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Evidence for the Presence of Complex High-Molecular Mass N-Linked Oligosaccharides in Intranuclear Glycoproteins From HeLa Cells

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Abstract Nonhistone proteins were extracted in 0.4 M NaCl from membrane-depleted nuclei of HeLa cells grown in the presence or the absence of [5,6-^{3}H]fucose. Control experiments strongly suggest that most extracted proteins were indeed nuclear components. Several proteins, present in the 0.4 M NaCl nuclear extract, with M_r ranging from 35,000 to 115,000 were identified on Western blots as fucosylated glycoproteins owing to their binding to the fucose-specific lectin, Ulex europeus agglutinin I. Results of experiments involving mild alkaline treatment and peptide N-glycosidase F digestion showed that the carbohydrate moieties of these fucosylated nuclear glycoproteins were N-linked to the polypeptide backbone. Analysis of the N-glycans revealed the presence of two populations of sialylated oligosaccharides on the basis of their relative molecular masses. The sensitivity of the high-M_r oligosaccharides to endo-β-galactosidase and their incorporation of [^{3}H]glucosamine suggest that they could contain repeating N-acetyllactosamine units. [^{3}H]Fucose incorporated into nuclei was confined to the nucleoli, as judged by autoradiography of sections cut through cells grown in the presence of [^{3}H]fucose. Electron microscopy autoradiography showed that the fibrillar centers were never labeled, while silver grains were observed on the dense and the granular components of nucleoli. Taking into account of these data most nuclear fucosylated glycoproteins extracted in 0.4 M NaCl might be nucleolar ribonucleoproteins.

Key words: nucleus, nucleolus, ribonucleoproteins, N-glycosylation, HeLa cells

Over the past 10 years, evidence has been accumulated in favor of the presence of glycoproteins in the animal cell nucleus [1–3]. Most of the data currently available arose from cytological studies by using lectin histochemistry [4–8], labeled glycosidases [9], or autoradiography after incorporation of radioactive sugars into cells [10]. Accordingly, many questions concerning the biosynthesis, structure, and sugar composition of nuclear glycoproteins remain unanswered. In fact, some nuclear pore proteins [11–16], a nuclear antigen [17], and a human RNA polymerase II transcription factor [18,19] are the only nuclear glycoproteins that have been identified unambiguously. Indeed, except for a nuclear pore glycoprotein [15,16] it is well established that these glycoproteins bear single N-acetylglucosamine residues linked to the polypeptide backbone by unusual O-glycosidic bonds [2]. The presence in the cell nucleus of other glycoproteins bearing complex oligosaccharide chains has been reported [20–29] but, in most cases, the linkage of the sugar moieties to the polypeptide backbone remain unknown. However, some data suggest the existence of N-glycosylated intranuclear proteins bearing complex oligosaccharide chains. For instance, biochemical analysis of purified high-mobility group (HMG) proteins 14 and 17 indicated that these proteins are N-glycosylated (28). A recent report showing that N-glycosylated proteins containing high-mannose type chains can be crosslinked

Abbreviations used: MEM, Minimum essential medium; UEA-I, Ulex europeus agglutinin I; PBS, Phosphate-buffered saline; PMSF, Phenylmethanesulfonylfluoride; EDTA, Ethylenediaminetetracetic acid; DTT, Dithiothreitol; SDS, Sodium dodecyl sulfate; PAGE, Polyacrylamide gel electrophoresis; PGNase F, Peptide N-glycosidase F; BSA, Bovine serum albumin.

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to DNA in intact cells [29] strongly argues for the existence of nuclear N-glycosylated proteins, even if the biosynthesis of such nuclear glycoproteins has not yet been elucidated.

To obtain further information on the existence of N-glycosylated intranuclear glycoproteins, we investigated their presence in a nonhistone protein fraction extracted from membrane-depleted HeLa cell nuclei. In light of previous data suggesting the existence of fucosylated glycoproteins in HeLa cell nuclei [20], we chose to analyze these glycoproteins. Keeping in mind that nuclei can be contaminated with extranuclear proteins during the isolation procedure, a protocol known to minimize this possibility greatly was used [30], and control experiments were carried out to assess the purity of the nuclear fraction.

The data reported here confirm that HeLa cell nuclei contain fucosylated glycoproteins. Moreover, they show that these glycoproteins bear N-linked glycans and we provide arguments supporting the presence of repeating N-acetyllactosamine units in the sugar moieties of nuclear fucosylated glycoproteins. Autoradiographic data suggesting that the nuclear fucosylated proteins extracted in 0.4 M NaCl might be nucleolar ribonucleoproteins are also given.

MATERIALS AND METHODS

Cell Culture and Radiolabeling

HeLa cells were grown as monolayers in Eagle's MEM supplemented with 7% heat-inactived fetal calf serum and 1% glutamine. Cells were harvested by mild trypsin digestion by using 50 μg/ml of trypsin–TPCK (Worthington Biochemical Corp., La Jolla, CA) for 10 min in Ca^{2+}–Mg^{2+} free PBS. The reaction was stopped by adding soybean trypsin inhibitor (at a concentration of 1 mg/ml). Cell viability assessed by trypan blue dye exclusion was above 95%. When required, 5×10^6 cells per 175 cm^2 flask were radiolabeled for 24 h with either 50 μCi/ml of L-[5,6-^{3}H]fucose (spec. act. 72 Ci/mmol, Radiochemical Amersham Centre, Buckinghamshire, UK), or with 20 μCi/ml of [^{35}S]methionine (spec. act. 1 300 Ci/mmol, Radiochemical Amersham Centre). In some experiments, cells were double labeled with 50 μCi/ml of D-[1,6-^{3}H]glucosamine (spec. act. 39 Ci/mmol, Radiochemical Amersham Centre) and with 10 μCi/ml of L-[1-^{14}C]fucose (spec. act. 38.7 mCi/mmol, Radiochemical Amersham Centre).

Autoradiographic Methods

Semithin (2-μm) and ultrathin (60-nm) sections of pellets of radiolabeled HeLa cells (see above) embedded in Epon 812 were used. For light microscopic observations, semithin sections were disposed in a drop of water on slides placed on hot plate. After drying, slides were dipped in Ilford emulsion L4 diluted 1 : 1 with distilled water [31]. After 15 days in the dark at 4°C, autoradiographs were treated with D19b (Kodak). Sections were subsequently stained with 0.5% toluidine blue in 1% sodium borate and observed with a Zeiss microscope.

For electron microscopy, ribbons of sections collected on grids were coated with L4 Ilford emulsion (1 v) diluted ½ in distilled water according to the procedure developed by Haase and Yung [32]. After suitable exposure time (about 2 months), two emulsion treatment procedures were used. For routine samples, Microdol X was used. For a better localization and a higher efficacy, the phenidon development [33] was associated with gold latensification [34]. Sections were stained with uranyl acetate and lead citrate and examined with a Siemens electron microscope at 80 kV.

Isolation of Membrane-Depleted Nuclei

Membrane-depleted nuclei were isolated according to Hancock's method [30] slightly modified [35]. This procedure, known to yield very clean membrane-depleted nuclei from HeLa cells [30,35], was applied either to whole cells or to karyoplasts, i.e., cells deprived of the majority of their cytoplasm and plasmic membrane by centrifugation through a discontinuous Ficoll gradient, as described elsewhere [37]. Briefly, karyoplasts or whole cells were washed for 10 min at 4°C in 0.2 mM phosphate buffer (pH 7.2) containing 0.2 mM EDTA, 100 mM sucrose and 0.5 mM PMSF (solution A). The cells were resuspended by gentle stirring in solution A containing 0.5% Nonidet P40 and membrane-depleted nuclei were centrifuged at 1,000*g* for 10 min.

Isolation of Subnuclear Fractions

The nuclear shell, composed of the lamina associated with a thin layer of peripheral condensed chromatin [38], and the internal components including nucleoli were isolated according to the method developed by Hubert and coworkers [35] and successfully applied to HeLa cell membrane-depleted nuclei [36,38,39].

Protein Extraction

Glycoproteins analyzed in this study belong to a nonhistone protein fraction extracted either from membrane-depleted nuclei isolated from whole cells or from karyoplasts in PBS, pH 7.4, containing 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.4 M NaCl (final concentration). The protein concentration was determined according to [40], using bovine serum albumin (BSA) as the standard.

SDS–PAGE Electrophoresis

Nuclear ^{35}S-labeled proteins, extracted as described above, were solubilized in electrophoresis sample buffer according to Laemmli's method [41] in the presence of 0.1 M DTT. The samples were heated at 100°C for 5 min before being applied to the 5–18% polyacrylamide gel and electrophoresed (SDS–PAGE). The gel was fixed, dried, and prepared for fluorography [42].

Enzyme-Linked Lectin Analysis

Nuclear glycoproteins (75 μg of protein per lane) were resolved after SDS–PAGE and transferred onto a nitrocellulose membrane. The blot was stained with 0.2% Ponceau red in 0.3% trichloroacetic acid (TCA) and then washed in 0.3% TCA. Transfer membranes were incubated with blocking buffer (PBS, pH 7.4, containing 3% BSA) for 30 min at room temperature and washed twice in PBS containing 0.05% Tween 20. Then, the membrane was incubated with 20 μg/ml of biotinylated-UEA I (Biocarb, Lund, Sweden) for 2 h at room temperature in the presence or in the absence of the specific inhibitor sugar (0.1 M fucose). The membrane was soaked in 1% BSA in PBS at room temperature. Streptavidin–horseradish peroxidase was added at 1 μg/ml for 30 min at room temperature. The sheets were washed 10 times in PBS containing 0.05% Tween 20 and incubated with hydrogen peroxide and 4-chloro-1-naphthol (Sigma) [43].

Analysis of Glycosidic Linkage

Mild alkaline treatment of labeled glycoproteins. Glycoproteins containing [^{3}H]fucose were subjected to mild alkaline treatment in 0.05 M NaOH in the presence of 1 M $NaBH_4$ for 16 h at 45°C [44]. After acidification to pH 5.0 with 2 M acetic acid, the solution was loaded onto a Sephadex G-50 column (Pharmacia, Uppsala, Sweden).

Peptide N-glycosidase F treatment of labeled glycoproteins. Tritiated fucose-labeled glycoproteins were treated overnight at room temperature with 10 U/ml of PGNase F (*Flavobacterium meningosepticum*), (Genzyme, Boston USA) in 0.2 M phosphate buffer (pH 8.6) containing 0.17% SDS, 10 mM 1–10 phenanthroline hydrate, 1.25% Noninet P40 [45]. After enzymatic treatment oligosaccharides were chromatographed on a column of Sephadex G-50 (see below).

Molecular Sleving of [^{3}H]Fucose-Labeled Oligosaccharides

Tritiated fucose-labeled oligosaccharides, obtained as described above, were dissolved in 0.1 M pyridine acetate (pH 5.0) and loaded onto a Sephadex G-50 column (100 cm × 1 cm) and eluted at a flow rate of 20 ml/h. The column was calibrated by using BSA (void volume, Vo), dextran T10 (10,000 M_r) asialotetraantennary complex oligosaccharides obtained from orosomucoid, (MW: 2 400), N-acetyllactosamine (MW: 384) and mannose (included volume, Vt); 1-ml fractions were collected and the radioactivity was determined in a Maxi β 4000 counter (Packard, Zurich, Switzerland) equipped with a dpm calculation program.

Oligosaccharide Analysis

Oligosaccharides were resolved into two peaks after chromatography on Sephadex G-50. Each peak was divided into two equal pools and treated as follows: the first one was treated with 20 mU/ml of endo-β-galactosidase from *Bacteroides fragilis* (Genzyme) for 24 h at 37°C in 0.1 M sodium acetate (pH 5.8). The reaction products were chromatographed on a Sephadex G-50 column as described above.

The second pool was desalted on a Bio-Gel P2 column (Bio-Rad) in water and applied to a 1.0 ml QAE–Sephadex (Pharmacia) column equilibrated with 2 mM Tris (pH 9.5). After elution with the starting buffer, the column was washed with a stepwise gradient of NaCl: 20, 70, 140, and 200 mM, and 1 M) as previously described [46]. The same chromatographic procedure was applied after treatment of oligosaccharides with 50 mU/ml of sialidase from *Vibrio cholerea* (Behring, Marburg Germany) in 0.05 M sodium acetate (pH 5.5) containing 0.15 M NaCl and 0.009 M $CaCl_2$ at 37°C for 16 h. Samples were boiled for 5 min at 100°C.

Preparation of Crude Microsomal Fractions

Cells were removed by scraping and resuspended in ice-cold Tris/mannitol buffer (2 mM Tris–HCl, 50 mM mannitol), pH 7.1, containing 1 mM PMSF. Cells were disrupted by 5 × 10 s sonication bursts by using an immersion probe (Branson Sonifier 250) at 4°C. After dilution with Tris/mannitol buffer, the homogenate was centrifuged at 2,500*g* for 10 min at 4°C. The supernatant was further centrifuged at 100,000*g* for 1 h at 4°C. The resulting pellet containing a crude microsomal fraction was resuspended in distilled water.

RESULTS

Purity of the Nuclear Protein Extract

If the isolation procedure were to contaminate the nuclei with extranuclear proteins, the amount of proteins should be different in membrane-depleted nuclei isolated from whole cells and in nuclei isolated from karyoplasts (see Materials and Methods). Comparative analysis according to the origin of the isolated nuclei strongly argued against nuclear contamination. Indeed, membrane-depleted nuclei isolated from whole cells or from karyoplasts contained the same amount of proteins (1.2 mg vs. 1.1 mg, respectively). In addition, when isolated plasma membranes prepared from cells labeled with [^{3}H]fucose were added to unlabeled cells before isolation of membrane-depleted nuclei, no significant level of radioactivity was detected in the membrane-depleted nuclei, regardless of their origin.

When cells were grown in the presence of [^{3}H]fucose for 24 h, radioactivity was detected in membrane-depleted nuclei, and comparable levels were found in nuclei from whole cells and from karyoplasts (55,200 dpm vs. 46 200 dpm, respectively). This finding added to the data reported above clearly showed that the results were not dependent upon the origin of the nuclei. Therefore, we decided to analyze fucosylated nuclear glycoproteins of membrane-depleted nuclei isolated from whole cells.

Intracellular Localization of [^{3}H]Fucose Incorporation

Autoradiography of semithin sections of cells grown in the presence of [^{3}H]fucose (Fig. 1) revealed a labeling over the whole cytoplasm. However, a paranuclear compartment appeared to be much more labeled than the remainder of cytoplasm. As concerns the nucleus, the nucleoplasm was very weakly labeled, while the grains present were almost completely restricted to the nucleoli. About 99% of nucleoli were covered by a significant number of silver grains. The nucleolar labeling was estimated to be sevenfold higher than the nucleoplasmic labeling per unit area. Obviously, if the nuclear labeling were to the background or to a mere diffusion of [^{3}H]fucose, silver grains would be randomly distributed. Furthermore, as judged by the absence of silver grains on the large intercellular areas, the background was negligible. Electron microscopy examination enabled us to establish that the strongly labeled cytoplasmic paranuclear compartment corresponded to the Golgi apparatus (Fig. 2). Furthermore, careful analysis of a number of sections passing through nucleoli showed that the dense fibrillar and the granular components were usually labeled while no silver grains were observed on the fibrillar center (Fig. 3).

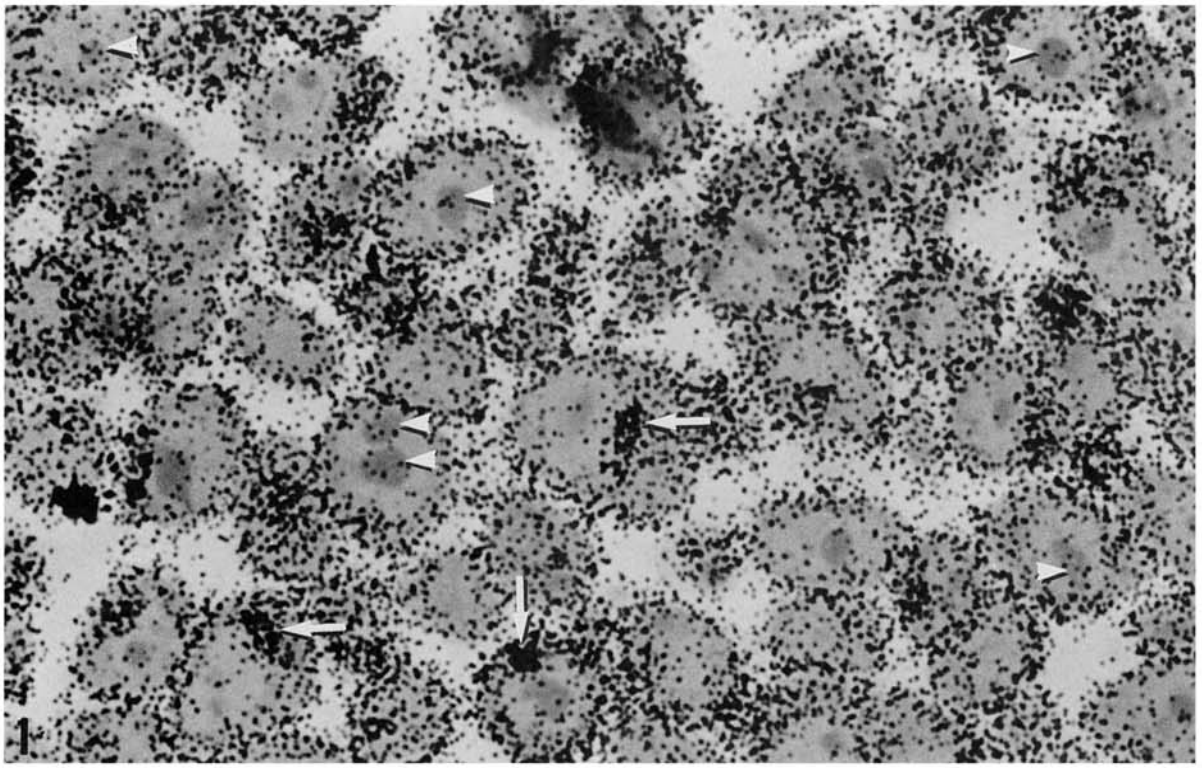
Nuclear Shell and Internal Components

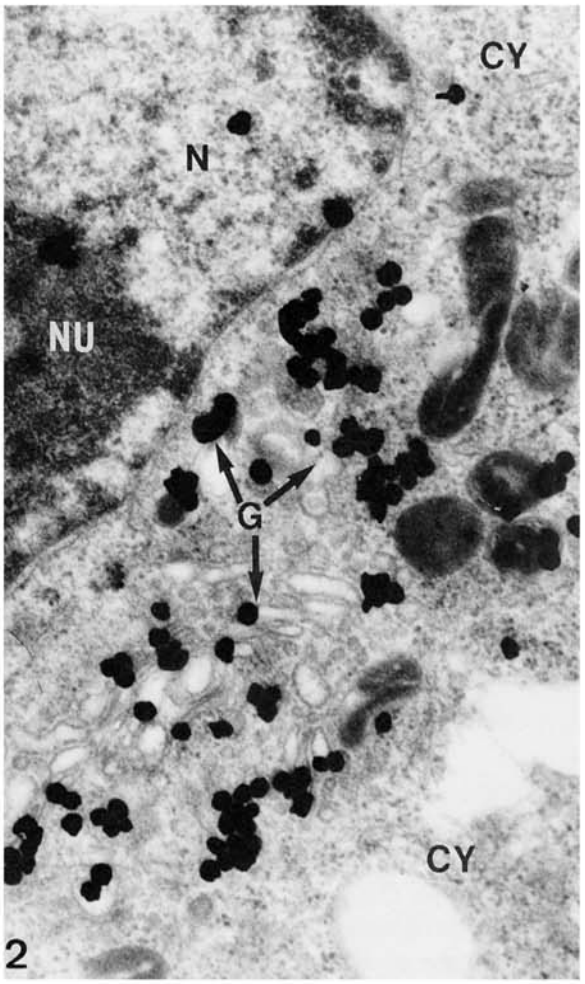
After depletion of the nuclear envelope, nuclear structures could be divided into nuclear shell and chromatin [35,36]. In agreement with the ultrastructural study described above, nearly 80% of the [^{3}H]fucose-labeled material was recovered in the chromatin fraction containing nucleoli.

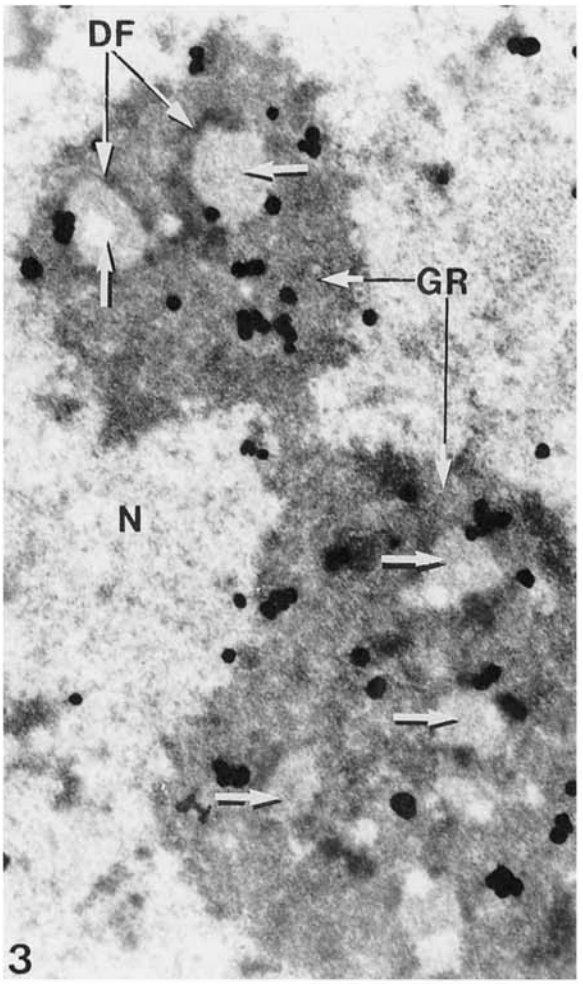
Fig. 1. Autoradiograph of a semithin section of a pellet of HeLa cells. Cells were incubated in the presence of 50 μCi/ml L-[5,6-^{3}H]fucose for 24 h. Sections of cells embedded in Epon were dipped in ilford emulsion L4. After 15 days of exposure in the dark, the emulsion was treated with D19b (see Materials and Methods). The cytoplasm is strongly labeled and especially over a paranuclear compartment (arrows). The nucleoli (arrowheads) are clearly labeled, unlike the nucleoplasm (×1,200).

Fig. 2. Electron microautoradiograph of HeLa cells. Cells were labeled as described for Figure 1 but developed with phenidon associated with gold latensification. This electron micrograph shows that the highly labeled paranuclear compartment (see legend to Fig. 1) corrresponds to the Golgi apparatus (G), which is labeled more strongly than the remainder of the cytoplasm (CY). N, nucleoplasm; NU, nucleolus (×30,000).

Fig. 3. Electron microautoradiograph of an HeLa cell nucleolus. Cells were labeled with [^{3}H]fucose (see legend to Fig. 1). Autoradiographs were treated as described in the legend to Figure 2). The nucleolar labeling appears to be localized over the granular component (GR) and over the dense fibrillar component (DF) surrounding unlabeled fibrillar centers (arrows) (×20,000).







Figures 1–3.

0.4 M NaCl Extracted Proteins

Proteins were extracted from membrane-depleted nuclei by using different concentrations of NaCl up to 2 M. The extraction of tritiated fucose-labeled material was almost complete with 0.4 M NaCl. This radioactivity accounted for ~1% of the [^{3}H]fucose incorporation into the cellular glycoproteins. However, the specific activity of nuclear [^{3}H]fucose labeled material was only 10 times lower than the specific activity of the cellular [^{3}H]fucose glycoproteins (1,230 dpm/μg protein vs. 11,650 dpm/μg protein).

Identification of Fucosylated Glycoproteins in the Nuclear Protein Extract by Using a Lectin-Binding Assay

The electrophoretic pattern of proteins extracted in 0.4 M NaCl from membrane-depleted nuclei of cells grown in the presence of [^{35}S] methionine showed a lot of polypeptides with M_r ranging from 14,000 to 190,000 (Fig. 4, lane 1). To detect fucosylated glycoproteins among these proteins, unlabeled extracted proteins were separated by SDS–PAGE, transferred onto a nitrocellulose membrane and incubated with the biotinylated lectin UEA I, which is specific to L-fucose. The incubation of the Western blot in the presence of streptavidin–horseradish peroxidase revealed that proteins with M_r ranging from 35,000 to 115,000 were recognized by the lectin (Fig. 4, lane 2). The same result was obtained by using proteins extracted in 2 M NaCl (not shown). Inhibition of lectin binding either by competition by using 0.1 M fucose or by Pronase digestion before electrophoresis demonstrated the specificity of the lectin binding to fucose associated with proteins.

Analysis of Glycosidic Linkage

Proteins were extracted from the membrane-depleted nuclei of cells grown in the presence of both [^{14}C]fucose and [^{3}H]glucosamine. The proteins were subsequently subjected to PGNase F digestion, which specifically degrades N-linked glycans, and to a mild alkaline treatment known to release O-linked glycans. The results of this comparative analysis showed that the majority of [^{14}C]fucose was released upon PGNase F digestion (78%) while very little radioactivity related to [^{3}H]glucosamine (12%) was released during this enzymatic treatment. The [^{14}C]fucose-labeled fraction resistant to PGNase F treatment was found to be also resistant to mild alkaline

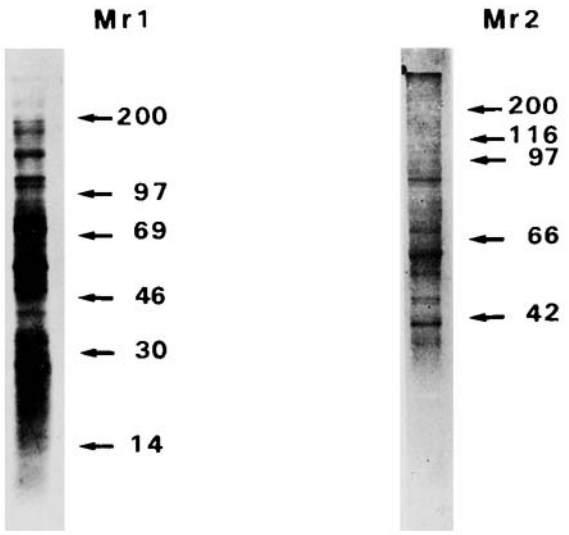


Fig. 4. Identification of fucosylglycoproteins extracted with 0.4 M NaCl from membrane-depleted nuclei of HeLa cells. Material was obtained after either cell labeling with [^{35}S]methionine (left lane) or from unlabeled cells (right lane). Unlabeled material (75 μg) was blotted onto a nitrocellulose membrane, incubated with biotinylated-UEA I and revealed with streptavidin–horseradish peroxidase. The samples were analyzed by SDS–PAGE (5–18% gradient acrylamide gel) followed by fluorography (left lane) or by Western blot (right lane). The mobility of radioactive standards (Mr1) and of unlabeled standards (Mr2) after transfer to nitrocellulose membrane, are indicated.

treatment. In contrast, the [^{3}H]glucosamine-labeled fraction was sensitive to mild alkaline treatment (not shown). Therefore, these results demonstrated that the majority of [^{14}C]fucose was incorporated into N-linked glycans, while [^{3}H]glucosamine was incorporated in O-linked sugar moieties. It is interesting to note that the results of similar experiments carried out by using the microsomal fraction of the same cells were quite different concerning [^{3}H]glucosamine. In fact, the ratio of fucose/glucosamine incorporation into N-linked glycoproteins was found to be sevenfold higher in the nuclear extract (78/12) than in the microsomal fraction (61/68).

Analysis of N-Linked [^{3}H]Fucose-Labeled Oligosaccharides

[^{3}H]Fucose-labeled oligosaccharides were resolved in two peaks after chromatography on a Sephadex G-50 column (Fig. 5). These peaks accounted for 35% (peak I) and 65% (peak II) of the recovered radioactivity. Regarding the elution pattern of the standards, [^{3}H]fucose was incorporated into oligosaccharides of M_r ranging from 2,400 to 10,000. However, these M_r could be overestimated by the presence of acidic groups

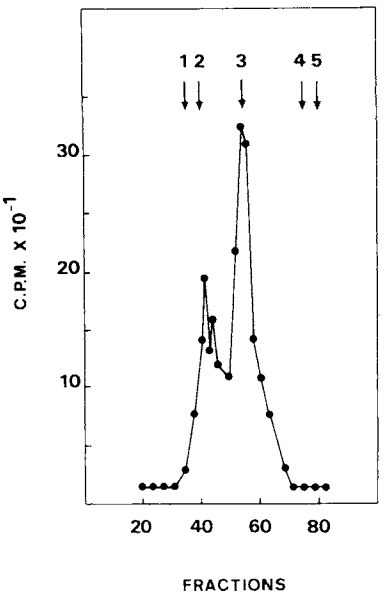


Fig. 5. Gel filtration of [^{3}H]fucose-labeled nuclear oligosaccharides released from glycoproteins by peptide N-glycosidase F treatment. HeLa cells were incubated for 24 h with 50 μCi/ml of L-[5,6-^{3}H]fucose. At the end of the incubation period, nuclear glycoproteins were obtained as described in Materials and Methods. Tritiated fucosylglycoproteins were loaded onto a Sephadex G-50 column (100 cm × 1 cm) and eluted with 0.1 M pyridine acetate, pH 5.0. One ml fractions were collected. Under these conditions, all the radioactivity was eluted together with BSA in the void volume (arrow 1). After PGNase F treatment, the resulting [^{3}H]fucose-labeled oligosaccharides were chromatographed on a Sephadex G-50 column under the same conditions. Arrows indicate the elution of standard used to calibrate the column: BSA (arrow 1, void volume): dextran T10 (arrow 2, M_r: 10,000): asialotetraantennary complex oligosaccharides (arrow 3, M_r: 2,400): N-acetyllactosamine (arrow 4, M_r: 384) and mannose (arrow 5, included volume). Peaks were collected as follows: Peak I from fraction 35 to fraction 48 and peak II from fraction 52 to fraction 68.

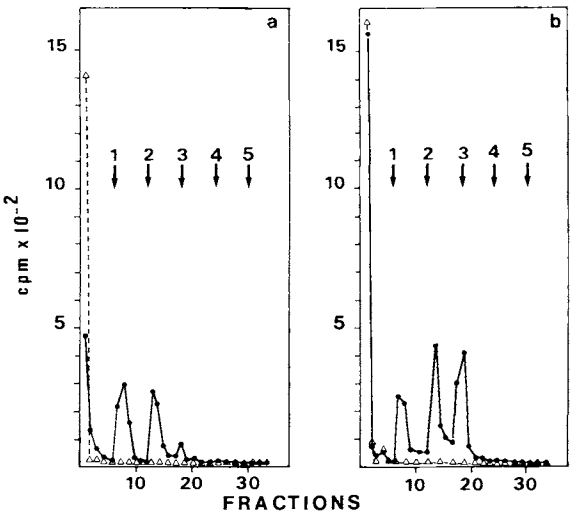


Fig. 6. Ion-exchange chromatography of [^{3}H]fucose-labeled nuclear oligosaccharides released from glycoproteins by peptide N-glycosidase F treatment. Oligosaccharides contained in peaks I (a) and II (b) after Sephadex G-50 chromatography (see Fig. 5) were independently loaded onto a column of QAE–Sephadex equilibrated with 2 mM Tris, pH 9.5. The column was eluted with a stepwise gradient of NaCl in the starting buffer. Arrow 1, 20 mM NaCl; arrow 2, 70 mM NaCl; arrow 3, 140 mM NaCl; arrow 4, 200 mM NaCl; arrow 5, 250 mM NaCl; arrow 6, 1 M NaCl. The experiment was performed before (●) and after (△) sialidase treatment. Note that after sialidase treatment all the recovered radioactivity in both peak I (a) and peak II (b) was eluted with the starting buffer.

on these glycans. Since the presence of sialic acid in nuclear glycoproteins has been suggested by lectin binding [29], we analyzed the behavior of both peaks I and II on QAE–Sephadex by stepwise salt elution before and after sialidase treatment. The results of this analysis (Figure 6) demonstrated that both peaks contained acidic [^{3}H]fucose oligosaccharides attributable to the presence of sialic acid. The results summarized in Table I indicate that peak II contains a higher proportion of highly negatively charged oligosaccharides than peak I (22 vs. 4%), while peak I contains fewer neutral species (31 vs. 45%). These data are in agreement with the observation that the elution pattern of [^{3}H]fucose-labeled oligosaccharides on a Sephadex G-50 column was slightly modified after sialidase treatment (data not shown). The majority of the

TABLE 1. QAE–Sephadex Chromatography of Tritiated Fucose-Labeled Nuclear Oligosaccharides

	Total oligosaccharide radioactivity (%)				
	2	20	70	140	200
Peaks[a]	(mM NaCl)				
I	31	36	26	4	0
II	45	13	20	22	0

[a]Peaks I and II obtained after chromatography on a Sephadex G-50 column were subjected to QAE–Sephadex chromatography as described in the legend to Figure 6.

radioactivity (peak II) was eluted with a calculated $M_r \simeq 1{,}800$ after sialidase treatment, whereas peak I still co-eluted with dextran T 10 after the enzymatic treatment.

Endo-β-Galactosidase Treatment of Labeled Oligosaccharides

A potential explanation for the high relative molecular mass of N-linked oligosaccharides would be the presence of repeating Gal-GlcNAc

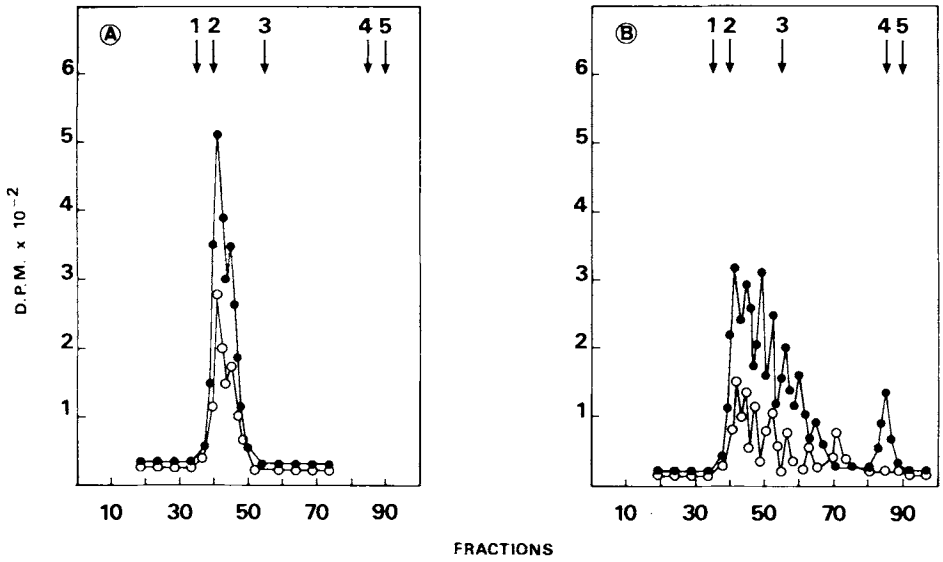


Fig. 7. Effect of endo-β-galactosidase on the chromatographic behavior of high relative molecular mass nuclear oligosaccharides. The oligosaccharides were obtained after PGNase F treatment of [^{3}H]glucosamine (●) and [^{14}C]fucose (○) double-labeled nuclear glycoproteins and chromatography on Sephadex G-50 (peak I) (see for details the legend to Figure 5). **A:** Untreated oligosaccharides. **B:** Endo-β-galactosidase treated oligosaccharides. Arrows indicate the elution of standards used to calibrate the column (see the legend to Figure 5).

units. In order to investigate the possible occurrence of N-linked polylactosaminyl chains in our nuclear extract, HeLa cells were incubated for 24 h with D-[1,6-^{3}H]glucosamine and L-[1-^{14}C]fucose and N-linked oligosaccharides were resolved after chromatography on a Sephadex G-50 column as detailed in the preceeding paragraph. Treatment of oligosaccharides contained in peak I with endo-β-galactosidase from *B. fragilis* released 75–80% and 70–75% of the radioactivity associated with [^{3}H]glucosamine and [^{14}C]fucose, respectively (Fig. 7). In both cases, oligosaccharides were degraded to a heterogeneous mixture of low M_r products. This heterogeneous pattern of degradation was still observed when the enzymatic treatment lasted for 48 h. Endo-β-galactosidase treatment of [^{3}H]glucosamine- and [^{14}C]fucose-labeled N-linked oligosaccharides from peak II did not release any radioactivity (data not shown).

DISCUSSION

The existence of extranuclear glycoproteins bearing complex N-linked oligosaccharide chains is well established. Consequently, the detection of such glycoproteins in membrane-depleted nuclei raises the question of whether they arise from contamination of the nuclei during the isolation procedure. Although the possibility of a slight contamination cannot be totally ruled out, several arguments are in favour of the purity of the nuclear extracts used in the present study. First, the nuclear isolation procedure used here has previously been shown to yield clean HeLa cell membrane-depleted nuclei without noticeable contamination by extranuclear proteins [30]. Second, if such a contamination occurred, the amount of proteins should be different in nuclei isolated from karyoplasts which had been depleted of 90–95% of their cell cytoplasm and plasma membrane before the lytic treatment. Third, experiments by using unlabeled cells and labeled membranes did not show contamination of nuclei. Finally, comparative analysis of the osidic linkages by using double-labeling revealed that the majority of the [^{3}H]glucosamine present in our nuclear extract was incorporated into O-linked glycoproteins, while the majority of the [^{14}C]fucose was associated with N-linked oligosaccharides. This result is concordant with data showing that the nuclear compartment is enriched, compared to the cytoplasmic compartment, in glycoproteins bearing O-linked N-acetylglucosamine residues [47]. Therefore, taken together, these data strongly suggest that most proteins present in the 0.4 M NaCl nuclear extract were indeed nuclear components. Accordingly, most fucosylated glycoproteins detected in this extract can reasonably be thought to be actual nuclear glycoproteins. The

biochemical analysis of these glycoproteins clearly shows that they contain oligosaccharide chains N-linked to the polypeptide backbone. The results presented herein supplement data recently published [29] and strongly support the existence in the cell nucleus of N-linked glycoproteins substituted with sialylated complex glycans. The most striking feature of these nuclear oligosaccharides containing fucose is the presence of high-M_r species and their partial sensitivity to endo-β-galactosidase treatment. These data suggest the presence of N-acetyllactosaminyl repeats in these oligosaccharides containing fucose. The partial sensitivity of glycans to this enzyme treatment could be due to either the presence of branched structures or of fucose residues near the enzyme cleaving site which have been shown to generate linkages resistant to the action of endo-β-galactosidase [48,49]. To the best of our knowledge, this is the first report suggesting the presence of N-acetyllactosaminyl repeats in N-glycan chains of nuclear glycoproteins. To date, polylactosaminyl chains have been shown to be attached to plasma membrane proteins [50] and lysosomal proteins [51]. The presence of nuclear glycosaminoglycans could not contribute to the high-molecular mass of oligosaccharides containing fucose because (1) the nuclear glycosaminoglycans identified so far did not contain fucose [52,53] and (2) the acidity of fucose-containing oligosaccharides was exclusively associated with sialic acid and the glycans were eluted from QAE–Sephadex with 140 mM NaCl, whereas glycosaminoglycans usually require higher ionic strength to be eluted [46].

The nucleolar labeling observed by autoradiography on sections of cells grown in the presence of [^{3}H]fucose does not demonstrate that [^{3}H]fucose was incorporated into glycoproteins. However, it is tempting, based on the following data, to postulate that most [^{3}H]fucose labeled glycoproteins detected in the 0.4 M NaCl extract correspond to nucleolar proteins. Taking into account the negligible background, the low nuclear labeling is in agreement with the low rate of radioactivity detected in the 0.4 M NaCl extract with respect to the cellular radioactivity. The observation that the nuclear labeling was confined to nucleoli is concordant with the finding that internal nuclear components were responsible for [^{3}H]fucose radioactivity. Moreover, the presence of silver grains only over certain nucleolar components suggests that the labeling is directly related to the incorporation of [^{3}H]fucose into nucleolar glycoproteins rather than to the diffusion of free fucose. The number and the amount of glycoproteins revealed on Western blot could appear to be paradoxical with regard to the very low nucleolar labeling. However, it must kept in mind that the amount of proteins used for the Western blot analysis was much greater than that present in an ultrathin section about 60 nm thick. The labeling of the dense fibrillar and the granular components of nucleoli known to be mainly constituted of ribonucleoproteins associated with ribosomal RNA [54, 55] could indicate that fucosylated glycoproteins in the 0.4 M NaCl nonhistone protein fraction are preribosomal ribonucleoproteins.

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