring contraction which does not result in chain cleavage (7). Another common procedure, employed to determine the ratio of *N*- to

TABLE V

Number and distribution of sulfate residues in heparan sulfate

Purified cell surface heparan sulfate was digested with a crude heparinase preparation as shown in Fig. 9. The number of *N*-sulfate and 6-*O*-sulfate groups/100 disaccharide units was determined directly from the percentages of ³H radioactivity present in peaks 2 and 3 and in peaks 2 and 4, respectively. The number of 2-*O*-sulfate groups/100 disaccharide units was calculated by subtracting these two values from the total number of sulfate groups/100 disaccharide units determined as described in Table IV.

Residue	210C	215CSC	219CT
<i>no. residues/100 disaccharide units</i>			
<i>N</i> -sulfate	44.5	44.0	50.1
6- <i>O</i> -sulfate	16.6	11.1	11.2
2- <i>O</i> -sulfate	25.7	23.6	22.7
Total sulfate	86.8	78.7	83.9

TABLE VI

Distribution of sulfate groups in the cell surface chondroitin sulfate

Chondroitin sulfate released by trypsin from the surface of cells labeled with glucosamine was isolated by ion exchange chromatography. The material was digested with chondroitinase ABC and subjected to descending paper chromatography in butan-1-ol/acetic acid/1 M ammonium hydroxide (2:3:1). The percentage distribution of radioactivity among the various unsaturated disaccharides was determined. About 12% of the radioactivity in each sample remained at the origin and was not susceptible to chondroitinase ABC. The results are shown as the mean ± standard deviation of three separate preparations of chondroitin sulfate for 210C and two for 215CSC and 219CT.

Disaccharide	Distribution in chondroitin sulfate		
	210C	215CSC	219CT
% radioactivity			
ΔDi-DiS	3.3 ± 0.5	4.8 ± 0.2	6.5 ± 1.5
ΔDi-6S	14.8 ± 1.5	4.4 ± 1.9	6.2 ± 0.7
ΔDi-4S	76.0 ± 3.7	85.2 ± 0.6	82.1 ± 1.1
ΔDi-OS	5.9 ± 0.6	5.6 ± 1.3	5.2 ± 0.3

O-sulfates, has been to exploit the sensitivity of *N*-sulfate groups to mild acid hydrolysis. However, under the conditions normally used this reaction has been shown to release as much as 45% of the O-sulfate groups of chondroitin sulfate (30) and is, therefore, not appropriate for quantitative analysis of heparan sulfate structure.

In this report we degraded cell surface heparan sulfate with nitrous acid at low pH under conditions where the *N*-sulfate group is quantitatively released (7) and where ring contraction resulting in maintenance of the glycosidic link occurs at only about 7% of the *N*-sulfated glucosamine residues of carboxyl-reduced heparin (13). Values for the number of *N*-sulfated glucosamine residues determined by this method agreed well with those obtained by enzymatic degradation with a mixture of bacterial enzymes (see below) and showed that approximately half of the glucosamine residues of the heparan sulfate of all three cell lines were *N*-sulfated, with the tumor cell line consistently showing a slightly higher degree of *N*-sulfation than the parent and the SV40-transformed clones. The *N*-sulfated residues were not uniformly distributed but, in agreement with other studies (8, 29, 31, 32), were located in blocks separated by regions of repeating disaccharide units containing only *N*-acetylated glucosamine residues. This was indicated by the finding that more than two-thirds of the glucosamine residues were in oligosaccharides other than tetrasaccharides; if alternate glucosamine residues had been *N*-sulfated only tetrasaccharides would be obtained. A slightly less random distribution of *N*-sulfate groups was apparent in 215CSC as judged by the higher proportion of oligosaccharides larger than tetrasaccharides, when compared with heparan sulfate from 210C. The uneven distribution of charged groups in the heparan sulfate isolated from all three cell lines was even more pronounced for the O-sulfate groups which were found to be highly concentrated in the regions of contiguous *N*-sulfated glucosamine containing disaccharide units (Tables III and IV). This result is consistent with other studies (31, 33) and supports certain aspects of the proposed mechanism of biosynthesis of heparin-like glycosaminoglycans (34).

The major finding of this study, detected by the discrimination between O- and *N*-sulfate groups by nitrous acid degradation, was the common difference between the parent clone and both tumor- and SV40-transformed cell lines, showed by a reduction in the amount of O-sulfation in both derivative cell lines. In addition, the cleavage patterns showed that this reduction in O-sulfation was not in the densely sulfated regions of the chain but rather in those regions where sulfate groups were more sparse. Thus, heparan sulfate from both types of transformed derivative cell lines had only one-half as many O-sulfate groups compared with the parent clone in oligosaccharides larger than disaccharides released by nitrous acid cleavage, whereas in the disaccharide fraction, which contains most of the O-sulfate groups, similar numbers of O-sulfate groups were found in heparan sulfate from the three cell lines (Table IV). The reduced O-sulfation was confirmed by anion exchange chromatography of the nitrous acid degradation products (Fig. 8).

It has previously been shown that a crude enzyme preparation from *F. heparinum* is capable of degrading heparin or heparan sulfate to monosaccharides (11). This crude preparation contains a variety of enzymes: two (35) or three (36) eliminases which in combination are capable of degrading heparin and heparan sulfate to disaccharides, a sulfatase acting on sulfate groups at position 2 of unsaturated uronic acid residues, and a glucuronidase which liberates the variously sulfated glucosamine residues (37). Two sulfatases acting on the 6-O-sulfated and *N*-sulfated glucosamine, respectively, complete the degradation to free glucosamine (37).

Under the conditions used in this study the latter two sulfatases were inactive, as judged by the absence of detectable levels of free glucosamine in the digestion products of heparin or heparan sulfate. Thus, this enzyme preparation has provided a method for discriminating between the two positions of O-sulfation in heparan sulfate. The results indicate a specific reduction of about 30% in the total amount of 6-O-sulfation, with apparently little change in amounts of 2-O-sulfation of iduronic acid residues in the heparan sulfate of the two types of derivative cell lines. As this reduction occurred mostly in regions of the chains containing relatively small numbers of sulfate groups (see above), it is clear that certain sequences of charged groups present in heparan sulfate from 210C will be found only rarely in heparan sulfate from both 215CSC and 219CT. This observation is relevant because the sequence of the various possible disaccharide units in such glycosaminoglycans is a decisive factor in determining their biological activity (26, 27).

The above data on tissue-cultured cells on the detailed distribution of sulfate groups in heparan sulfate are similar to data on gross distribution of sulfate groups in heparan sulfate from various tissues, e.g. by-products of industrial production of heparin from bovine lung (29, 33, 38, 39) and heparan sulfate isolated from liver (8, 38) and from brain (40). It differs from heparan sulfate isolated from aorta (31, 38) and umbilical cord (41), both of which had low levels of total sulfate.⁴

It has previously been reported that heparan sulfate from SV3T3 had a reduced overall degree of sulfation compared to heparan sulfate from 3T3 as determined by cellulose acetate electrophoresis at pH 1 (42). Our results indicate a gross reduction in sulfation is also found after SV40 transformation of 210C; however, it should be noted that such a change was not apparent in cells selected from 210C on the basis of high tumorigenicity (Fig. 5). In a recent paper, which appeared during the preparation of our manuscript, these authors presented the results of the analysis by ion exchange and exclusion chromatography of the nitrous acid degradation products of heparan sulfate from 3T3 and SV3T3 (43). Our results confirm and extend their conclusion that the reduced sulfation after SV40 transformation occurs predominantly in the O-sulfate groups of heparan sulfate. These authors showed an altered distribution of O- and *N*-sulfate groups and calculated that SV3T3 had 8% less sulfate groups as O-sulfates compared to the distribution of sulfate groups in 3T3 (43). Our results support this altered distribution, although the extent is somewhat less, 5% fewer sulfate groups by proportion at the O positions of heparan sulfate from 215CSC compared to 210C (Table III). However, such altered proportions are consistent not only with reduced absolute amounts of O-sulfation, but also with unchanged O-sulfation and increased *N*-sulfation. The ion exchange elution profiles of nitrous acid-degraded SV3T3 heparan sulfate indicated that the altered distribution of sulfate groups was in fact due to an absolute reduction in O-sulfate groups; however, the authors did not quantitate this reduction (43). In this report we determined, by two independent methods, the number of glucosamine residues which bore *N*-sulfate groups. Having established the link between [³⁵S]sulfate and [³H]glucosamine radioactivity, it was possible to estimate the number of sulfate residues at various positions in a given number of repeating disaccharide units of the heparan sulfate chain. The results indicated an absolute reduction of about 20% in the total number of O-sulfate residues

⁴ Iduronic acid represented from 35 to 50% of the total uronic acid (unpublished results) and is therefore similar to some values previously reported for heparan sulfate from liver (8), lung (33), and umbilical cord (41), although values below this range have been reported for liver and lung heparan sulfates (38).

in heparan sulfate from SV40-transformed and tumor cells and that this reduction is concentrated in the regions of the chain which carry relatively few *O*-sulfate groups (Table IV). In addition the results of heparinase degradation indicate that this reduction occurs mostly at only one of the two *O*-sulfate positions, the 6-*O*-sulfates (Table V).

It is probable that the correlation between this altered sulfation and SV40 transformation is close, as essentially the same results have been found by two independent groups working with different cell lines (Refs. 2-4, 43, and this paper). The specific reduction in 6-*O*-sulfation reported here was found both in SV40-transformed cells and in cells selected from a clone by their high tumorigenicity. The possibility that this change in heparan sulfate metabolism may also correlate well with neoplastic transformation is supported by the finding that an independently obtained second tumor cell line (220CT) selected *in vivo* from an independent clone (216C) also showed the altered anion exchange properties. The simplest explanation for the high tumorigenicity of the 219CT and 220CT lines is that spontaneously transformed variant (mutant?) cells appeared at a very low frequency from the corresponding clonal parents 210C and 216C, and these cells were then selected by the *in vivo* passage as tumor lines. It would seem unlikely that the specific biochemical change in heparan sulfate metabolism occurred by chance in both cases, independent from the observed heritable change in tumorigenicity. In studies of other tumor cells, it has been shown that heparan sulfate from an ascites hepatoma had a lower degree of sulfation than heparan sulfate from normal liver (44), and it has been suggested independently (8) that heparan sulfate from this hepatoma (45) had a lower degree of *O*-sulfation than that from normal liver (8).

The biochemical findings have to be correlated with the biological properties judiciously. It might be considered that the altered metabolism of heparan sulfate is related to the ability to grow to high cell densities, as SV40 transformants such as SV3T3 (3, 4, 43) are selected for this ability and the SV40-transformed cells studied here, although isolated without bias to their growth properties, were found to have high saturation densities (Table I). However, the altered anion exchange property was also observed in the tumor cell line 220CT compared to its parent 216C even though the cell lines have similar high saturation densities (Table I). It should be recalled that the cell growth properties in culture reported in Table I did not correlate with tumorigenicity (*cf.* also Ref. 46). This is particularly true for the plating efficiency in methocel which is generally considered to correlate more closely with tumorigenicity than other cell parameters and yet was very low for both tumor cell lines. In addition transformation by SV40 does not necessarily result in increased tumorigenicity in mouse cells (2, 46) as the SV40-coded T and tumor-specific transplantation antigen (47) on the cell surface (48) may cause recognition and rejection in the syngeneic mouse. However, after SV40 infection of nonpermissive cells, such as murine cells, in coincidence with the expression of T-antigens, there is invariably an increased synthesis of cellular DNA accompanied by increased activities of the appropriate enzymes (1). It is also commonly thought that the tumorigenic transformation is associated with some loss of control of DNA synthesis. Perhaps it is in this respect, over-riding the normal control of cellular DNA replication, that the common alteration in heparan sulfate metabolism, a putative element in cell growth control, may somehow be connected with the two different forms of transformation studied in this report, and we are pursuing these leads.

Analysis of the chondroitin sulfate, which also had altered anion exchange properties, most noticeably in the tumor cell

lines, showed that both derivative cell lines had substantial reductions in the amounts of the minor isomer, chondroitin 6-sulfate. The reasons for the difference in the ion exchange elution positions between the virally transformed and the tumor cell lines relative to the parent clone is not clear from the distribution of sulfate groups (Table VI), but conceivably could be due to differences in amounts of iduronic acid or in sequence of charged groups in copolymers of chondroitin sulfate.

The cause of these structural alterations in cell surface glycosaminoglycans could be due either to the presence of inhibitors or to reductions in specific sulfotransferases or in the levels of 3'-phosphoadenosine 5'-phosphosulfate. Recent evidence has suggested that the control of glycosaminoglycan sulfation is predominantly at the level of availability of 3'-phosphoadenosine 5'-phosphosulfate (49, 50). Studies on solubilised microsomal enzymes from mouse mastocytoma suggesting that sulfotransferases involved in the *O*-sulfation of heparin have a higher apparent K_m for 3'-phosphoadenosine 5'-phosphosulfate than the *N*-sulfotransferase (51) indicate how reduced levels of sulfate activation could result in reductions of sulfation at specific positions in heparan sulfate, as reported here. These possibilities are being investigated in studies on sulfate activation and sulfotransferases in the various cell lines.

Although the function of heparan sulfate is not known, it is probable that this molecule plays a crucial role in modulating interactions between the cell surface and its environment. Such properties will depend on its interaction with other components present at or acting on the cell surface. The recent findings of subpopulations of heparin with widely differing biological activity yet having similar gross chemical structure (52-54) and the requirement for specific sequences in heparin for such activity (26, 27) indicate that the fine structure of heparan sulfate will be important in its interactions with other molecules and therefore that the metabolic control of this structure may be critical in the control of such phenomena as cell growth and adhesion.

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REFERENCES

1. Tooze, J., ed (1980) *Molecular Biology of Tumor Viruses*, Cold Spring Harbor Laboratory, New York
2. Winterbourne, D. J., and Mora, P. T. (1978) *J. Biol. Chem.* **253**, 5109-5120
3. Underhill, C. B., and Keller, J. M. (1975) *Biochem. Biophys. Res. Commun.* **63**, 448-454
4. Underhill, C. B., and Keller, J. M. (1977) *J. Cell. Physiol.* **90**, 53-60
5. McFarland, V. W., Mora, P. T., Schultz, A., and Pancake, S. (1975) *J. Cell. Physiol.* **85**, 101-111
6. Saito, H., Yamagata, T., and Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1536-1542
7. Shively, J. E., and Conrad, H. E. (1976) *Biochemistry* **15**, 3932-3942
8. Oldberg, Å., Höök, M., Öbrink, B., Pertoft, H., and Rubin, K. (1977) *Biochem. J.* **164**, 75-81
9. Lindahl, U., Bäckström, G., Jansson, L., and Hallén, A. (1973) *J. Biol. Chem.* **248**, 7234-7241
10. Wessler, E. (1971) *Anal. Biochem.* **41**, 67-69
11. Linker, A., and Hovingh, P. (1965) *J. Biol. Chem.* **240**, 3724-3728
12. Treveleyn, W. E., Procter, D. P., and Harrison, J. S. (1950) *Nature (Lond.)* **166**, 444
13. Shively, J. E., and Conrad, H. E. (1976) *Biochemistry* **15**, 3943-3950
14. Jacobsson, I., Höök, M., Pettersson, I., Lindahl, U., Larm, O., Wirén, E., and von Figura, K. (1979) *Biochem. J.* **179**, 77-87

15. Reed, L. J., and Muench, M. (1938) *Am. J. Hyg.* **27**, 493-497
16. Oldberg, Å., Kjellén, L., and Höök, M. (1979) *J. Biol. Chem.* **254**, 8505-8510
17. Rollins, B. J., and Culp, L. A. (1979) *Biochemistry* **18**, 141-148
18. Rollins, B. J., and Culp, L. A. (1979) *Biochemistry* **18**, 5621-5629
19. Dietrich, C. P., Sampaio, L. O., Toledo, O. M. S., and Cássaro, C. M. F. (1977) *Biochem. Biophys. Res. Commun.* **75**, 329-336
20. Chiarugi, V. P., and Vannucchi, S. (1976) *J. Theor. Biol.* **61**, 459-475
21. Ohnishi, T., Ohshima, E., and Ohtsuka, M. (1975) *Exp. Cell Res.* **93**, 136-142
22. Sampaio, L. O., Dietrich, C. P., and Giannotti Filho, O. (1977) *Biochim. Biophys. Acta* **498**, 123-131
23. Kraemer, P. M., and Smith, D. A. (1974) *Biochem. Biophys. Res. Commun.* **56**, 423-430
24. Long, W. F., and Williamson, F. B. (1979) *IRCS J. Med. Sci.* **7**, 429-434
25. Kraemer, P. M. (1979) in *Surfaces of Normal and Malignant Cells* (Hynes, R. O., ed) pp. 149-198, Wiley, Chichester
26. Rosenberg, R. D., Armand, G., and Lam, L. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 3065-3069
27. Lindahl, U., Bäckström, C., Höök, M., Thunberg, L., Fransson, L., and Linker, A. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 3198-3202
28. Winterbourne, D. J. (1979) in *Glycoconjugates* (Schauer, R., Buddecke, E., Kraemer, M. F., Vliegenthart, J. F. G., and Wiegandt, H., eds) pp. 629-630, Georg Thieme, Stuttgart
29. Cifonelli, J. A. (1968) *Carbohydr. Res.* **8**, 233-242
30. Bhavanandan, V. P., Breindel, D., and Davidson, E. A. (1977) *Biochim. Biophys. Acta* **498**, 250-254
31. Höök, M., Lindahl, U., and Iverius, P. (1974) *Biochem. J.* **137**, 33-43
32. Linker, A., and Hovingh, P. (1975) *Biochim. Biophys. Acta* **385**, 324-333
33. Cifonelli, J. A., and King, J. A. (1977) *Biochemistry* **16**, 2137-2141
34. Lindahl, U., Höök, M., Bäckström, G., Jacobsson, I., Riesenfeld, J., Malmström, A., Roden, L., and Feingold, D. S. (1977) *Fed. Proc.* **36**, 19-23
35. Hovingh, P., and Linker, A. (1970) *J. Biol. Chem.* **245**, 6170-6175
36. Silva, M. E., Dietrich, C. P., and Nader, H. B. (1976) *Biochim. Biophys. Acta* **437**, 129-141
37. Dietrich, C. P., Silva, M. E., and Michelacci, Y. M. (1973) *J. Biol. Chem.* **248**, 6408-6415
38. Linker, A., and Hovingh, P. (1973) *Carbohydr. Res.* **29**, 41-62
39. Dietrich, C. P., and Nader, H. B. (1974) *Biochim. Biophys. Acta* **343**, 34-44
40. Margolis, R. H., and Atherton, D. M. (1972) *Biochim. Biophys. Acta* **273**, 368-373
41. Cifonelli, J. A., and King, J. A. (1975) *Connect. Tissue Res.* **3**, 97-104
42. Johnston, L. S., Keller, K. L., and Keller, J. M. (1979) *Biochim. Biophys. Acta* **583**, 81-94
43. Keller, K. L., Keller, J. M., and Moy, J. N. (1980) *Biochemistry* **19**, 2529-2536
44. Nakamura, N., Hurst, R. E., and West, S. S. (1978) *Biochim. Biophys. Acta* **538**, 445-457
45. Funakoshi, I., Nakada, H., and Yamashina, I. (1974) *J. Biochem. (Tokyo)* **76**, 319-333
46. Mora, P. T., Chang, C., Couvillion, L., Kuster, J. M., and McFarland, V. W. (1977) *Nature* **269**, 36-40
47. Chang, C., Martin, R. G., Livingston, D. M., Luborsky, S. W., Hu, C. P., and Mora, P. T. (1979) *J. Virol.* **29**, 69-75
48. Chandrasekaran, K., Winterbourne, D. J., Luborsky, S. J., and Mora, P. T. (1981) *Int. J. Cancer* **27**, in press
49. Conrad, G. W., and Woo, M.-L. (1980) *J. Biol. Chem.* **255**, 3086-3091
50. Sugahara, K., and Schwartz, N. B. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 6615-6618
51. Jansson, L., Höök, M., Wasteson, Å., and Lindahl, U. (1975) *Biochem. J.* **149**, 49-95
52. Höök, M., Björk, I., Hopwood, J., and Lindahl, U. (1976) *FEBS Lett.* **66**, 90-93
53. Bengtsson, G., Olivecrona, T., Höök, M., and Lindahl, U. (1977) *FEBS Lett.* **79**, 59-63
54. Niewiarowski, S., Rucinski, B., James, P., and Lindahl, U. (1979) *FEBS Lett.* **102**, 75-78