The Plant Cell, Vol. 7, 1459–1471, September 1995 © 1995 American Society of Plant Physiologists

Plant Nuclear Pore Complex Proteins Are Modified by Novel Oligosaccharides with Terminal *N*-Acetylglucosamine

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Only a few nuclear pore complex (NPC) proteins, mainly in vertebrates and yeast but none in plants, have been well characterized. As an initial step to identify plant NPC proteins, we examined whether NPC proteins from tobacco are modified by *N*-acetylglucosamine (GlcNAc). Using wheat germ agglutinin, a lectin that binds specifically to GlcNAc in plants, specific labeling was often found associated with or adjacent to NPCs. Nuclear proteins containing GlcNAc can be partially extracted by 0.5 M salt, as shown by a wheat germ agglutinin blot assay, and at least eight extracted proteins were modified by terminal GlcNAc, as determined by in vitro galactosyltransferase assays. Sugar analysis indicated that the plant glycans with terminal GlcNAc differ from the single O-linked GlcNAc of vertebrate NPC proteins in that they consist of oligosaccharides that are larger in size than five GlcNAc residues. Most of these appear to be bound to proteins via a hydroxyl group. This novel oligosaccharide modification may convey properties to the plant NPC that are different from those of vertebrate NPCs.

INTRODUCTION

The nuclear pore complex (NPC) is the site for nucleocytoplasmic transport of macromolecules, such as proteins, T-DNA, and different RNA species. The architecture and the major components of this complex have been investigated extensively in amphibians, using different electron microscopic techniques (for review, see Panté and Aebi, 1993). The NPC is a supramolecular structure of ~124 MD (Reichelt et al., 1990) and exhibits an eightfold rotational symmetry. It consists of a cytoplasmic and nucleoplasmic ring, and these rings are connected to the central channel complex via sets of spokes. It is thought that the spokes form eight aqueous channels of ~9 nm that allow passive diffusion of some ions and small molecules (Hinshaw et al., 1992; Akey and Rademacher, 1993), whereas the central channel complex with a functional diameter of up to 26 nm is proposed to be involved in the specific transport of macromolecules (Feldherr et al., 1984). Recently, other morphological peripheral structures of the NPC have been observed, such as cytoplasmic fibrils that are attached to the cytoplasmic ring and the nuclear basket that extends from the nucleoplasmic ring into the nucleoplasm (for reviews, see Akey, 1992; Panté and Aebi, 1993). In some species, the nuclear basket has been shown to be attached to a fibrous lattice termed the nuclear envelope lattice (Goldberg and Allen, 1992).

Based on scanning transmission electron microscopy mass analysis, the NPC is proposed to consist of at least 100 different proteins (Reichelt et al., 1990). To date, only a small number of NPC proteins have been identified and characterized at the biochemical and molecular levels. These have come mainly from vertebrates and yeast, and none have been identified in plants. Little is known about the function of the NPC proteins in nucleocytoplasmic transport, NPC organization, and assembly in any system studied (for reviews, see Forbes, 1992; Fabre and Hurt, 1994; Rout and Wente, 1994; Hicks and Raikhel, 1995b). Of particular interest with respect to protein import is a family of vertebrate NPC glycoproteins that are modified by the addition of single O-linked *N*-acetylglucosamine (O-GlcNAc) residues (for reviews, see Hart et al., 1989; Forbes, 1992). This post-translational modification is distinct from other protein sugar modifications; the sugar is an unmodified monosaccharide moiety whose addition occurs in the cytoplasm (for reviews, see Hart et al., 1989; Haltiwanger et al., 1992). In vertebrate nuclei, the O-GlcNAc NPC proteins have been identified by their ability to bind to wheat germ agglutinin (WGA), a lectin that binds to GlcNAc and sialic acids (Goldstein and Hayes, 1978), and by monoclonal antibodies raised against nuclear fractions (Davis and Blobel, 1986; Finlay et al., 1987; Hanover et al., 1987; Holt et al., 1987; Park et al., 1987; Schindler et al., 1987; Snow et al., 1987). The most abundant O-GlcNAc–bearing NPC protein, denoted p62, has been sequenced from several vertebrate species, and its glycosylation has been studied in detail (Starr et al., 1990; Carmo-Fonseca et al., 1991; Cordes et al., 1991; Cordes and Krohne, 1993).

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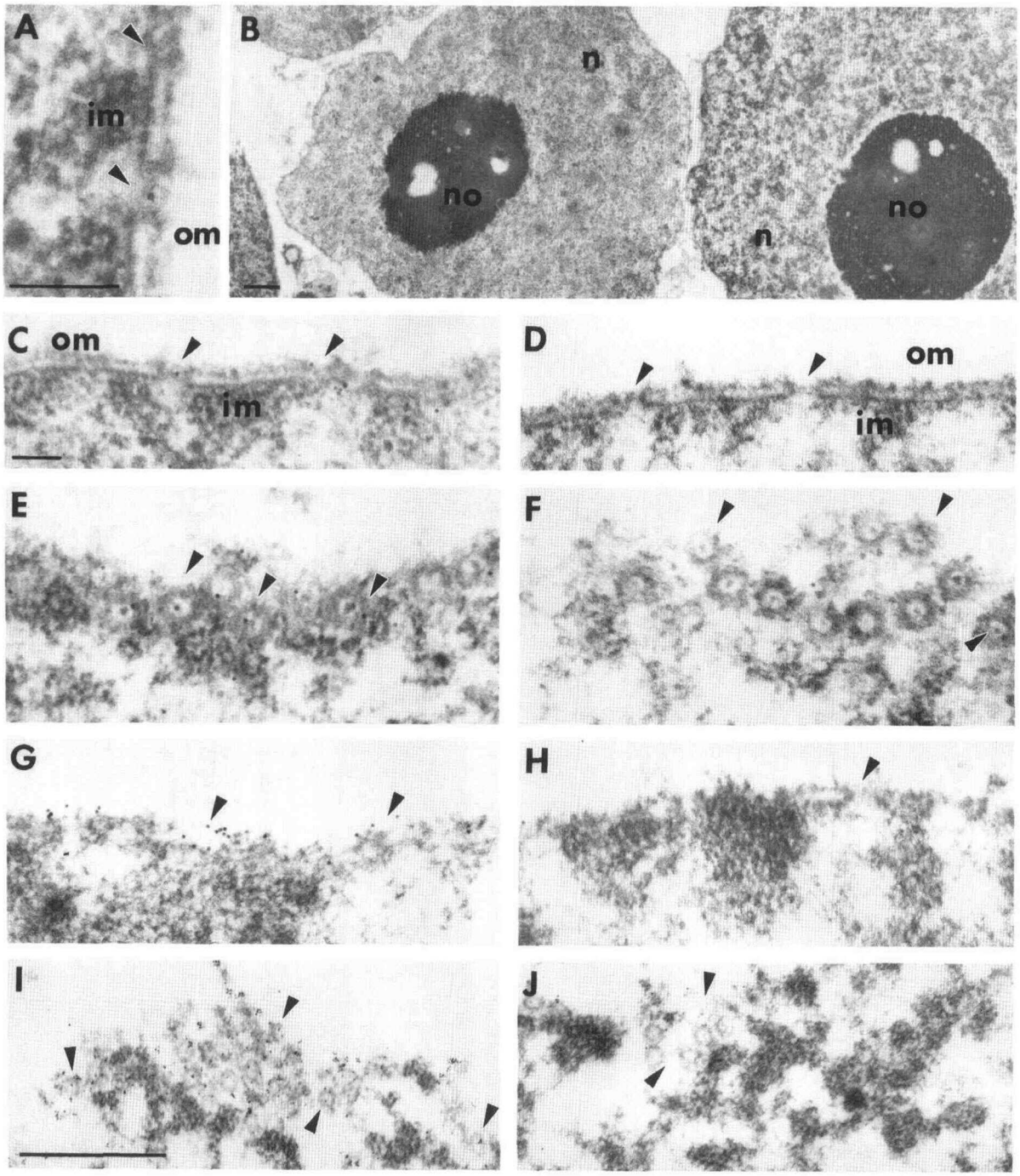

Figure 1. Ultrastructural Localization of NPC Proteins Modified by GlcNAc in Isolated Tobacco Nuclei.

(A) to **(F)** Nuclei isolated in the presence of 0.01% Triton X-100.
(G) to **(J)** Nuclei isolated in the presence of 0.6% Triton X-100.
In **(A)** to **(C)**, **(E)**, **(G)**, and **(I)**, purified nuclei were incubated with WGA-conjugated colloidal gold particles in the absence of chitotriose before fixation. In **(D)**, **(F)**, **(H)**, and **(J)**, these nuclei were incubated with the WGA probe in the presence of chitotriose before fixation. **(A)** to **(D)**, **(G)**, and **(H)** show longitudinal sections; **(E)**, **(F)**, **(I)**, and **(J)** show tangential sections. Arrowheads indicate NPCs. Bars in **(A)** to **(C)** = 0.1 μm; scale in **(D)** to **(H)** is the same as in **(C)**. The bar in **(I)** = 0.5 μm; scale is the same for **(J)**. im, inner membrane; om, outer membrane; n, nucleus; no, nucleolus.

Several studies have demonstrated that the O-GlcNAc NPC proteins are involved in protein import. Agents that bind to these glycoproteins, such as WGA, antibodies that recognize this protein family, and antibodies specific for p62 block nucleocytoplasmic transport (Finlay et al., 1987; Yoneda et al., 1987; Dabauvalle et al., 1988; Featherstone et al., 1988; Newmeyer and Forbes, 1988; Wolff et al., 1988). Moreover, depletion of O-GlcNAc proteins from NPCs reconstituted in Xenopus extracts inhibits transport in vitro by preventing the formation of transport-competent nuclei and NPCs (Dabauvalle et al., 1990; Finlay and Forbes, 1990; Finlay et al., 1991; Miller and Hanover, 1994). Studies by Sterne-Marr et al. (1992) suggest that a cytosolic factor essential for nuclear import binds to Nup153p, a mammalian O-GlcNAc NPC protein. The WGA binding protein, Nup98p, has also been implicated to function as a docking protein for cytosol-mediated binding of import substrate in vitro (Radu et al., 1995a, 1995b). Although the family of O-GlcNAc proteins has been shown to be involved in import, the exact role of O-GlcNAc sugar modification in nuclear transport is not fully understood (Miller and Hanover, 1994). Interestingly, the O-GlcNAc modification has not been observed in any yeast NPC protein (Rout and Wente, 1994). In addition, WGA has no effect on nuclear import in yeast (Kalinich and Douglas, 1989). These observations indicate that differences exist in nuclear transport and NPCs of different organisms.

In plants, little is known about the nuclear transport machinery (for review, see Raikhel, 1992; Hicks and Raikhel, 1995b) or about NPC proteins and their function in nuclear transport. Although the plant NPC has been morphologically described for several decades (Roberts and Northcote, 1970; for review, see Jordan et al., 1980), almost no further characterization of plant NPC components has been accomplished. Scofield et al. (1992) reported that a 100-kD protein, identified using antibodies raised against the yeast NPC protein Nsp1p (Hurt, 1988), is enriched in nuclear matrix preparations of carrot suspension-cultured cells and is located at the plant NPC. Recently, binding of nuclear localization signals (NLSs) was observed to a low-affinity binding site that is proteinaceous and firmly associated with the nuclear envelope (NE) and NPCs of isolated dicot and monocot nuclei (Hicks and Raikhel, 1993; Hicks et al., 1995). Cross-linking studies under conditions similar to those used for NLS binding identified several polypeptides that bind specifically to functional NLSs. The affinities and the biochemical properties of these polypeptides are similar to those of the NLS binding site (Hicks and Raikhel, 1995a). These data indicate that in plants, components that recognize functional NLSs may be located at the plant NPC.

Using an immunofluorescence in vitro nuclear import system, we have found that WGA does not inhibit protein import into the plant nucleus, although the lectin binds to the nuclear surface (Hicks and Raikhel, 1995b). In the current study, we investigated the WGA binding sites at the nuclear surface in plants and demonstrated that proteins at the NPC of tobacco suspension-cultured cells are detected by WGA and therefore are modified by GlcNAc. The plant glycoproteins have unusual oligosaccharide modifications that are discussed and compared with those of vertebrate NPC proteins modified by a single O-GlcNAc.

RESULTS

Proteins at the NPC Are Modified by GlcNAc

Nuclei were purified from tobacco suspension-cultured cells in the presence of either 0.01% or 0.6% Triton X-100 by multiple Percoll gradients (see Methods). As shown by electron microscopy, the structural integrity of the purified nuclei was preserved (Figure 1B). Nuclei treated with 0.01% detergent were surrounded by two distinct membranes, the outer and inner membrane of the NE (Figures 1A to 1D; longitudinal sections). In longitudinal sections, the NPCs were visible as channels embedded in the NE (Figures 1A, 1C, and 1D). The typical cylindrical shape of the NPCs was apparent in tangential sections (Figures 1E and 1F), and the central transporter was present as a dark central granule in some NPCs. Because some other organelles were present in the nuclear fraction isolated with 0.01% detergent, 0.6% Triton X-100 was included in the nuclei isolation buffer. As previously reported by Willmitzer and Wagner (1981), the addition of high-detergent concentration eliminates contaminating organelles by lysis. Because the nuclear integrity does not rely on the NE, nuclei remain intact under these conditions (Aaronson and Blobel, 1974). Based on light and electron microscopy, the nuclear preparation treated with 0.6% Triton X-100 appeared to be free of contaminating organelles, such as vesicles, chloroplasts, and mitochondria, and was of purity equivalent to that achieved in other studies (Willmitzer and Wagner, 1981; Saxena et al., 1985). Treatment with 0.6% detergent did not appear to change the ultrastructure of the nuclei (Figures 1G to 1J). As expected, most of the outer and inner NEs were removed by the detergent (Figures 1G and 1H). The absence of the NE made it difficult to observe the NPCs in longitudinal sections (Figures 1G and 1H). However, the NPCs were visible in tangential sections (Figures 1I and 1J), and their overall morphology was preserved. This confirms observations from studies that examined animal nuclei isolated in the presence of high detergent (Aaronson and Blobel, 1974; Reichelt et al., 1990). It is possible, however, that some peripheral components of the NPC may be lost during this isolation procedure.

To examine the WGA binding sites, purified nuclei were incubated with a WGA probe. In contrast with animals, plants do not appear to contain sialic acid moieties (Corfield and Schauer, 1982). Therefore, in plants, the WGA probe is specific for GlcNAc and its oligomers, such as chitotriose. The tobacco nuclei were incubated with WGA-conjugated colloidal gold particles, fixed, and examined by electron microscopy (Figure 1). Most of the WGA labeling was associated with the periphery of nuclei treated with low or high detergent (Figures 1A, 1C, and 1G; longitudinal sections). Little binding of WGA

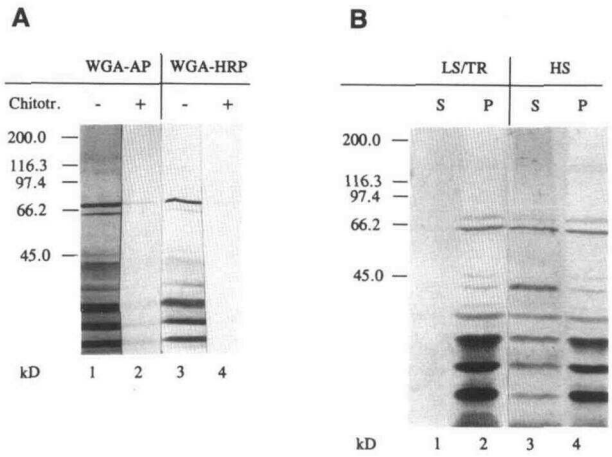

Figure 2. Biochemical Characterization of Tobacco Nuclear Proteins Modified by GlcNAc.

(A) Nuclear proteins were treated with DNase I and subjected to protein gel blot analysis using WGA-AP and WGA-HRP. Equal numbers of nuclei (1.5×10^6) were loaded in each lane. Lane 1 contains WGA-AP; lane 2, WGA-AP plus chitotriose (Chitotr.); lane 3, WGA-HRP; lane 4, WGA-HRP plus chitotriose. (−), without chitotriose; (+), plus chitotriose.

(B) Nuclear proteins treated with DNase I were solubilized in either a 0.02 M KCl/2% Triton X-100 (LS/TR) or a 0.5 M NaCl (HS) buffer and separated into a soluble (S) and an insoluble (P) fraction. The equivalent of 5×10^6 nuclei per treatment was subjected to protein gel blot analysis using WGA-AP. Lanes 1 and 2, LS/TR treatment; lanes 3 and 4, HS treatment.

Proteins were resolved by electrophoresis on 10% SDS–polyacrylamide gels. Mass standards in kilodaltons are as indicated.

was observed at internal nuclear structures. As specifically apparent in nuclei surrounded by the NE, WGA binding was often found at or adjacent to NPCs (Figure 1C); an enlarged NPC labeled by WGA is shown in Figure 1A. In particular, the rings and the central regions of the NPCs appeared to be labeled by WGA, using either nuclear preparation (Figures 1E and 1I; tangential sections). Individual NPCs were often labeled by several gold particles, suggesting multiple binding sites at each NPC. The association of WGA to the nuclear periphery and, in particular, to the NPCs seemed to be higher in nuclei treated with 0.6% Triton X-100 (Figures 1G and 1I). Higher concentrations of detergent may have solubilized some components of the NPC and thereby exposed proteins modified by GlcNAc that were not accessible to the probe following low-detergent isolation. WGA binding was specific because it was reduced in the presence of chitotriose, a specific competitor of WGA binding (Figures 1D, 1F, 1H, and 1J). These results indicate that proteins modified by GlcNAc are present at the periphery of the tobacco nucleus, and some of these glycoproteins are components of the NPC.

Nuclear Proteins Modified by GlcNAc Are Extracted by Salt

Nuclei isolated in the presence of 0.6% Triton X-100 were used as material for all biochemical studies for two reasons. As shown by electron microscopy, these nuclei were devoid of contaminating organelles. In addition, their NPCs appeared to be structurally preserved and contained proteins modified by GlcNAc. To obtain more information about these glycoproteins, nuclei were treated with DNase I, and proteins were subjected to protein gel blot analysis (Figure 2A). Proteins of similar mass were detected using two different WGA probes: WGA–alkaline phosphatase (WGA-AP; Figure 2A, lane 1) and WGA–horseradish peroxidase (WGA-HRP; Figure 2A, lane 3). WGA binding was greatly reduced in the presence of 2 mM chitotriose (Figure 2A, lanes 2 and 4), but it was unaffected by 250 mM mannose (data not shown), indicating that binding was specific. These results demonstrate that several nuclear proteins are modified by GlcNAc.

The nuclear glycoproteins were characterized further by the following treatment. Nuclear proteins were solubilized by either a low-salt/Triton X-100 (LS/TR) buffer (0.02 M KCl, 2% Triton X-100) or a high-salt (HS) buffer (0.5 M NaCl) and separated into soluble and insoluble fractions by centrifugation. The protein equivalent of 5×10^6 nuclei per treatment was analyzed by protein gel blot analysis, using WGA-AP as a probe (Figure 2B). Seven of eight detected proteins were partially extracted by the HS buffer (Figure 2B, lane 3), although the majority of the proteins with lower apparent mass (~20 to 30 kD) were still associated with the insoluble fraction (Figure 2B, lane 4). None of the proteins was extracted by the LS/TR buffer (Figure 2B, lane 1). These fractionation data show that exposure to salt destabilized the interaction of these glycoproteins with other nuclear components, whereas exposure to a high percentage of nonionic detergent had no effect. A similar HS buffer containing 0.5 M salt is used to extract the O-GlcNAc NPC proteins from vertebrates. These O-GlcNAc proteins are also only partially solubilized under these conditions (Davis and Blobel, 1986; Snow et al., 1987), with an efficiency similar to that of nuclear glycoproteins from plants. The basis of this extraction is not understood (Davis and Blobel, 1986), and a higher salt concentration does not seem to solubilize a much higher percentage of NPC glycoproteins (Davis and Blobel, 1986; Snow et al., 1987). Overall, the results obtained by extraction and electron microscopic analysis indicate that at least some of the plant nuclear proteins modified by GlcNAc are located at the NPC.

Nuclear Proteins Are Modified by Terminal GlcNAc

Because WGA can bind to terminal as well as internal GlcNAc residues (Ebisu et al., 1977), a different approach was used to obtain more information about the sugar modification. We employed an in vitro–labeling procedure using galactosyltransferase (GalTF; Roquemore et al., 1994). This enzyme specifically

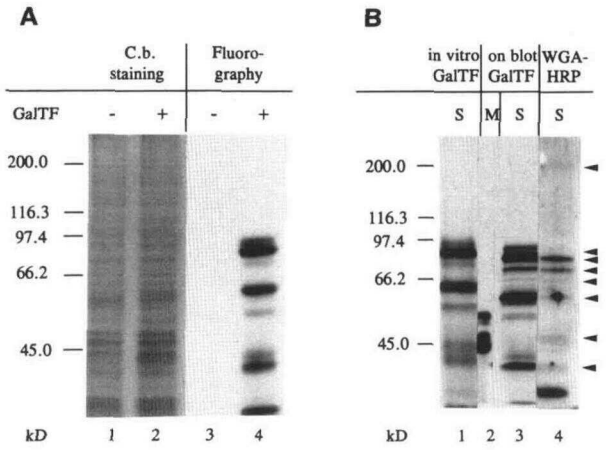

Figure 3. Detection of Salt-Extracted Nuclear Proteins Modified by Terminal GlcNAc Using GalTF Labeling Assays and WGA Blot Assay.

(A) *In vitro* GalTF labeling assay. Nuclear proteins were treated with DNase I and solubilized with 0.5 M NaCl. Equal amounts of solubilized proteins (30 μg) were incubated in the presence (+) or absence (−) of GalTF with UDP-^{3}H-galactose. Radiolabeled proteins were visualized by fluorography for 12 hr. Labeling in the absence of the GalTF is shown in lane 1 (Coomassie-blue–stained gel) and lane 3 (fluorography). Labeling in the presence of the GalTF is shown in lane 2 (Coomassie blue–stained gel) and lane 4 (fluorography). C.b., Coomassie blue.

(B) In vitro, on-the-blot GalTF labeling assays and WGA blot analysis. Nuclear proteins were solubilized as described in **(A)** and treated as follows. In lane 1, proteins were subjected to the in vitro GalTF labeling assay as given in **(A)**. Lane 2 shows the results of the on-the-blot GalTF labeling assay with protein mass standards. Proteins were immobilized on Immobilon-P membranes. One-half of the lane was subjected to the on-the-blot GalTF labeling assay in the presence of GalTF and then visualized by fluorography for 43 hr. The other half of the lane was subjected to the on-the-blot GalTF labeling assay in the absence of GalTF; no labeling of ovalbumin was observed (data not shown). Ovalbumin (which runs as a doublet on SDS–polyacrylamide gels, positive control), 45 kD; BSA (negative control), 66.2 kD. Lane 3 shows the results of the on-the-blot GalTF labeling assay with salt-extracted nuclear proteins. Solubilized proteins of 2 × 10^6 nuclei were treated as described for lane 2. The entire lane was subjected to the on-the-blot GalTF labeling assay in the presence of GalTF and visualized as given for lane 2. Lane 4 shows the results of the WGA blot analysis with salt-extracted nuclear proteins. Solubilized proteins (equal amount as given for lane 3) were subjected to protein blot analysis and detected by WGA-HRP using chemiluminescence. Arrowheads indicate proteins detected by both the on-the-blot GalTF labeling assay and WGA blot analysis.

Proteins were resolved by electrophoresis on 7.5% SDS–polyacrylamide gels. Mass standards in kilodaltons are as indicated. M, protein mass standard; S, salt-extracted proteins.

transfers the ^{3}H-galactose moiety of UDP-^{3}H-galactose to terminal GlcNAc residues of glycoproteins (Whiteheart et al., 1989; Roquemore et al., 1994). Nuclear proteins were extracted with the HS buffer and labeled in vitro with ^{3}H-galactose by using GalTF (Figure 3A, lanes 2 and 4). A control reaction in the absence of GalTF was performed to show that labeling was specific to GalTF and not to endogenous GalTF activity associated with the nuclei (Figure 3A, lanes 1 and 3). The radiolabeled proteins were visualized by fluorography, and at least eight proteins were specifically labeled by the GalTF (Figure 3A, lane 4). In particular, two proteins having apparent masses of 60 and 90 kD (after ^{3}H-galactose incorporation) did not appear to be highly abundant proteins by the Coomassie Brilliant Blue R 250–stained protein profile (Figure 3A, lane 2), suggesting the presence of multiple terminal GlcNAc moieties on these proteins.

Due to the incorporation of ^{3}H-galactose, the mobility of proteins decreases following SDS-PAGE after in vitro GalTF labeling (Snow et al., 1987; Miller and Hanover, 1994). Hence, it was not possible to align directly glycoproteins detected by the in vitro GalTF labeling assay with proteins detected by WGA blot analysis. We therefore established an on-the-blot GalTF labeling assay in which proteins immobilized on membranes were incubated with UDP-^{3}H-galactose and GalTF under conditions that were similar to those used in the in vitro GalTF labeling assay (Figure 3B). Under these conditions, ^{3}H-galactose was incorporated after immobilization of proteins on the blot; thus, the apparent mass of the radiolabeled proteins did not change. Proteins present in the mass standards served as positive and negative controls for the on-the-blot GalTF labeling assay (Figure 3B, lane 2). Ovalbumin is a 45-kD protein modified by N-linked terminal GlcNAc moieties and functioned as a positive control (see also Holt and Hart, 1986; Hanover et al., 1987). BSA (66.2 kD) is not modified by terminal GlcNAc and served as a negative control. The pattern of the galactosylation products using the on-the-blot GalTF labeling assay (Figure 3B, lane 3) was similar to that of the in vitro GalTF labeling assay (Figure 3B, lane 1).

These results demonstrated that the on-the-blot GalTF labeling assay is an easy and fast method for detecting proteins modified by terminal GlcNAc. More importantly, it allowed a direct comparison of proteins containing terminal GlcNAc (Figure 3B, lane 3) with the glycoproteins detected by WGA blot analysis (Figure 3B, lane 4). The majority of the proteins were detected by both methods but with different intensities (indicated by arrowheads). The glycoproteins with a lower apparent mass (~20 to 30 kD) and detected by the WGA probes were also labeled in the on-the-blot GalTF assay, although some with low intensity (data not shown). These correlative studies indicate that the majority of the nuclear proteins detected by WGA are modified by terminal GlcNAc, and some of these proteins with terminal GlcNAc are likely to be NPC proteins.

Salt-Extracted Nuclear Proteins Are Modified by Oligosaccharides with Terminal GlcNAc

The ^{3}H-galactose–labeled saccharides can be attached to proteins via the hydroxyl group of a serine, threonine, tyrosine, or hydroxyproline (O linked) or via the amido group of an

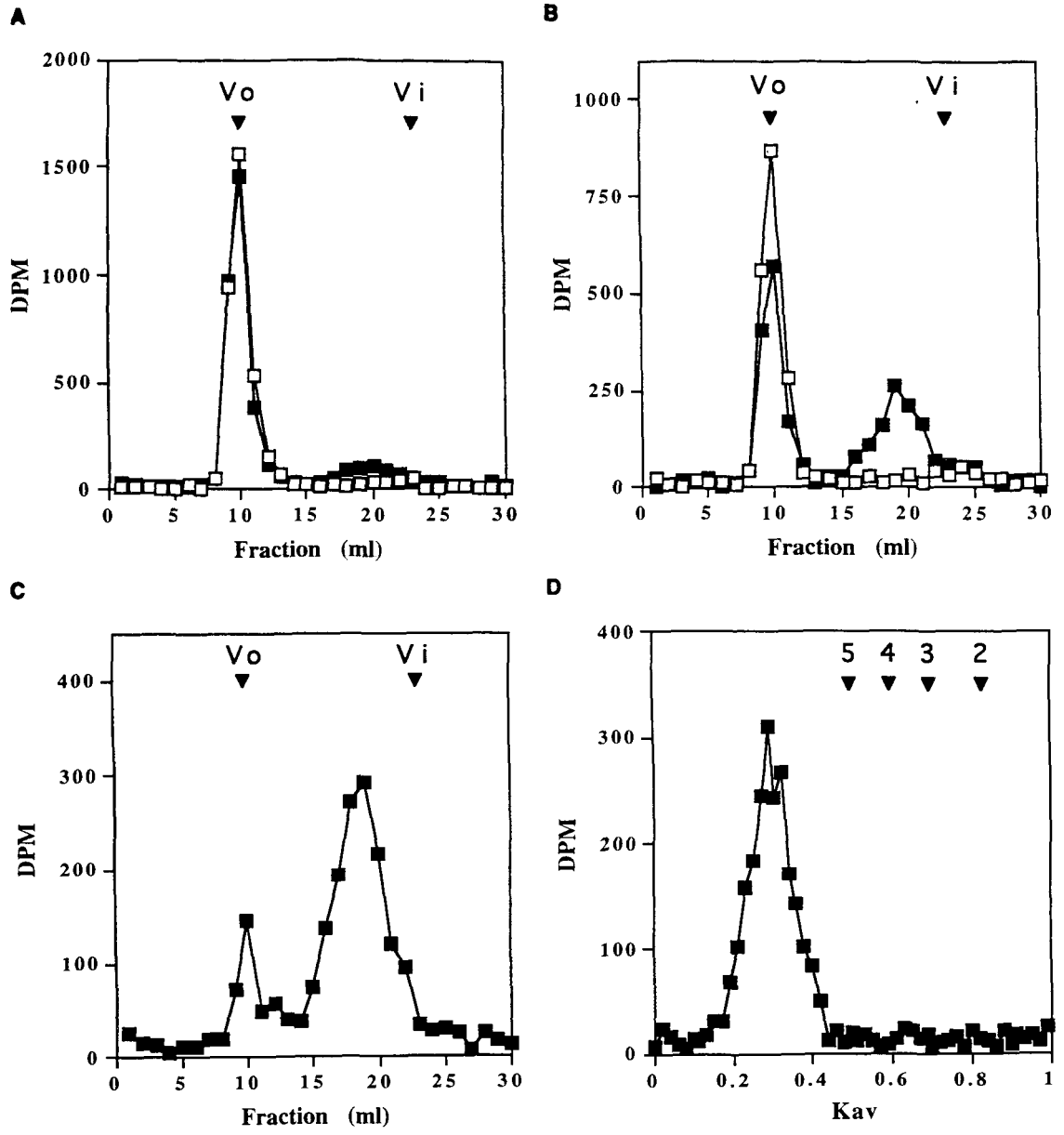

Figure 4. Characterization of Carbohydrates with Terminal GlcNAc on Salt-Extracted Nuclear Glycoproteins.

(A) Carbohydrate linkages resistant to PNGase F. Salt-extracted, ^{3}H-galactose–labeled nuclear proteins (5 × 10^{5} dpm) were incubated with (filled squares) or without (open squares) PNGase F and fractionated over a Sephadex G-50 column. Fractions (0.5%) were assayed for radioactivity by liquid scintillation counting.

(B) Plant protein samples not inhibiting PNGase F activity. A 1:1 ratio (by radioactivity) of ^{3}H-galactose–labeled ovalbumin and ^{3}H-galactose–labeled nuclear proteins was incubated with (filled squares) or without (open squares) PNGase F, and the products were analyzed as given in **(A)**.

(C) β Elimination released saccharides from PNGase F–resistant glycoproteins. PNGase F–resistant and PNGase F–labeled nuclear proteins (fractions 8 to 13 from **[A]**) were pooled and subjected to alkaline-induced β elimination. Products were fractionated over a Sephadex G-50 column. One percent of the fractions were assayed for radioactivity.

(D) Saccharides released from glycoproteins by β elimination that were larger than five GlcNAc residues. Released labeled saccharides (fractions 14 to 23 from **[C]**) were pooled and fractionated on a Toyopearl HW 40 column. Five percent of the fractions were assayed for radioactivity. Numbers above the solid triangles represent migration positions of oligosaccharide standards: 2, Gal-GlcNAcitol; 3, Gal-(GlcNAc)-GlcNAcitol; 4, Gal-$(GlcNAc)_2$-GlcNAcitol; 5, Gal-$(GlcNAc)_3$-GlcNAcitol.

Kav, volume fraction (Ve – Vo/Vi – Vo); Vi, inclusion volume; Vo, void volume.

asparagine (N linked). To examine these alternatives, nuclear proteins extracted with the HS buffer were used for the subsequent sugar analysis for the following reasons. Seven of eight glycoproteins were present in the solubilized fraction, even though their extraction was not complete. In addition, the subsequent in vitro GalTF labeling assay requires that the protein sample be fully solubilized and of an ionic strength of <0.2 M (Roquemore et al., 1994). The extracted proteins were radiolabeled during the in vitro GalTF labeling assay, and the protein sample was divided into equal aliquots and subjected to the following treatments. One aliquot was digested with peptide: *N*-glycosidase F (PNGase F; Figure 4A), an enzyme that releases the common classes of N-linked glycans (Tarentino et al., 1985). The second aliquot was treated similarly except without PNGase F (Figure 4A) to control for proteolysis during the PNGase F experiment. The elution profiles of these samples over a Sephadex G-50 column were identical, indicating that virtually no radioactivity associated with the proteins was removed by the PNGase F (Figure 4A). Parallel experiments using ^{3}H-galactose–labeled ovalbumin, a protein containing N-linked terminal GlcNAc, were performed as a positive control for PNGase F activity. PNGase F removed 75% of the radioactivity associated with ^{3}H-galactose–labeled ovalbumin (data not shown). Furthermore, PNGase F activity was not inhibited by any component in the plant sample because the enzyme released 32% of the radioactivity associated with a 50:50 mixture (by radioactivity) of ^{3}H-galactose–labeled ovalbumin and salt-extracted nuclear proteins (Figure 4B). This value was consistent with a 64% cleavage of the ovalbumin glycans in this control group.

O-glycosidic linkages are sensitive to alkaline-induced β elimination (Spiro, 1972). Radiolabeled proteins resistant to PNGase F treatment (Figure 4A) were subjected to alkaline borohydride to release O-linked sugars from the proteins (Figure 4C). Approximately 80% of the ^{3}H-galactose–labeled sugars were released from the nuclear proteins as determined by Sephadex G-50 chromatography (Figure 4C, fractions 14 to 23). Twenty percent of the radioactivity remained in the void volume (Figure 4C), indicating that these labeled sugars were on large polysaccharides or still associated with the proteins. These labeled sugars may belong to a small group of plant N-glycans that appear to be resistant to PNGase F treatment (Tretter et al., 1991). The same profile of β-eliminated sugars was observed, whether the samples were subjected to β elimination with or without prior digestion with PNGase F. These results indicate that at least 80% of the ^{3}H-galactose–labeled sugars are attached to the salt-extractable proteins via an O-linkage.

To determine the size of these labeled glycans, β-eliminated sugars (Figure 4C, fractions 14 to 23) were fractionated on a Toyopearl HW 40 size exclusion column (Figure 4D). Oligosaccharide standards composed of Gal-GlcNAc$_{(1-3)}$-GlcNAcitol were used to assess size (but not to indicate structure). The radiolabeled sugars migrated at a position larger than five saccharides, consisting of Gal-GlcNAc$_3$-GlcNAcitol. None of the radioactivity comigrated with the disaccharide Gal-GlcNAcitol, as would be expected if the sugars were a single O-GlcNAc modification typical of vertebrate NPC proteins. The elution profile appeared to consist of more than one peak, suggesting the presence of multiple-size populations of ^{3}H-galactose–labeled oligosaccharides. Forty percent of these labeled oligosaccharides adsorbed to a Sep-Pak (C18) cartridge, indicating the presence of a hydrophobic group (data not shown). The remaining 60% were not retained on the column and were resistant to Pronase digestion and dansylation, indicating the absence of peptides or primary amines (data not shown). These results are consistent with those labeled oligosaccharides associated with the nuclear glycoproteins via an O-linkage.

These studies indicate that the proteins extracted by high salt from tobacco nuclei are modified by oligosaccharides larger in size than five GlcNAc residues, including the terminal GlcNAc. Most of these are bound to the proteins via an O-linkage. Fractionation and electron microscopy data indicate that at least some of these glycoproteins are located at the NPC.

DISCUSSION

We identified and characterized a subset of nuclear proteins from tobacco suspension-cultured cells that contain novel carbohydrate modifications. Probing isolated nuclei with WGA revealed the presence of GlcNAc-containing proteins that are associated with the rings or central structures of plant NPCs. Seven of eight glycoproteins detected by WGA were partially extracted by 0.5 M salt, and the majority of these glycoproteins were modified by terminal GlcNAc(s), as shown in GalTF labeling assays. Carbohydrate analysis demonstrated that the glycans with terminal GlcNAc consist of sugars that are larger in size than five GlcNAc residues, and most of these oligosaccharides appear to be attached to the proteins via an O-linkage.

Proteins Modified by GlcNAc Are Located at the NPC

In vertebrates, WGA is a potent inhibitor of protein import into the nucleus (Finlay et al., 1987; for review, see Forbes, 1992). This lectin binds to the single O-GlcNAc present on several NPC proteins and was successfully used to identify and purify some of these NPC glycoproteins (Finlay et al., 1991; Hallberg et al., 1993; Kita et al., 1993; Sukegawa and Blobel, 1993; Powers et al., 1995; Radu et al., 1995b). Interestingly, WGA does not appear to bind to yeast NPCs (Davis and Fink, 1990; Carmo-Fonseca et al., 1991; Rout and Blobel, 1993), and so far, none of the identified NPC proteins in yeast has been shown to be modified by O-GlcNAc. In plants, the nature of sugar modification on NPC proteins is not known.

Using isolated nuclei from tobacco cells, several nuclear proteins were detected by WGA. At least some of these nuclear glycoproteins are probably NPC proteins using the following criteria: (1) WGA labeling was associated with or adjacent to the NPC, with little binding to other structural components of

the nucleus; (2) treatment of nuclei with DNase I did not release any of the nuclear proteins modified by GlcNAc (A. Heese-Peck and N.V. Raikhel, unpublished results); (3) the proteins detected by WGA were enriched in the nuclear fraction but were not detectable in the cytoplasmic fraction (A. Heese-Peck and N.V. Raikhel, unpublished results); and (4) the plant nuclear glycoproteins were solubilized under the same salt conditions and with a similar efficiency as the O-GlcNAc NPC proteins from vertebrate nuclei (Davis and Blobel, 1986; Snow et al., 1987).

Based on electron microscopy using WGA as a probe, the plant NPC glycoproteins were found mainly at the rings and the center of the NPCs. As was apparent in longitudinal sections, these glycoproteins appeared to be present at the cytoplasmic and, though in lower amounts, the nucleoplasmic sides of the NPCs. Using a similar embedding/thin sectioning preparation, WGA labels the cytoplasmic and the nucleoplasmic centers of rat NPCs (Hanover et al., 1987) and almost exclusively the cytoplasmic center of Xenopus oocyte NPCs (Finlay et al., 1991). Quick freezing/freeze drying/rotary metal shadowing of Xenopus oocyte nuclei showed WGA labeling at the terminal ring of the nuclear baskets as well as at the center and inner annulus of the cytoplasmic face (Panté et al., 1994). An O-GlcNAc protein from vertebrates, Nup153p, has been shown to be a constituent of nucleoplasmic filaments and the terminal ring of the nucleoplasmic baskets (Cordes et al., 1993; Panté et al., 1994). Nup153p contains four Cys-Cys–type zinc fingers capable of binding DNA in vitro and has been proposed to function in chromatin organization (Sukegawa and Blobel, 1993) or in opening/closing of the baskets (Panté et al., 1994). Recently, another nucleoplasmic NPC protein with the ability to bind to WGA, Nup98p, has been identified and shown to interact with cytosolic import factors in vitro (Radu et al., 1995b). However, Powers et al. (1995) showed, by using reconstituted Xenopus nuclei depleted of Nup98p, that this NPC protein is not essential for nuclear import in vivo. A putative O-GlcNAc protein, p250, has been localized to the cytoplasmic filaments of the NPC using a polyclonal antibody (Panté et al., 1994) and may correspond to the rat Nup214p/CAN protein (Kraemer et al., 1994).

It is possible that several of the plant glycoproteins are homologs of these vertebrate NPC proteins, although the apparent mass of most plant proteins is different from that of the O-GlcNAc NPC proteins. The mass of the 55-kD plant GlcNAc protein (60 kD after ^{3}H-galactose incorporation) raises the possibility that it may be the plant homolog of vertebrate p62. p62 is found in multiple copies on the cytoplasmic and/or the nucleoplasmic faces of the central NPC regions (Davis and Blobel, 1986; Dabauvalle et al., 1990; Cordes et al., 1991; Wilken et al., 1993). However, a monoclonal antibody against rat p62 (mAb414; Davis and Blobel, 1986) did not detect plant NPC proteins of similar mass (A. Heese-Peck and N.V. Raikhel, unpublished results). In fact, mAb414 recognized an antigen associated with tobacco chromatin but not with the NPC (A. Heese-Peck, O.N. Borkhsenious, and N.V. Raikhel, unpublished results). This monoclonal antibody has also been shown to detect NPC as well as non-NPC proteins in yeast (Aris and Blobel, 1989; Davis and Fink, 1990). A study using higher plants reported the detection of a 100-kD protein by mAb414 in nuclear matrix fractions of carrot suspension-cultured cells after extensive extraction with 1 M salt (Scofield et al., 1992); however, no electron microscopy data using mAb414 were provided. We investigated whether other antibodies against known NPC proteins recognized plant nuclear proteins, specifically the nuclear proteins modified by terminal GlcNAc. However, no specific labeling of the tobacco NPC was observed by using electron microscopy (A. Heese-Peck, O.N. Borkhsenious, and N.V. Raikhel, unpublished results).

Oligosaccharides with Terminal GlcNAc

We demonstrated that the 0.5 M salt-extracted nuclear proteins from tobacco contain a novel sugar modification that is different from the O-GlcNAc modification found on vertebrate NPC proteins (Figure 5). The vertebrate NPC proteins are solubilized under similar salt conditions and are modified by single O-GlcNAc(s) attached to the proteins via an O-linkage (Hanover et al., 1987; Holt et al., 1987). Sugar analysis indicates that salt-extractable plant nuclear proteins are modified by oligosaccharides consisting of saccharides larger in size than five GlcNAc residues, and most of these oligosaccharides are also attached to the proteins via an O-linkage. At least two size populations of oligosaccharides possessing terminal GlcNAc were attached to the nuclear proteins. Currently, it is not known whether these oligosaccharides share a common core structure. To perform such detailed carbohydrate analysis, the purification of individual proteins in large quantities is required and will be pursued in the future. Thus, probing plant nuclear proteins with the GalTF indicates that the single O-GlcNAc modification present on vertebrate NPC proteins is not found on salt-extracted nuclear proteins from plants.

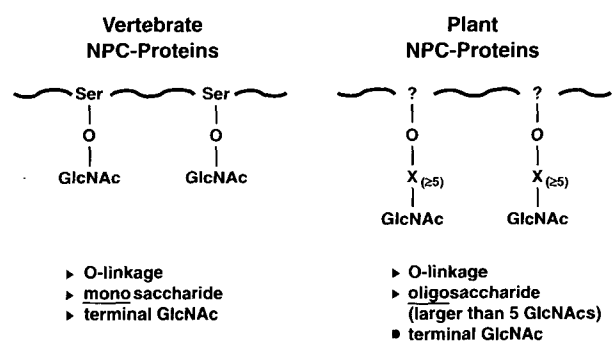

Figure 5. Comparison of O-GlcNAc from Vertebrate NPC Proteins and O-Linked Oligosaccharides with Terminal GlcNAc from Plant Nuclear and NPC Proteins.

Question mark, unknown amino acid (probably serine, threonine, or hydroxyproline); X, unknown saccharides (in size, larger than or equal to five GlcNAcs).

We can only speculate about the function of the oligosaccharides in plant NPC proteins. The O-linked oligosaccharides may have a function similar to that proposed for the O-GlcNAc found in vertebrate NPC proteins. These include the assembly of multimeric protein complexes, resistance to proteolysis, and regulation of protein function (Hart et al., 1989; Haltiwanger et al., 1992). In addition, O-GlcNAc modification of vertebrate NPC proteins has been proposed to function in nucleocytoplasmic transport because WGA inhibits transport of protein and RNA by binding to O-GlcNAc (Finlay et al., 1987; Yoneda et al., 1987; Dabauvalle et al., 1988; Featherstone et al., 1988; Newmeyer and Forbes, 1988; Wolff et al., 1988). Interestingly, Miller and Hanover (1994) have shown in reconstitution assays that NPC proteins altered by the addition of galactose to O-GlcNAc residues are still able to form NPCs. These NPCs are morphologically normal and are competent to import proteins into the nucleus. Thus, it has been suggested that a specific recognition of the GlcNAc moiety may not play a direct role in nuclear protein import and in NPC assembly. This implies that the protein portion rather than the carbohydrate moiety of these glycoproteins may function in nuclear import. Furthermore, yeast nuclei are able to import proteins, although the NPC proteins do not appear to be modified by O-GlcNAc. In plants, Harter et al. (1994) reported that the nuclear import of endogenous G-box binding factors (GBFs) is inhibited in the presence of WGA in permeabilized parsley cells. In these studies, imported GBFs are detected by an antibody cotranslocation assay that is indirect and based on the detection of protease-resistant GBF antibody associated with the nuclei.

An in vitro import system was developed in our laboratory in which the accumulation of import substrate can be visualized by immunofluorescence. Using this import assay, WGA (0.7 mg/mL) does not block nuclear import in vitro (Hicks and Raikhel, 1995b; G.R. Hicks, S. Lobreaux, and N.V. Raikhel, unpublished results). However, as demonstrated in the current study, proteins at the plant NPC were modified by GlcNAc. These NPC proteins appeared to be modified by carbohydrates that are larger than the single O-GlcNAc of vertebrate NPC proteins. The larger carbohydrate moieties may extend farther away from the NPC center, so that the binding of WGA to the GlcNAc does not hinder protein import into the plant nucleus, although import of larger substrates such as immunoglobin (Harter et al., 1994) could be affected. Although the carbohydrates with terminal GlcNAc do not appear to be involved in nuclear import, it cannot be excluded that the proteins themselves may play a role in the import process. At this point, the significance of the GlcNAc modification is not understood in any system studied. However, this modification has been proven to be a useful tool to identify and isolate NPC proteins involved in nucleocytoplasmic transport.

The oligosaccharides with terminal GlcNAc may also aid in purification of the plant NPC glycoproteins. Preliminary results indicate that several of the plant glycoproteins can be purified by lectin affinity chromatography. The purification of the glycoproteins will enable us to produce specific antibodies and to localize the glycoproteins in situ. We will also investigate whether any of the plant NLS binding proteins that may be localized to the plant NPC (Hicks and Raikhel, 1993, 1995a) correspond to one of the glycoproteins identified in this study.

METHODS

Materials

All chemicals were purchased from Sigma unless otherwise noted.

Cell Culture and Nuclear Purification

Nicotiana tabacum suspension-cultured cells were maintained and subcultured, and protoplasts were prepared from 6- to 7-day cell cultures essentially as described by Bednarek et al. (1990). The purification of tobacco nuclei was based on the methods of Willmitzer and Wagner (1981) and Saxena et al. (1985). Isolation of nuclei in the presence of 0.01% Triton X-100 was performed as described by Hicks and Raikhel (1993), and the isolation of nuclei in the presence of 0.6% Triton X-100 was performed as follows: protoplasts from two 250-mL cultures were pelleted at 50*g* for 4 min at room temperature. All subsequent steps were performed at 4°C. All buffers containing Percoll were adjusted to a pH of 5.6. The following concentrations of protease inhibitors were present during all steps: 5 μg/mL pepstatin, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 5 μg/mL aminocaproic acid, 0.4 mM phenylmethylsulfonyl fluoride. The pellet was resuspended in 50 mL of 0.01% Triton X-100 (Boehringer Mannheim) in nuclear isolation buffer (NIB; 10 mM 2-(*N*-morpholino)ethanesulfonic acid-KOH, pH 5.6, 0.2 M sucrose, 10 mM NaCl, 10 mM KCl, 2.5 mM EDTA, 2.5 mM DTT, 0.1 mM spermine, 0.5 mM spermidine) and placed on ice for 10 min. Protoplasts were ruptured by passage through a 21-gauge needle six times. The lysate was centrifuged for 4 min onto a cushion of 67% Percoll in NIB at 1000*g*, and the interface material was diluted with 100 mL of 0.6% Triton X-100 in NIB. The lysate was incubated on ice for 20 min and filtered through a 20-μm nylon mesh; it was then divided and layered onto six-step gradients consisting of 1 mL of 67% Percoll in NIB and 5 mL of 7.5% Percoll in NIB. The gradients were centrifuged at 1000*g* for 4 min, and the interfaces between the Percoll phases were combined, diluted with 100 mL of 0.6% Triton X-100 in NIB, and incubated on ice for 10 min. The step gradient was repeated, and the interfaces were combined and diluted with 50 mL of 0.6% Triton X-100 in NIB and loaded onto another step gradient. The interfaces containing the purified nuclei were washed in nuclear storage buffer (20% glycerol in NIB) and pelleted at 750*g* for 5 min. After another washing and pelleting step, the nuclei were quantitated as described by Hicks and Raikhel (1993) and stored at −80°C. Yields were 4 to 6 × 10^8 nuclei.

Isolation and Fractionation of Nuclear Proteins

Isolated nuclei were incubated with DNase I (Boehringer Mannheim) at a concentration of 30 units per 10^6 nuclei in the presence of 5 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$ for 30 min at room temperature and pelleted at 12,000*g* for 5 min. The DNase I–treated nuclear pellet was resuspended in SDS sample buffer or subjected to further extraction by incubation in a low-salt plus detergent (LS/TR) buffer (2% Triton

X-100, 10% sucrose, 20 mM Tris-ethanolamine, pH 7.4, 20 mM KCl, 5 mM $MgCl_2$, 1 mM DTT) or in a high-salt (HS) buffer (0.5 M NaCl, 5 mM $MgCl_2$, 20 mM Tris, pH 7.5) for 30 min on ice. Solubilized proteins were separated from the insoluble fraction by a 15-min centrifugation step (12,000*g*) at 4°C and were precipitated with 10% trichloroacetic acid followed by an acetone wash. The samples were resuspended in SDS sample buffer (Laemmli, 1970).

Protein Blot Analysis Using WGA Probes

Proteins solubilized in SDS sample buffer were electrophoresed on SDS–polyacrylamide gels and transferred to Immobilon-P membranes (Amersham) according to standard methods (Harlow and Lane, 1988). Unless noted otherwise, blots were blocked overnight in 5% nonfat dry milk in Tris-buffered saline plus 0.1% Tween 20 (TBST), incubated for 2.5 hr with 1 μL/mL wheat germ agglutinin–alkaline phosphatase (WGA-AP; EY-Laboratories, San Mateo, CA) or 1 μg/mL WGA–horseradish peroxidase (WGA-HRP; EY-Laboratories) in TBST, washed twice in TBST and twice in TBS for 10 min each, and developed according to standard methods (Harlow and Lane, 1988). Control blots were treated identically except that 2 mM chitotriose (EY-Laboratories) or 250 mM mannose was added during the incubation with the WGA probes. For increased sensitivity, WGA-HRP in combination with the chemiluminescence kit (Boehringer Mannheim) was used to detect proteins. In these experiments, blots were blocked overnight in 3% BSA in TBST, incubated with 0.5 μg/mL WGA-HRP in TBST, washed as described earlier, and developed according to the manufacturer's specifications. The monoclonal antibody mAb414 was a generous gift from L.I. Davis (Duke University Medical Center, Durham, NC).

Electron Microscopy

Purified tobacco nuclei (5×10^5) were diluted with binding buffer (50 mM Tris, pH 7.3, 25 mM KCl, 2.5 mM $MgCl_2$, 3 mM $CaCl_2$, 20% glycerol) to 10^5 nuclei and incubated with WGA-conjugated colloidal gold particles (16 μg/mL; 10-nm diameter; EY-Laboratories) for 20 min at room temperature. Control samples contained 5 mM chitotriose (EY-Laboratories). The nuclei were briefly pelleted, fixed in 2% paraformaldehyde, 1% glutaraldehyde, 50 mM sodium phosphate buffer, pH 7.2, 500 mM sucrose for 1 hr at room temperature, and then postfixed in 1% OsO_4 in 10 mM sodium phosphate buffer, pH 7.2, 50 mM sucrose for 1 hr at room temperature. Sections were stained and visualized as described in Hicks and Raikhel (1993).

Galactosyltransferase Labeling Assays

Bovine milk *N*-acetylglucosamine (GlcNAc) β-1,4-galactosyltransferase (GalTF) was purchased from Sigma or Oxford GlycoSystem (Rosedale, NY). Prior to use, the GalTF from Sigma was autogalactosylated (Roquemore et al., 1994) and stored at −20°C. The GalTF from Oxford GlycoSystem was used as provided by the manufacturer. Equivalent results were obtained with GalTFs from either source. DNase I–treated tobacco nuclei were incubated with 0.5 M NaCl, 50 mM Hepes, pH 6.8, for 1 hr on ice and centrifuged at 12,000*g* for 5 min at 4°C. In vitro GalTF labeling of solubilized proteins was performed essentially as described by Roquemore et al. (1994), using 100 milliunits of GalTF and 3 μCi of UDP-^{3}H-galactose (1.0 mCi/mL; 17.3 Ci/mmol; Amersham) in a 100-μL reaction. After incubation at 37°C for 3 hr, aliquots were taken for protein size analysis on 7.5% SDS–polyacrylamide gels. The gel was treated with Fluoro-Hance (Research Products International Corp., Mt. Prospect, IL), according to the manufacturer's specification, and dried. Labeled proteins were visualized by fluorography, using ReflectionA film (New England Nuclear Research Products, Boston, MA) at −80°C. The remaining samples were used for further carbohydrate analysis and separated from unincorporated label by desalting over a Sephadex G-50 (3-mL) gel filtration column. Radiolabeled proteins were pooled, lyophilized, resuspended in a small volume of deionized water, aliquoted for further analysis, and precipitated with 8 volume acetone for at least 6 hr at −20°C.

For the on-the-blot GalTF labeling assay (Parchment et al., 1986), proteins were solubilized as described for the in vitro GalTF labeling assay. To lower the salt concentration, the sample was diluted with 50 mM Hepes, pH 6.8, and concentrated by Centriprep 10 Concentrator (Amicon, Beverly, MA) at 4°C. SDS sample buffer was added, and the sample was treated as described for protein blot analysis. Blots were blocked overnight with 3% BSA in TBS and equilibrated four times for 10 min in labeling buffer (10 mM Hepes-NaOH, pH 7.3, 10 mM galactose, 5 mM $MnCl_2$). After incubation with 50 milliunits of GalTF and 10 μCi of UDP-^{3}H-galactose in labeling buffer (1-mL reaction) for 3 hr at 37°C, blots were rinsed for 5 min in 10 mM EDTA, 1% SDS, washed four times for 10 min in labeling buffer, dried, sprayed with EN^{3}HANCE (New England Nuclear Research Products), and dried. Radiolabeled proteins were visualized by fluorography as described earlier.

Peptide:*N*-Glycosidase F Treatment

Peptide:*N*-glycosidase F (PNGase F) treatment and Sephadex G-50 column chromatography of salt-extracted, ^{3}H-galactose–labeled nuclear proteins and ^{3}H-galactose–labeled ovalbumin (used as a positive control) were performed as described by Roquemore et al. (1994), with the addition of the following protease inhibitors to the PNGase F reaction buffer: 0.2 mM phenylmethylsulfonyl fluoride, 2 μg/mL antipain, 10 units per mL aprotinin, 10 μg/mL benzamidine, 1 μg/mL leupeptin, 1 μg/mL pepstatin. Fractions (0.5%) from the Sephadex G-50 column were assayed for radioactivity by liquid scintillation counting. PNGase F–resistant proteins in the void volume were lyophilized, resuspended in <300 μL of double-distilled H_2O, and precipitated in 80% cold acetone.

β Elimination of PNGase F–Resistant Nuclear Proteins

Acetone-precipitated PNGase F–resistant, ^{3}H-galactose–labeled nuclear proteins were subjected to alkaline borohydride for 48 hr and fractionated over a Sephadex G-50 column as described by Roquemore et al. (1994). One percent of the fractions from the Sephadex G-50 column were assayed for radioactivity by liquid scintillation counting. Released ^{3}H-galactose–labeled saccharides in the inclusion volume were lyophilized and resuspended in 1 mL of distilled H_2O. The SDS in the resuspended samples was precipitated by adding 300 μL of 20% KCl, incubating on ice for 15 min, and centrifuging at 10,000*g* for 15 min at 4°C. The resulting supernatant was fractionated on a 1.5 × 200 cm Toyopearl HW 40 (TosoHaas, Philadelphia, PA) column equilibrated with 200 mM ammonium acetate and 10% ethanol at 55°C. One percent dextran (40K) and 1% galactose were added to the samples for internal standards. The void volume (dextran) and the inclusion volume (galactose) were determined by a phenol–sulfuric acid assay

(Dubois et al., 1956). The Toyopearl HM 40 column was calibrated with ^{3}H-galactose-$(GlcNAc)_n$-GlcNAcitol oligosaccharides (where n = 0 to 3). Five percent of fractions from the Toyopearl column were assayed for radioactivity by liquid scintillation.

The ^{3}H-galactose–labeled oligosaccharides released by β elimination were analyzed for the presence of peptide fragments or single amino acids. The labeled oligosaccharides were concentrated by lyophilization, passed over a Sep-Pak C18 column (Waters, Milford, MA), equilibrated with 0.1% trifluoroacetic acid, and eluted with 60% acetonitrile in 0.1% trifluoroacetic acid. Both the oligosaccharides that passed freely through the Sep-Pak column and those that were eluted with acetonitrile were treated with Pronase according to Fukuda (1989) and refractionated on the 1.5 × 200 cm Toyopearl HW 40 column. These two groups of oligosaccharides were also dansylated to detect primary amines according to Tapuhi et al. (1981), and the products were analyzed by reverse phase HPLC on a Microsorb-MV C18 column (Rainin Instrument Co., Woburn, MA) according to Kaneda et al. (1982).

ACKNOWLEDGMENTS

We thank Harley Smith and Drs. Stephane Lobreaux and Mark Shieh for many interesting discussions and in particular Dr. Glenn Hicks for critical reading of the manuscript. We also acknowledge Kurt Stepnitz for photography and Marlene Cameron for artwork. This work was supported by a grant from the U.S. Department of Energy (No. DE-FG02-90ER20021) to N.V.R. and a National Research Service Award fellowship (No. F32-NS09415) to R.N.C.

Received May 22, 1995; accepted July 6, 1995.

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