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O-Linked Oligosaccharides Are Acquired by Herpes Simplex Virus Glycoproteins in the Golgi Apparatus

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Summary

The O-linked oligosaccharides on mature forms of herpes simplex virus type 1 (HSV1) glycoproteins were characterized, and were found to account largely for the lower electrophoretic mobilities of these forms relative to the mobilities of immature forms. Other posttranslational modifications of HSV1 glycoproteins (designated gB, gC, gD and gE) were related temporally to the discrete shifts in electrophoretic mobilities that signal acquisition of the O-linked oligosaccharides. Fatty acid acylation (principally of gE) could be detected just prior to the shifts, whereas conversion of high-mannose-type N-linked oligosaccharides to the complex type occurred coincident with the shifts. The addition of O-linked oligosaccharides did not occur in cells treated with the ionophore monensin or in a ricin-resistant cell line defective in the processing of N-linked oligosaccharides. We conclude that extension of O-linked oligosaccharide chains on HSV1 glycoproteins, and probably also attachment of the first O-linked sugars, occurs as a late posttranslational modification in the Golgi apparatus.

Introduction

Considerable attention has been focused on defining the intracellular location and functional significance of various steps in the synthesis and processing of membrane-bound and secreted glycoproteins. The site of synthesis of most glycoproteins is on membrane-bound ribosomes of the rough endoplasmic reticulum, where high-mannose-type oligosaccharides are transferred en bloc from dolichol phosphate donors to asparagine residues on nascent chains (Struck and Lennarz, 1980) and small hydrophobic leader sequences are removed (Blobel et al., 1979). The polypeptides are then transported by unknown mechanisms, perhaps involving clathrin-coated vesicles (Rothman et al., 1980), to the Golgi apparatus, where fatty acid molecules may be attached (Schmidt and Schlesinger, 1980; Dunphy et al., 1981) and high-mannose asparagine-linked (N-linked) oligosaccharides are processed to complex-type oligosaccharides (Tabas and Kornfeld, 1979; Bretz et al., 1980; Bennett and O'Shaughnessy, 1981; Roth and Berger, 1982). From the Golgi apparatus the glycoproteins move to the cell surface or are targeted to cellular organelles such as the lysosomes (Neufeld and Ashwell, 1980).

Much less is known about the structure, assembly and attachment of O-linked oligosaccharides to gly-

coproteins than is known for N-linked oligosaccharides. The occurrence of O-linked oligosaccharides was first recognized in glycoproteins from mucous secretions (Carlson, 1968). Subsequently, glycoproteins such as fetuin (Spiro and Bhoyroo, 1974), human chorionic gonadotropin (Bahl, 1969) and erythrocyte glycophorin (Thomas and Winzler, 1969) were found to contain both O-linked and N-linked oligosaccharides. The O-linked oligosaccharides were attached to serine or threonine via an N-acetylgalactosamine (GalNAc) residue. The structures of these oligosaccharide chains vary from disaccharides composed of GalNAc and sialic acid to large oligosaccharides of 20 sugar residues containing GalNAc, galactose, N-acetylglucosamine, fucose and sialic acid (Kornfeld and Kornfeld, 1980). Several studies have addressed the question of the stage and location at which O-linked oligosaccharides are attached to glycoproteins during their intracellular transport. Strous (1979) concluded that GalNAc could be added in O-glycosidic linkage to growing polypeptides in the rough endoplasmic reticulum, whereas others (Kim et al., 1971; Ko and Raghupathy, 1972; Hanover et al., 1980) have found that enzymes capable of mediating such reactions are enriched in smooth endoplasmic reticulum and Golgi membranes rather than in rough endoplasmic reticulum membranes.

Cells infected with herpes simplex virus provide a useful system for investigating the processes discussed above. Previous studies have shown that glycoproteins specified by herpes simplex virus type 1 (HSV1) contain O-linked oligosaccharides (Oloffson et al., 1981) as well as N-linked oligosaccharides (Pizer et al., 1980; Serafini-Cessi and Campadelli-Fiume, 1981; Person et al., 1982; Wenske et al., 1982). We have confirmed these findings, further characterized the O-linked oligosaccharides and shown that ³H-palmitate is incorporated into one of the HSV1 glycoproteins. We have also shown that the relatively large difference in electrophoretic mobilities of mature and immature forms of each HSV1 glycoprotein (Spear, 1976; Baucke and Spear, 1979; Eisenberg et al., 1979; Eberle and Courtney, 1980) is due primarily to the presence of O-linked oligosaccharide chains on the mature forms. Using the discrete shifts in electrophoretic mobilities that occur during posttranslational processing of these glycoproteins as a marker for the acquisition of O-linked oligosaccharides, we have investigated the temporal order in which other posttranslational modifications (fatty acid acylation and processing of N-linked oligosaccharides) occur relative to these shifts. These studies were carried out not only under conditions designed to allow normal glycoprotein processing, but also in the presence of monensin, an ionophore known to cause accumulation of secreted and membrane proteins and virions in Golgi-derived vacuoles (Tartakoff and Vassalli, 1978; Uchida et al., 1979; Johnson and

Schlesinger, 1980; Johnson and Spear, 1982), and in ricin-resistant cells defective in the processing of N-linked oligosaccharides.

Our results indicate that extension of O-linked oligosaccharides to yield chains of sufficient number or size to affect electrophoretic mobilities of the glycoproteins, and probably also addition of the first amino acid-linked GalNAc residues, occur in the Golgi apparatus subsequent to addition of fatty acid and coincident with the processing of high-mannose-type N-linked oligosaccharides to complex-type oligosaccharides.

Results

Oligosaccharides Released from HSV1 Glycoproteins by Mild Alkaline Borohydride

HSV1 glycoproteins were labeled with ^{14}C -glucosamine in infected cells. Glucosamine can be converted to galactosamine and sialic acid, all of which are incorporated into oligosaccharides in eucaryotic cells (Kornfeld and Ginsberg, 1966; Oloffson et al., 1981). The ^{14}C -labeled HSV1 glycoproteins gC and gD were isolated on preparative SDS-polyacrylamide gels and treated with 0.05 M NaOH, 1 M NaBH₄ at 45°C for 14–20 hr (mild alkaline borohydride), conditions shown to selectively release O-linked oligosaccharides (Spiro, 1966; Marshall and Neuberger, 1977). The mixtures of released oligosaccharides and glycoprotein were chromatographed on Biorad P6 (Figures 1A and 1B). For both gC and gD approximately

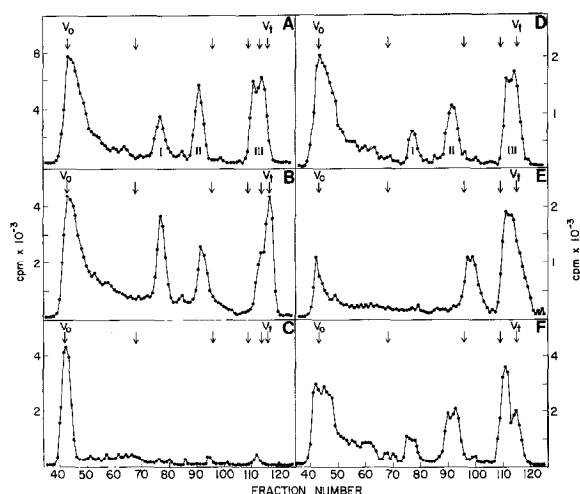


Figure 1. Oligosaccharides Released by Mild Alkaline Borohydride Treatment of HSV1 and VSV Glycoproteins

^{14}C -glucosamine-labeled HSV1 gC (A and D), HSV1 gD (B), HSV1 gC treated with neuraminidase (E) and VSV G protein (C) and ^{14}C -galactose-labeled gC (F) were extracted from SDS-polyacrylamide gels and treated with 0.05 M NaOH, 1.0 M NaBH₄ at 45°C for 14–20 hr. The mixtures of glycoprotein and released oligosaccharides were chromatographed on a column of Biorad P6, and fractions were collected and counted. Arrows: elution positions of blue dextran (V₀), the glycopeptides derived from fetuin (3180 daltons) and ovalbumin (1550 daltons), stachyose, lactose and N-acetylglucosamine (V).

40%–60% of the total label was observed as a peak at the void volume (V₀), presumably due to N-linked oligosaccharides that remain attached to the glycoprotein. Labeled material released from gC and gD and included in the P6 column was eluted principally in three peaks: peak I, containing material of approximately 2750 daltons; peak II, of approximately 1800 daltons; and peak III, which contained material as large as stachyose (666 daltons) and as small as glucosamine (215 daltons). A similar distribution of label was observed when preparations of HSV1 gC isolated by radioimmunoprecipitation were examined (results not shown). No labeled material was included on the column if gC was chromatographed directly after purification or if treated with only 1.0 M NaBH₄ (results not shown). No oligosaccharides were released under these conditions from isolated vesicular stomatitis virus (VSV) G protein (Figure 1C), which contains only N-linked oligosaccharides (Moyer et al., 1976).

Two kinds of experiments were carried out to characterize further the gC oligosaccharides released by β elimination. First, the isolated glycoprotein was digested with exoglycosidases prior to treatment with alkaline borohydride. The elution profile of the oligosaccharides released from neuraminidase-treated gC (Figure 1E) was markedly different from that of untreated gC (Figure 1D). Peaks I and II were absent, and a new peak of ^{14}C -labeled material appeared, with a smaller apparent molecular weight than the glycopeptide of ovalbumin (1550 daltons). Therefore, the oligosaccharides in peaks I and II are highly sialated. Further treatment of desialated gC with β -galactosidase produced only a very small shift in elution profile of the oligosaccharides released by alkaline borohydride (results not shown).

Second, gC was isolated from cells labeled with ^{14}C -galactose, instead of ^{14}C -glucosamine, and subjected to alkaline borohydride treatment. The elution profile, shown in Figure 1F, indicates that the oligosaccharides of peaks I, II and III all contain galactose. We observed a preferential labeling of the larger molecular weight material in peak III, consistent with the idea that peak III is composed of multiple oligosaccharides differing in molecular weight and that the larger molecular weight forms contain relatively more galactose.

GalNAc Oligosaccharidase Releases Oligosaccharides from HSV1 Glycoproteins, Resulting in Decreased Electrophoretic Mobilities of the Glycoproteins

A preparation of the enzyme α -D-N-acetylgalactosamine oligosaccharidase (GalNAc oligosaccharidase) purified from *Clostridium perfringens* has been found to release O-linked oligosaccharides from porcine submaxillary mucins by cleavage between serine or threonine residues and GalNAc (Huang and Aminoff,

1972; Pomato and Aminoff, 1978). Under appropriate conditions this enzyme released oligosaccharides from the mature form of HSV1 gC, and the released material had an elution profile very similar to that of the oligosaccharides released by alkaline borohydride (Figure 2A). The distribution of label in the three peaks was somewhat different in the GalNAc oligosaccharidase digest, perhaps because the larger oligosaccharides were more resistant to release by the enzyme. It has been suggested that this enzyme is less active in removing sialated oligosaccharides than desialated oligosaccharides (N. Pomato, personal communication). GalNAc oligosaccharidase did not release ^{14}C -glucosamine-labeled oligosaccharides from VSV G protein under these conditions (Figure 2B). In addition, treatment of VSV G protein with this enzyme did not affect the mobility of the glycoprotein on SDS-polyacrylamide gels, as might have been observed if oligosaccharides or monosaccharides had been removed (Figure 3A).

HSV1 glycoproteins were labeled with ^{35}S -methionine in a pulse-chase experiment, and the mature and immature forms of the glycoproteins were precipitated with monoclonal antibodies prior to treatment with GalNAc oligosaccharidase. The results, presented in Figures 3A and 3B, illustrate a phenomenon that has been reported previously (Spear, 1976; Baucke and Spear, 1979; Eisenberg et al., 1979; Eberle and Courtney, 1980)—namely, that posttranslational processing of HSV1 glycoproteins is accompanied by discrete shifts in their electrophoretic mobilities (to lower mobility). Extracts prepared immediately after the 10 min pulse of ^{35}S -methionine contained only the faster-migrating immature forms of the glycoproteins as labeled species, whereas extracts prepared after the 3 hr chase contained principally the slowly mi-

grating mature forms. Both forms were observed after 1 hr of chase, although the immature forms tended to be more abundant.

The immature forms of the HSV1 glycoproteins were insensitive to GalNAc oligosaccharidase (Figures 3A and 3B). However, the mobilities of the mature forms of gB, gC, gD and gE (results not shown for gE) increased after treatment with GalNAc oligosaccharidase. In fact, the mobilities of the enzyme-treated, mature glycoproteins resembled closely those of their respective immature forms, suggesting that the shifts in mobility that occur during the maturation of these glycoproteins result primarily from the addition or extension of O-linked oligosaccharide chains as a posttranslational, rather than a cotranslational, modification. The shifts in electrophoretic mobilities of the glycoproteins occur some 20 min to 3 hr after the polypeptides are synthesized and partially glycosylated. It should be noted that the use of GalNAc oligosaccharidase at levels 10 times higher than used in these studies in the absence of ovalbumin produced shifts in the mobilities of VSV G protein and of the immature HSV1 glycoproteins, suggesting the presence of contaminating exoglycosidic or endoglycosidic activities. VSV G protein and the immature forms of the HSV1 glycoproteins served as internal controls to ensure that these contaminating activities had been diluted out or suppressed.

Effect of Neuraminidase on Electrophoretic Mobilities of the HSV1 Glycoproteins

To test the possibility that the addition of sialic acid residues to O-linked oligosaccharides is the principal cause of the decrease in electrophoretic mobilities associated with maturation of the HSV1 glycoproteins, we treated immunoprecipitated glycoproteins labeled with ^{35}S -methionine in a pulse-chase experiment with neuraminidase. The conditions used for enzymatic digestion were shown in a separate experiment to remove all of the ^{14}C -N-acetylmannosamine label incorporated into gC and gD (data not presented). The electrophoretic mobilities of the mature forms of ^{35}S -methionine-labeled gC and gD increased after neuraminidase treatment, but not to the extent observed after treatment with GalNAc oligosaccharidase (Figure 4). Electrophoretic mobilities of the immature forms were not affected by neuraminidase. Similar results were obtained in experiments carried out with gB and gE (results not shown) except that it was difficult to compare the very small shifts in mobility of gB obtained by use of either enzyme. With gE, however, there was only a slight increase in mobility after neuraminidase treatment, as was found for gC (Figure 4). It should be noted that neuraminidase removes sialic acid from both N-linked and O-linked oligosaccharides, so that the shifts in electrophoretic mobility observed overestimate the effect due to removal of sialic acid from O-linked oligosaccharides.

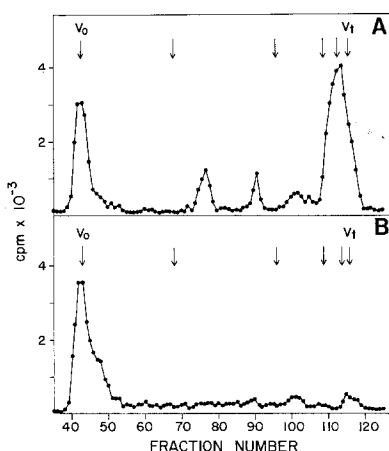


Figure 2. Oligosaccharides Released by GalNAc Oligosaccharidase Treatment of HSV1 gC and VSV G Protein

^{14}C -labeled HSV1 gC (A) and VSV G protein (B) extracted from SDS-polyacrylamide gels were treated with the enzyme GalNAc oligosaccharidase, and the reaction mixtures were chromatographed on a column of Biorad P6. The calibration of the column was described in the legend to Figure 1.

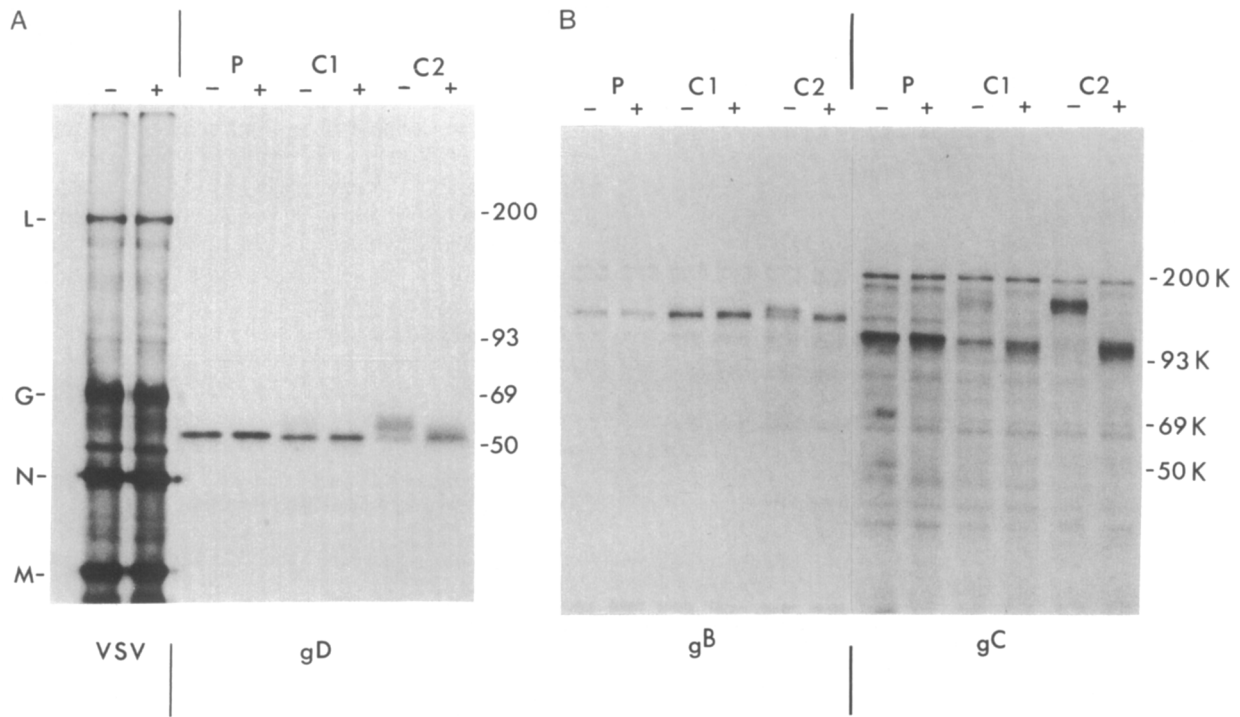


Figure 3. GalNAc Oligosaccharidase Treatment of VSV G Protein and HSV1 gD, gB and gC

VSV proteins were labeled with ^{35}S -methionine from 3–13 hr after infection and then extracted. HSV1 proteins were labeled for 10 min with ^{35}S -methionine, and then the proteins were immediately extracted (lanes P) or the label was chased for 1 hr (lanes C1) or 3 hr (lanes C2) before extraction. The HSV1 glycoproteins gB, gC and gD were immunoprecipitated with monoclonal antibodies, eluted with 2% SDS, 2% β -mercaptoethanol and dialyzed against 0.1% SDS. VSV proteins and the isolated HSV1 glycoproteins were mixed with ovalbumin (1 mg/ml final concentration) and either not treated (lanes -) or treated (lanes +) with GalNAc oligosaccharidase for 2 hr at 37°C, prior to analysis by electrophoresis.

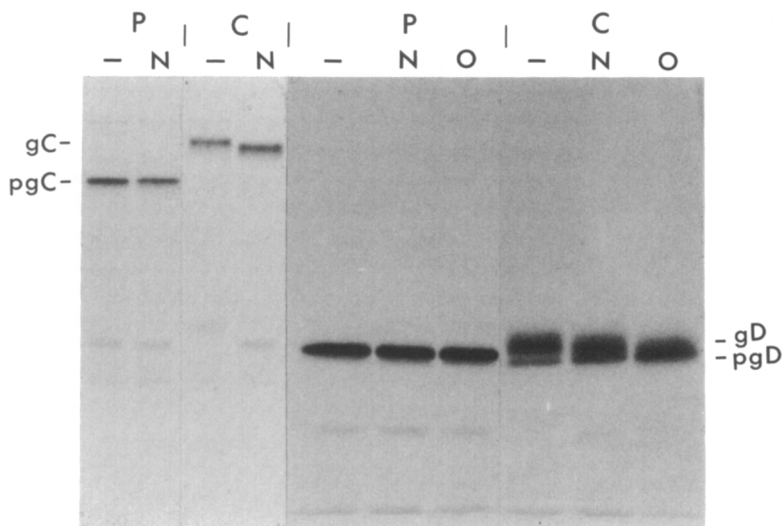


Figure 4. Neuraminidase Treatment of HSV1 Glycoproteins

HSV1 proteins were labeled for 10 min with ^{35}S -methionine, and then the proteins were immediately extracted (lanes P) or the label was chased for 3 hr (lanes C) before extraction. The HSV1 glycoproteins gC and gD were immunoprecipitated with monoclonal antibodies, and the *Staphylococcus aureus* complexes were washed twice with 0.1 M sodium acetate (pH 5.5), 1 mM CaCl_2 and then incubated with no enzyme (lanes -) or with neuraminidase (0.1 U/ml) for 2 hr at 37°C (lanes N). Alternatively, the complexes were washed twice with 0.1 M sodium phosphate (pH 6.4), 0.1% SDS, 1 mg/ml ovalbumin, and incubated with GalNAc oligosaccharidase (0.5 mU/ml) for 2 hr at 37°C (lanes O).

The mature forms after desialation retain sufficient O-linked oligosaccharide that they remain electrophoretically differentiable from the immature forms. This is consistent with our finding that desialated oligosaccharide released by alkaline borohydride treatment is of considerable size (Figure 1).

Monensin Blocks the Acquisition of O-Linked Oligosaccharides

We (Johnson and Spear, 1982) and others (Wenske et al., 1982) have shown that monensin prevents the posttranslational processing events that result in decreased electrophoretic mobilities of the HSV1 gly-

coproteins. To determine whether the abnormal forms of glycoproteins that accumulate in the presence of monensin contain O-linked oligosaccharides, we carried out two experiments. First, gD was isolated from monensin-treated infected cells and treated with mild alkaline borohydride, and the reaction mixture was chromatographed on P6. The results, shown in Figure 5, demonstrate that there were few if any oligosaccharides or monosaccharides released under these conditions. The second experiment was to determine whether GalNAc oligosaccharidase altered the elec-

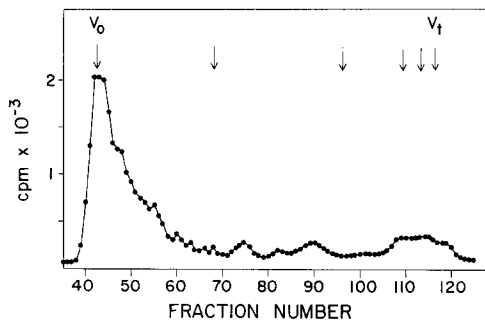


Figure 5. Oligosaccharides Released from HSV1 gD Isolated from Monensin-Treated Cells

¹⁴C-glucosamine-labeled gD extracted from monensin-treated cells and purified on SDS-polyacrylamide gels was treated with 0.05 M NaOH, 1.0 M NaBH₄ at 45°C for 18 hr. The mixture of glycoprotein and released oligosaccharides was chromatographed on Biorad P6, calibrated as described in the legend to Figure 1.

trophoretic mobilities of gD and gC isolated from monensin-treated cells by immunoprecipitation. The data in Figure 6 illustrate our previous finding that monensin blocks the modifications of the glycoproteins responsible for decreased electrophoretic mobility, and show also that the glycoproteins accumulating in monensin-treated cells were resistant to the enzyme. Taken together, these findings provide evidence that monensin blocks the attachment of O-linked oligosaccharides to herpes simplex virus glycoproteins and that these oligosaccharides are largely responsible for the shifts in electrophoretic mobilities normally observed. Moreover, these posttranslational modifications must occur late in the intracellular transport of these glycoproteins to the cell surface, at some stage during or after they pass through the Golgi apparatus.

Two Other Posttranslational Modifications Further Define the Time and Place of Attachment of O-Linked Oligosaccharides

The enzyme endo-β-N-acetylglucosaminidase H (endo H) has been shown to cleave selectively N-linked oligosaccharides of the high mannose type but not of the complex type (Tarentino and Maley, 1974). Acquisition of endo H insensitivity was examined to relate N-linked oligosaccharide processing to the posttranslational shifts in electrophoretic mobility associated with acquisition of O-linked oligosaccharides. Treatment of the immature forms of HSV1 gly-

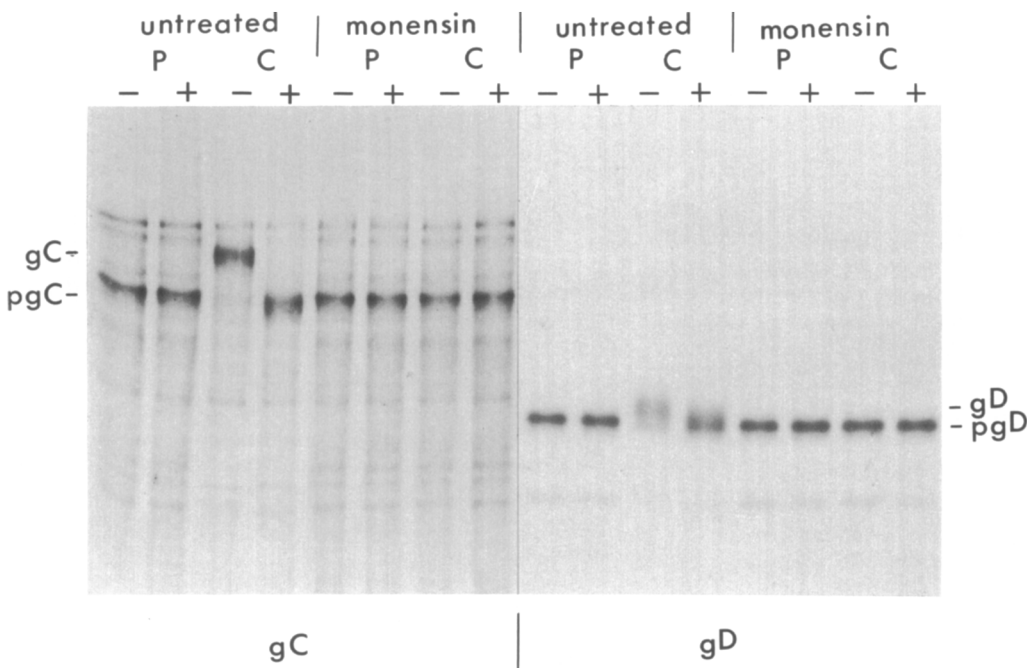


Figure 6. HSV1 Glycoproteins Extracted from Monensin-Treated Cells and Treated with GalNAc Oligosaccharidase

HSV1 proteins were labeled with ³⁵S-methionine in untreated (lanes untreated) or monensin-treated (lanes monensin) cells for 10 min, and then the proteins were immediately extracted (lanes P) or the label was chased for 3 hr (lanes C) before extraction. The glycoproteins gC and gD were isolated by immunoprecipitation with monoclonal antibodies, not treated (lanes -) or treated (lanes +) with GalNAc oligosaccharidase and subjected to electrophoresis on SDS-polyacrylamide gels. The mature forms of each of the glycoproteins are designated gC and gD and the immature forms are designated pgC and pgD.

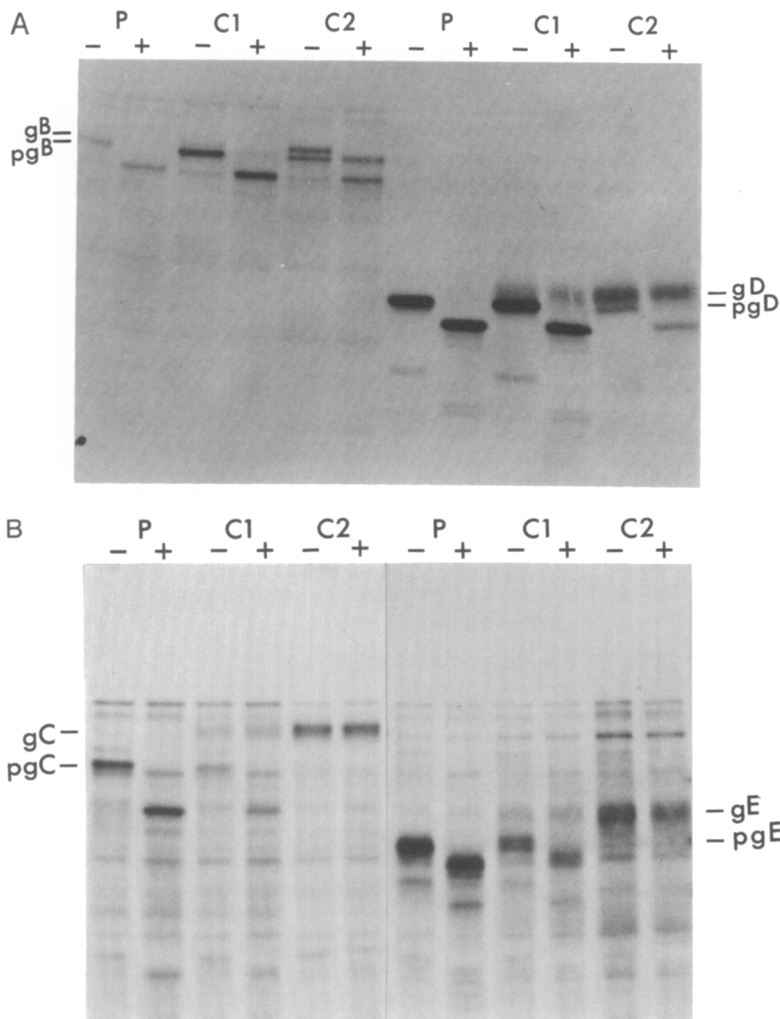


Figure 7. Endo H Treatment of HSV1 Glycoproteins

HSV1 glycoproteins were labeled with ^{35}S -methionine as described in the legend to Figure 3, and extracted immediately after the pulse (lanes P) or after chase periods of 1 hr (lanes C1) or 3 hr (lanes C2), followed by immunoprecipitation with monoclonal antibodies. The isolated glycoproteins were then treated with endo H (lanes +) or not treated (lanes -) and electrophoresed on SDS-polyacrylamide gels.

coproteins gB, gD, gC and gE with endo H resulted in a marked increase in electrophoretic mobility on SDS-polyacrylamide gels (Figure 7). As the glycoproteins were processed to the forms with slower electrophoretic mobility (during the 3 hr chase period) they became resistant to endo H, as reported by others (Serafini-Cessi and Campadelli-Fiume, 1981; Person et al., 1982; Wenske et al., 1982). We found, as did Wenske et al. (1982), that gB remained partially sensitive to endo H. Therefore, conversion of the immature forms of HSV1 glycoproteins to the mature forms parallels conversion of the endo H-sensitive form to an endo H-resistant form. It appears that the form of the glycoprotein that has O-linked oligosaccharides also has fully processed N-linked oligosaccharides.

The covalent attachment of fatty acid molecules to viral glycoproteins has been described to occur as they pass through the Golgi apparatus (Schmidt et al., 1979; Schmidt and Schlesinger, 1980; Dunphy et al., 1981). Of the four HSV1 glycoproteins studied here, only gE, the IgG Fc receptor (Baucke and Spear, 1979), was found to incorporate significant quantities

of label from ^3H -palmitate (Figure 8). When cells were pulse-labeled for 6 min with ^3H -palmitate, the immature forms of gE was preferentially labeled. Therefore, the attachment of fatty acid, which is thought to occur early in the transport of viral glycoproteins through the Golgi apparatus, appears to precede the attachment of O-linked oligosaccharides.

HSV1 Glycoproteins Produced in a Lectin-Resistant Cell Line Do Not Contain O-Linked Oligosaccharides

The ricin-resistant cell line CL6, isolated by Gottlieb and Kornfeld (1976), contains multiple defects in the processing of N-linked oligosaccharides such that high mannose oligosaccharides with the structure $[\text{Man}]_8[\text{GlcNAc}]_2\text{-Asn}$ accumulate (Tabas and Kornfeld, 1978). We infected CL6 cells and the parental cell line L929 with HSV1 to compare them with respect to the processing of viral glycoproteins. The immature forms of gB and gD were converted to the more slowly migrating, mature forms of gB and gD in L929 cells (Figure 9), as had been observed in HEP-2 cells.

However, mature forms of gB and gD did not appear during the chase period in CL6 cells, nor were the glycoproteins detected in CL6 cells sensitive to GalNAc oligosaccharidase (results not shown). This result is consistent with the idea that HSV1 glycopro-

teins are not glycosylated at serine or threonine residues in this lectin-resistant cell line.

Discussion

Our results confirm a previous report (Oloffson et al., 1981) on the presence of O-linked oligosaccharides in HSV1 glycoproteins, and demonstrate also that these O-linked oligosaccharides are largely responsible for the large differences in electrophoretic mobility between mature and immature forms of the glycoproteins. If the immature forms contain any O-linked carbohydrate, the number and size of these chains must be too small to affect the electrophoretic mobilities of the glycoproteins or the linkages must be resistant to GalNAc oligosaccharidase. Results obtained from analysis of gD made in monensin-treated cells also suggest that the addition of GalNAc to serine and threonine residues in this polypeptide is not normally a cotranslational or early posttranslational event, unless monensin can block such a pre-Golgi modification or unless insufficient radiolabel was incorporated into gD to permit detection of small monosaccharides or oligosaccharides released by alkaline borohydride. The effects of neuraminidase treatment on electrophoretic mobilities of the mature glycoproteins indicate that sialation of the O-linked oligosaccharides cannot account fully for processing-linked shifts in electrophoretic mobility, and therefore carbohydrate moieties other than sialic acid (that is, galactose, N-acetylglucosamine) must account partially for the decreased electrophoretic mobilities (larger apparent size) associated with the presence of O-linked chains.

We present evidence that the steps in synthesis of the O-linked oligosaccharides that result in decreased electrophoretic mobilities of the glycoproteins occur

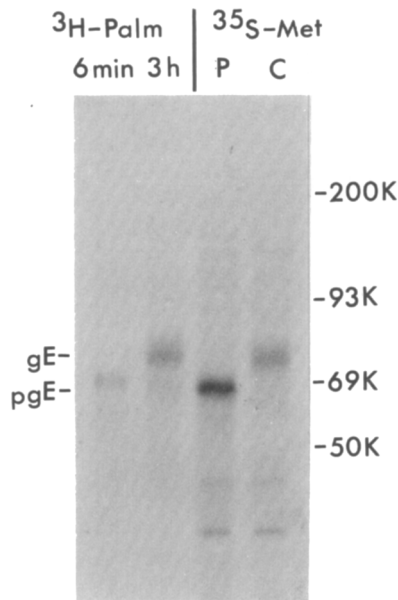


Figure 8. Attachment of Fatty Acid to HSV1 gE
HSV1-infected cells were labeled with ^3H -palmitate (lanes ^3H -Palm) for 6 min or 3 hr and then extracted with NP40-DOC buffer. In parallel, monolayers were labeled for 10 min with ^{35}S -methionine (lanes ^{35}S -Met), and the proteins were immediately extracted (lane P) or the label was chased for 3 hr (lane C) before extraction for precipitation with monoclonal antibody and electrophoresis on SDS-polyacrylamide gels. Approximately 50 times more of the extract from cells labeled for 6 min with ^3H -palmitate was required to visualize the pgE band relative to gE labeled with ^3H -palmitate for 3 hr.

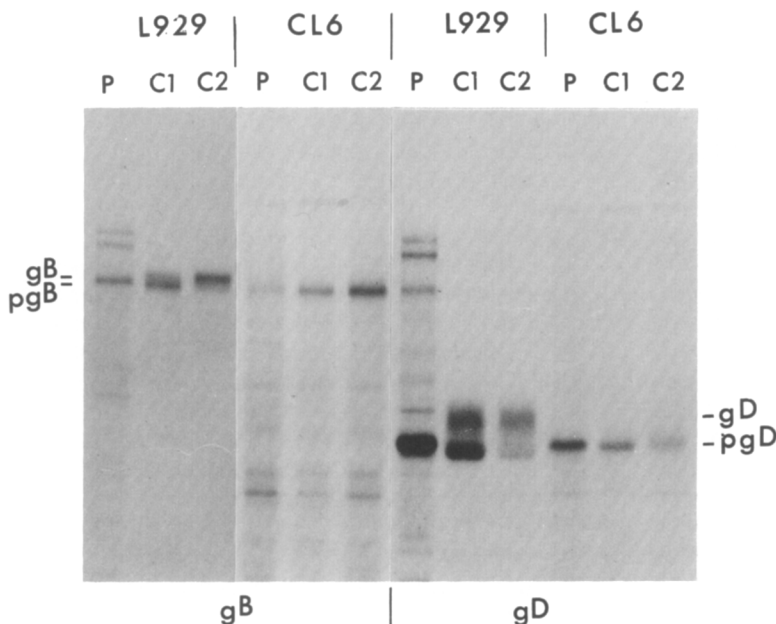


Figure 9. Posttranslational Processing of HSV1 Glycoproteins in a Ricin-Resistant Cell Line

L929 and CL6 cells infected with HSV1 were labeled in a pulse-chase experiment as described in the legend to Figure 3. Glycoproteins gB and gD were immunoprecipitated with monoclonal antibodies and subjected to electrophoresis on SDS-polyacrylamide gels.

in the Golgi apparatus. First, the shifts in electrophoretic mobility occurred after addition of fatty acid to gE and coincident in time with the processing of N-linked oligosaccharides from high-mannose to complex-type oligosaccharides. Both of these processes have been shown to occur in the Golgi apparatus (Schmidt and Schlesinger, 1980; Dunphy et al., 1981; Tabas and Kornfeld, 1979; Bretz et al., 1980). Second, the ionophore monensin, which interferes with Golgi function (Tartakoff and Vassalli, 1977, 1978), blocks both the processing of N-linked oligosaccharides on HSV1 glycoproteins (Wenske et al., 1982) and the addition of O-linked oligosaccharides. Our conclusion about the intracellular site for addition of O-linked oligosaccharides is in agreement with findings that transferases catalyzing attachment of GalNAc to serine and/or threonine residues in rat intestinal mucosa (Kim et al., 1971) and brain (Ko and Raghupathy, 1972) and hen oviduct (Hanover et al., 1980) are localized in smooth membranes. In contrast, Strous (1979) reported that GalNAc was attached to nascent epithelial polypeptides on polysomes. Our data argue against attachment of O-linked oligosaccharides to nascent HSV1 glycoproteins, although it is possible that a small amount of GalNAc is attached as a cotranslational modification and escaped detection.

The finding that O-linked oligosaccharides were not added to HSV1 glycoproteins in CL6 cells suggests either that enzymes necessary for this addition are defective in these cells, and are therefore of cellular genetic origin, or that attachment of O-linked oligosaccharides is contingent upon the processing of N-linked oligosaccharides. The CL6 cells fail to attach terminal sugars to N-linked oligosaccharides (Gottlieb and Kornfeld, 1976), processes known to occur in the Golgi apparatus (Tabas and Kornfeld, 1979; Bretz et al., 1980; Roth and Berger, 1982). It remains to be determined whether the same enzymes may participate in the addition of monosaccharides to both N-linked and O-linked oligosaccharide chains. Experiments carried out *in vitro* have shown, however, that enzyme preparations capable of adding sugars to O-linked oligosaccharides may not be able to attach these sugars to N-linked oligosaccharides (reviewed by Schachter and Roseman, 1980).

Campadelli-Fiume et al. (1982) recently reported that the conversion of high-mannose-type N-linked oligosaccharides on HSV1 glycoproteins to complex-type oligosaccharides is blocked in a ricin-resistant BHK cell line, and that infectious virions (containing immature glycoproteins) can be produced nonetheless. Whether O-linked oligosaccharides were attached in the mutant BHK cell line was not discussed by these authors; they reported, however, the absence of shifts in electrophoretic mobility, which we have shown here to signal the addition of O-linked oligosaccharides. We did not investigate the infectiv-

ities of virions produced in the mutant CL6 cells because, although mouse L cells are permissive for expression of most if not all herpes simplex virus genes, they are only semi-permissive for the production of infectious virus.

The O-linked oligosaccharides constituted a major fraction of the labeled carbohydrate on HSV1 glycoproteins and consisted principally of three size fractions. The largest of these oligosaccharides may be comparable in size and composition with blood-group substances described by Feizi et al. (1971). However, a large fraction of the ^{14}C -glucosamine and ^{14}C -galactose label released by mild alkaline borohydride from HSV1 glycoproteins chromatographed as smaller molecular weight oligosaccharides similar to the disaccharides, trisaccharides and tetrasaccharides observed in submaxillary mucins (Carlson, 1968; Aminoff et al., 1979), fetuin (Spiro and Bhoyroo, 1974), human IgA (Baenziger and Kornfeld, 1974) and certain tumor-cell glycoproteins (Bhavanandran and Davidson, 1976). Differences in electrophoretic mobilities between mature and immature forms of the glycoproteins suggest that a large amount of O-linked oligosaccharide is added to some species (gC, for example), although the exact number of oligosaccharide chains cannot be estimated from these differences. It is of interest that label from inorganic sulfate can be incorporated into gE (Hope et al., 1982), since O-linked oligosaccharides of certain submaxillary gland mucins have been found to contain sulfated sugars (Lombart and Winzler, 1974).

A list of possible functions of O-linked oligosaccharides would include some of those previously proposed for N-linked oligosaccharides: altering the initial folding or final conformation of polypeptides (Gibson et al., 1980); targeting of glycoproteins to specific intracellular organelles or the cell surface (Neufeld and Ashwell, 1980); protecting the glycoproteins from proteolytic degradation (Schwartz et al., 1976); and influencing binding or other properties of the glycoproteins. We can probably rule out effects of O-linked oligosaccharides on initial folding of glycoproteins because they seem to be added as relatively late posttranslational modifications. In addition, functions of the HSV1 glycoproteins essential for virion infectivity in cell culture do not depend on the presence of O-linked oligosaccharides. This conclusion emerged from the results presented here coupled with our previous findings (Johnson and Spear, 1982) that infectious virions are produced in the presence of monensin and that these virions, which accumulate in intracytoplasmic vacuoles, contain immature glycoproteins. The results of this study suggested that the virion envelope, acquired at the inner nuclear membrane, initially contains immature glycoproteins that are processed to the mature forms as the virions are transported through the Golgi apparatus. It seems unlikely that O-linked oligosaccharides play any role

in the targeting of HSV1 glycoproteins to the nuclear membrane, but they could conceivably play some role in the transport of viral glycoproteins or virions to the cell surface. Also, addition of oligosaccharides to serine and threonine residues on cellular and viral glycoproteins, already glycosylated at asparagine residues, may lead to increased hydration of the cell or virion surface and increased protection from proteolytic degradation.

O-linked oligosaccharides have also been reported on glycoproteins specified by vaccinia virus (Shida and Dales, 1981) and coronavirus (Nieman and Klenk, 1981). These viruses share in common with HSV1 the property of acquiring their envelopes at internal cellular membranes, rather than at the plasma membrane.

Experimental Procedures

Cells and Viruses

HEp-2 cells, L929 cells and CL6 cells, the latter provided by S. Schlesinger, were grown in monolayer culture in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% (v/v) fetal calf serum, both from KC Biologicals. The herpes simplex virus strain used was HSV1(HFEM), obtained from A. Buchan. VSV(San Juan) was obtained from M. J. Schlesinger. Both viruses were passaged at low multiplicity on HEp-2 cells.

Infection of Cells and Labeling with Radioactive Precursors

Monolayers of HEp-2 cells growing on plastic dishes or glass bottles were infected with HSV1 or VSV at 20 pfu/cell. HSV1 was adsorbed for 2 hr at 37°C in phosphate-buffered saline (PBS) containing 1% fetal calf serum and 0.1% (w/v) glucose. VSV was allowed to adsorb for 1 hr at 37°C in PBS containing 1% fetal calf serum. Medium 199 supplemented with 1% inactivated fetal calf serum (199V) was added to the monolayers after virus adsorption. Cells were labeled with ¹⁴C-glucosamine (50 mCi/mmol; New England Nuclear) at 10 μCi/ml, ¹⁴C-galactose (50 mCi/mmol; New England Nuclear) at 10 μCi/ml or ¹⁴C-N-acetylmannosamine (50 mCi/mmol) at 2.5 μCi/ml, 4–21 hr after infection with HSV1 or 3–13 hr after infection with VSV. Cells were labeled for 6 min or 3 hr with ³H-palmitate (22 Ci/mmol; New England Nuclear) at 100 μCi/ml, 8 hr after infection with HSV1. Pulse-chase experiments were performed at 8 hr after infection by labeling cells for 10 min with ³⁵S-methionine (1000 Ci/mmol; New England Nuclear) at 25 μCi/ml in 199V lacking methionine and containing 20 mM HEPES (pH 7.3); some of the cultures were harvested immediately, and 199V containing a tenfold excess of unlabeled methionine was added to the others for 1 hr or 3 hr. Cells were washed twice with PBS and scraped into PBS containing 1% NP40, 0.5% sodium deoxycholate (DOC) and ovalbumin at 1 mg/ml (NP40-DOC extraction buffer) if glycoproteins were to be immunoprecipitated or into 2% SDS, 50 mM Tris-HCl (pH 6.8) if glycoproteins were to be purified by SDS-polyacrylamide gel electrophoresis. Cells were treated with 0.2 μM monensin (from 1 mM stocks in absolute ethanol) 2 hr after infection with HSV1 (Johnson and Spear, 1982).

Purification of Radiolabeled Viral Glycoproteins

Glycoproteins were purified by preparative electrophoresis on 1.5 mm thick, 8.5% polyacrylamide gels crosslinked with N,N'-Diallyltartardiamide (DATD) (Heine et al., 1974). SDS extracts of ¹⁴C-glucosamine- or ¹⁴C-galactose-labeled cells were sonicated and boiled for 5 min before being electrophoresed for 4.5 hr at 15 mA per gel. Gels were immediately dried and subjected to autoradiography, and the autoradiographs were used to detect labeled glycoproteins. The labeled glycoprotein bands were excised from the dried gels, hydrated in 0.075 M Tris-glycine, 0.2% SDS, 10% glycerol, 0.05 mg/

ml ovalbumin, 0.05 mg/ml cytochrome c and electrophoresed into dialysis tubing (Stephens, 1975). The isolated glycoproteins were dialyzed against 0.1% SDS and lyophilized. Alternatively, HSV1 glycoproteins were isolated by immunoprecipitation with monoclonal antibodies (I-144, specific for gB; II-474, specific for gC; II-436, specific for gD; or II-481, specific for gE) as previously described (Johnson and Spear, 1982) and dialyzed against 0.1% SDS for treatment with enzymes or eluted into 2% SDS, 50 mM Tris-HCl (pH 6.8), 10% glycerol for electrophoresis on 8.5% polyacrylamide gels crosslinked with DATD (Heine et al., 1974). Analytical gels were impregnated with 2,5-diphenyloxazole (Bonner and Laskey, 1974) and then dried and placed in contact with Cronex Medical x-ray film at -70°C.

Mild Alkaline Borohydride Treatment of Glycoproteins and Gel Filtration of Oligosaccharides

Viral glycoproteins were incubated with 0.05 M NaOH, 1.0 M NaBH₄ for 14–20 hr at 45°C as described by Carlson (1968). High concentrations of NaBH₄ were found to be necessary to prevent destruction of GalNAc linked to serine or threonine (Carlson et al., 1970). Excess borohydride was destroyed by addition of 2 M HCl to pH 6.5. The mixture was chromatographed on a column (95 × 1.2 cm) of Biorad P6 (200–400 mesh) and eluted with 0.1 M NH₄HCO₃, 0.1% SDS. Fractions were collected, and aliquots were dried on glass filters and counted. The column was calibrated with blue dextran 2000 (V₀); the glycopeptides derived by pronase digestion of fetuin (3180 daltons) (Spiro, 1962) and ovalbumin (1550 daltons) (Spiro, 1966); stachyose; lactose; and N-acetylglucosamine (V₁).

Enzyme Incubations

Digestions with GalNAc oligosaccharidase (Bethesda Research Laboratories) were performed with enzyme at concentrations of 0.5 mU/ml on isolated viral glycoproteins in the presence of ovalbumin (1 mg/ml) and 0.1% SDS in 0.1 M Na₂PO₄ (pH 6.4) for 2 hr at 37°C. The digestions were immediately stopped by addition of 2% SDS and boiling for 5 min, and were loaded onto the P6 column or subjected to electrophoresis. Isolated HSV1 glycoproteins were treated with neuraminidase (type X; Sigma) at 0.1 U/ml in 50 mM sodium acetate (pH 5.5), 1 mM CaCl₂ for 20 hr at 37°C. Glycoproteins were treated with β-galactosidase (type VII; Sigma) at 100 U/ml in 200 mM Na₂PO₄ (pH 7.2) for 20 hr at 37°C. Endo H (Miles Laboratories) was used at 35 mU/ml in 0.1 M sodium citrate buffer (pH 5.5), 0.1% SDS.

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