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Glycoproteins Associated with Nuclei of Cells before and after Transformation by a Ribonucleic Acid Virus†

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ABSTRACT: A comparison was made by gel filtration of Pronase-digested glycopeptides derived from nuclei of baby hamster kidney fibroblasts (BHK_{21}/C_{13}) and the same clone transformed by the Bryan strain of Rous sarcoma virus (C_{13}/B_4). The comparison showed the presence of glycopeptides associated with the nuclei from the transformed cells labeled with radioactive D-glucosamine or L-fucose which were not seen to the same extent in the nuclei from nontransformed cells. Extraction of the nuclei with Triton X-100 from both cell lines removed most of the fucose- and glucosamine-containing glycoproteins. The nuclei appeared intact after this procedure suggesting that the glycoproteins are associated with the outer nuclear membrane. The ribosomal fractions from both cell lines showed a small amount of a heterogeneous population of fucose-containing glycopeptides. The glucosamine-containing glycopeptides of the nuclei differed somewhat in their patterns of elution from those derived from the surface membranes of the corresponding cells. However both the nucleus and surface membrane-derived glycoproteins from the transformed cells always showed the presence of glycopeptides which were not seen to the same extent in these cell fractions from the nontransformed fibroblasts.

The glycoprotein composition of surface membranes has been observed to change after transformation by RNA (Buck *et al.*, 1970) or DNA viruses (Buck *et al.*, 1971). These changes were demonstrated by gel filtration of surface membrane digests labeled with radioactive D-glucosamine or L-fucose. Further fractionation and chemical analyses showed that the glycoproteins which were more apparent after virus transformation actually contained in addition to fucose more sialic acid, mannose, and galactose suggesting a lengthening of a portion of the carbohydrate moiety (Glick, 1971). The appearance of these glycoproteins on the cell surface has been followed in hamster fibroblasts after viral infection and shown to correlate with the tumorgenicity of the cell population (Glick *et al.*, 1972).

Others when comparing 3T3 and SV40 transformed 3T3 mouse fibroblasts, have observed small (Wu *et al.*, 1969) or no (Sakiyama and Burge, 1972) differences in the membrane glycoproteins. The reason for this discrepancy is not apparent. Hamster embryo cells, transformed by SV40 virus, show the appearance of specific glycoproteins characteristic of the other transformed cells examined (M. C. Glick, unpublished observations).

In order to see if the glycoproteins expressed on the cell surface following virus transformation are present also in nuclei, a comparison was made of the glycopeptides from nuclei of cells before and after virus transformation. The results show that changes in the glycopeptides associated with the nucleus also accompany virus transformation.

Materials and Methods

Cell Culture. Baby hamster kidney fibroblasts (BHK_{21}/C_{13}) and the same clone transformed by the Bryan strain of Rous sarcoma virus (C_{13}/B_4) were grown as described previously (Buck *et al.*, 1970). The cells were of early passage stocks and in no case were used beyond the twelfth passage in our laboratory. Examination of the cultures at routine intervals for *Mycoplasma* showed them to be negative.

The cells were cultured for 72 hr in the presence of L-[1-^{14}C]fucose (50.8 Ci/mol), L-[G-^{3}H]fucose (4.3 Ci/mmol), D-[μ-^{14}C]glucosamine (10.7 Ci/mol), or D-[6-^{3}H]glucosamine (1.3 Ci/mmol) obtained from New England Nuclear Corp., Boston, Mass. These procedures as well as those used for

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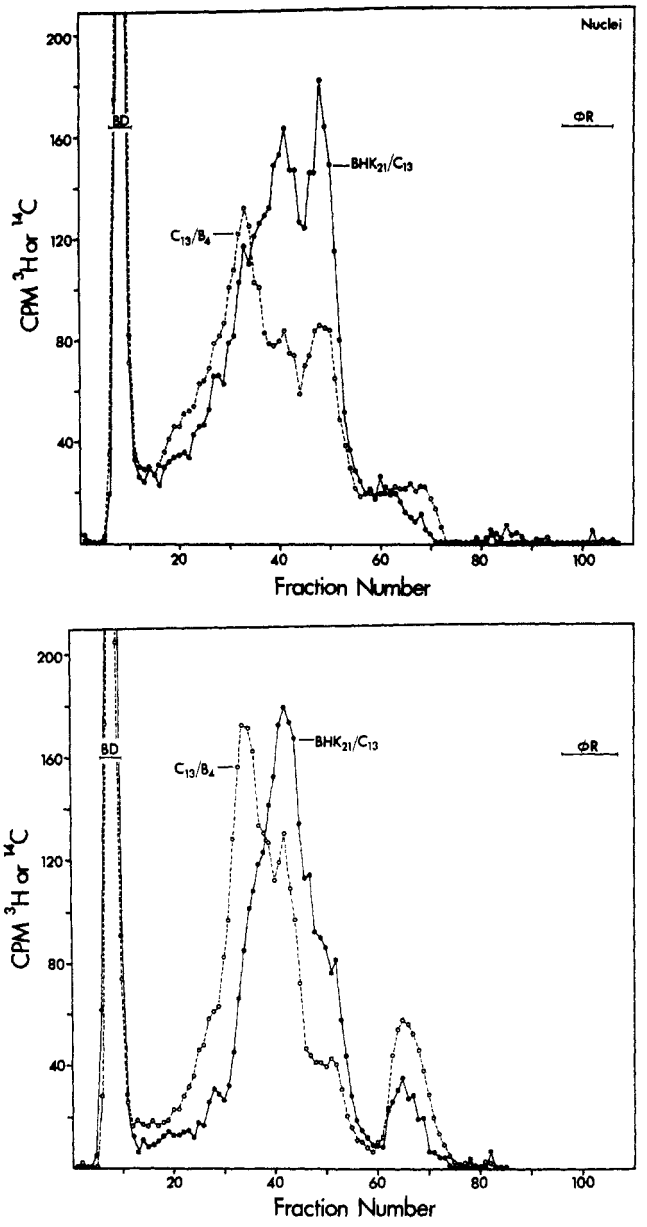

FIGURE 1: Chromatography on Sephadex G-50 of Pronase-digested nuclei and trypsinates isolated from BHK_{21}/C_{13} cells grown in the presence of D-[^{3}H]glucosamine (●) and C_{13}/B_4 cells grown in the presence of D-[^{14}C]glucosamine (○). Cochromatography of (a) nuclei and (b) trypsinates from BHK_{21}/C_{13} cells compared with nuclei and trypsinates from C_{13}/B_4 cells. All procedures are described in Materials and Methods. BD, fractions in which Blue Dextran was eluted; ϕR, fractions in which Phenol Red was eluted.

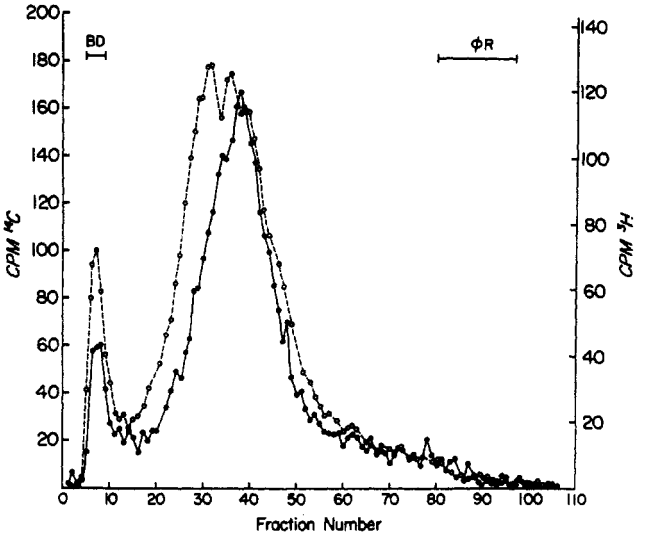

FIGURE 2: Chromatography on Sephadex G-50 of Pronase-digested nuclei isolated from BHK_{21}/C_{13} cells grown in the presence of L-[^{3}H]-fucose (●) and C_{13}/B_4 cells grown in the presence of L-[^{14}C]fucose (○). All procedures are described in Materials and Methods. BD, fractions in which Blue Dextran was eluted; ϕR, fractions in which Phenol Red was eluted.

harvesting the cells have been described in detail (Buck *et al.*, 1970).

Preparation of Cell Fractions. NUCLEI. Nuclei were prepared from radioactive BHK_{21}/C_{13} and C_{14}/B_4 cells as described for L cells (Glick *et al.*, 1971). The nuclei were obtained from cells from which the surface membranes were removed by the Zn ion procedure (Warren and Glick, 1969). Electron micrographs of nuclei prepared by this procedure showed the nuclei to be whole with the outer membrane intact. The preparations contained very little contaminating particulate matter (Glick *et al.*, 1971). The nuclei which can be counted in a hemocytometer contained approximately 3–6% of the total cell radioactivity when the cells were grown in the presence of L-[^{3}H]- or [^{14}C]fucose. All nuclei which were examined were prepared by this procedure.

TRYPSINATES AND SURFACE MEMBRANES. Radioactive BHK_{21}/C_{13} and C_{13}/B_4 cells were removed from the culture bottle with trypsin (1 mg/ml per 1×10^7 cells). The material removed by this procedure is referred to as "trypsinate." The trypsinization procedure and the processing of this material have been described (Buck *et al.*, 1970). Surface membranes were prepared from the trypsinized cells by the Zn ion procedure (Warren and Glick, 1969).

RIBOSOMES AND SOLUBLE MATERIAL. Ribosomal and soluble protein fractions were obtained from radioactive BHK_{21}/C_{13} and C_{13}/B_4 cells after the removal of the surface membranes. These procedures were as described for L cells (Glick *et al.*, 1971).

Pronase Digestion and Gel Filtration. Prior to chromatography on Sephadex G-50 the cell fractions to be compared were mixed and digested exhaustively with Pronase. All of these procedures as well as processing for radioactive counting have been described in detail (Buck *et al.*, 1970).

Triton Extraction of the Nuceli. Nuclei (1×10^7) were suspended in 0.25 ml of a solution containing 0.1 M Tris (pH 7.2), 0.002 M $CaCl_2$, 17% sucrose, and 5% Triton X-100 (Rohm and Haus, Philadelphia) for 5 min at 5° and subsequently centrifuged at 6000*g* for 10 min at 5°. The supernatant material was removed and the nuclei were resuspended in 0.2 ml of the extraction mixture, kept for 2 min at 5°, and centrifuged. The nuclei appeared intact when examined in the phase-contrast microscope after this procedure. No nuclei or fragments were seen in the supernatant solutions. After the extraction was repeated for the third time and the nuclei examined in the phase-contrast microscope some of them appeared to be lysed.

Incubation with Neuraminidase. Radioactive nuclei and surface membranes were incubated with neuraminidase (*Vibrio cholerae;* Calbiochem) as described (Glick *et al.*, 1970). After 60-min incubation at 37° the fractions were centrifuged at 6200*g* for 20 min. The supernatant solutions were

TABLE I: Triton Extraction (in cpm) of Nuclei from BHK_{21}/C_{13} and C_{13}/B_4 Cells.[a]

Fractions	D-Glucosamine		L-Fucose	
	BHK_{21}/C_{13} ^3H	C_{13}/B_4 ^{14}C	BHK_{21}/C_{13} ^3H	C_{13}/B_4 ^{14}C
Nuclei	7433 (100%)	7800 (100%)	4100 (100%)	4267 (100%)
Extract 1	5520 (74.3%)	6458 (82.8%)	3000 (73.2%)	3475 (81.5%)
Extract 2	621 (8.4%)	735 (9.4%)	250 (8.0%)	146 (3.4%)
Pellet	1314 (17.7%)	1062 (13.6%)	65 (2.2%)	401 (9.4%)

[a] Nuclei were isolated from BHK_{21}/C_{13} or the virus-transformed C_{13}/B_4 cells grown in the presence of D-[^{14}C]- or [^{3}H]glucosamine or L-[^{14}C]- or [^{3}H]fucose. The nuclei were extracted twice with Triton X-100. The radioactivity in two successive extractions (extracts 1 and 2) and the pellet remaining after the second extraction was determined as described in Materials and Methods. The numbers in parentheses represent the percentage of total radioactivity of the nuclei found in each subsequent fraction.

centrifuged at 6200*g* for 20 min and aliquots were removed for radioactive counting. The pellets were suspended in 0.5% sodium dodecyl sulfate and aliquots were removed for radioactive counting (Buck *et al.*, 1971). Controls containing no added neuraminidase were treated by the same procedure.

Results

Glucosamine-Containing Glycopeptides. Nuclei from BHK_{21}/C_{13} and C_{13}/B_4 which were made radioactive by the growth of the cells in D-[^{3}H]- or [^{14}C]glucosamine, respectively, were combined and digested with Pronase and the glycopeptides were examined by gel filtration on Sephadex G-50. Figure 1a represents the patterns obtained. Nuclei from both cell lines always showed the presence of material eluting in the void volume representing approximately 20% of the total radioactivity in the nuclei.

Differences were observed in the distribution of the glycopeptides eluted from the gel; that is, the nuclei from the virus-transformed cells (C_{13}/B_4) showed the presence of larger amounts of material which was eluted from the column soon after the void volume in comparison with the nontransformed cells (BHK_{21}/C_{13}). This material appeared in fractions 20–35 (Figure 1a) and is similar to the differences observed when the trypsinates from both cell lines were compared (Figure 1b). However, the nuclei from both cell lines contained larger amounts of material in fractions 43–53 than observed in the trypsinates.

Fucose-Containing Glycopeptides. Nuclei from BHK_{21}/C_{13} and the virus-transformed cells, C_{13}/B_4, radioactively labeled with L-[^{14}C]- or [^{3}H]fucose were combined, digested with Pronase, and examined by gel filtration on Sephadex G-50. Figure 2 represents the distribution of fucose-containing glycopeptides from the nuclei. As observed for the glucosamine-containing glycopeptides (Figure 1) the more rapidly migrating glycopeptides were always found in the nuclei from the virus-transformed cells, C_{13}/B_4 (fractions 20–35). This is similar to the distribution of the fucose-containing glycopeptides from the trypsinates of these cells (Buck *et al.*, 1970); 5–10% of the total radioactivity of both cell lines eluted with the void volume of the gel and was less than that found when radioactive D-glucosamine was used as a precursor (compare Figure 1a and Figure 2). When the radioactive labels were reversed, similar results were obtained.

Glycopeptides in the Triton Extract of Nuclei. Isolated nuclei from BHK_{21}/C_{13} or the virus-transformed, C_{13}/B_4, cells made radioactive by growth of the cells in the presence of L-[^{14}C]- or [^{3}H]fucose or D-[^{14}C]- or [^{3}H]glucosamine were combined and extracted twice with Triton X-100. Table I shows the distribution of radioactivity in the extracts. The Triton extract from nuclei of both cells lines contained 80–85% of the radioactive L-fucose. When the nuclei were labeled with radioactive D-glucosamine, 85–90% of radioactivity was extracted with the Triton. A third extraction removed an additional 5–10% of the radioactivity but also lysed some of the nuclei.

Triton extracts of the radioactive nuclei from BHK_{21}/C_{13} and C_{13}/B_4 cells were digested with Pronase and examined by gel filtration on Sephadex G-50. Figure 3a,b shows the distribution of the glucosaminine- and fucose-containing glycopeptides. In both cases, the distribution of the glycopeptides was similar to that obtained when the whole nuclei were analyzed by the same procedure (Figures 1a and 2).

Comparison of the Triton Extracts of Nuclei with the Trypsinates. A comparison of the Triton extract of the nuclei from the virus-transformed cells with the material removed from the cell surface (trypsinate) of the nontransformed cells, as well as a comparison of the Triton extract of nuclei of BHK_{21}/C_{13} cells with the trypsinate from C_{13}/B_4 are shown in Figure 4a,b. As a reference the pattern obtained with the trypsinates from both cell lines can be seen in Figure 1b. With both cell lines, the distribution of glucosamine-containing glycopeptides of the nuclei was different from that found in the material removed from the cell surface (trypsinate). Glucosamine-containing glycopeptides which eluted less rapidly from the Sephadex gel (fractions 43–53) were more abundant in the patterns obtained from the Triton extracts of nuclei than in the trypsinates (Figures 4 and 1). However, the glycopeptides which eluted more rapidly from Sephadex G-50 were always observed to a greater extent in the virus-transformed cells than in the nontransformed cells, whether comparing the nuclei, Triton extracts of nuclei, or the trypsinates.

Fucose-Containing Glycopeptides in Other Fractions of the Cells. Ribosomes were isolated from radioactive BHK_{21}/C_{13} and C_{13}/B_4 cells after removal of the surface membranes and nuclei. The ribosomal fractions contained less than 0.4% of the total radioactivity of the cell. The distribution on Sephadex G-50 of this radioactivity as fucose-containing glycopeptides from the ribosomal preparations is shown in Figure 5a. Although the glycopeptides appear as a heterogeneous population, the pattern showing this distribution was reproducible. Reversal of the radioactive labels gave the same result. More than 50% of the total radioactivity found in the ribo-

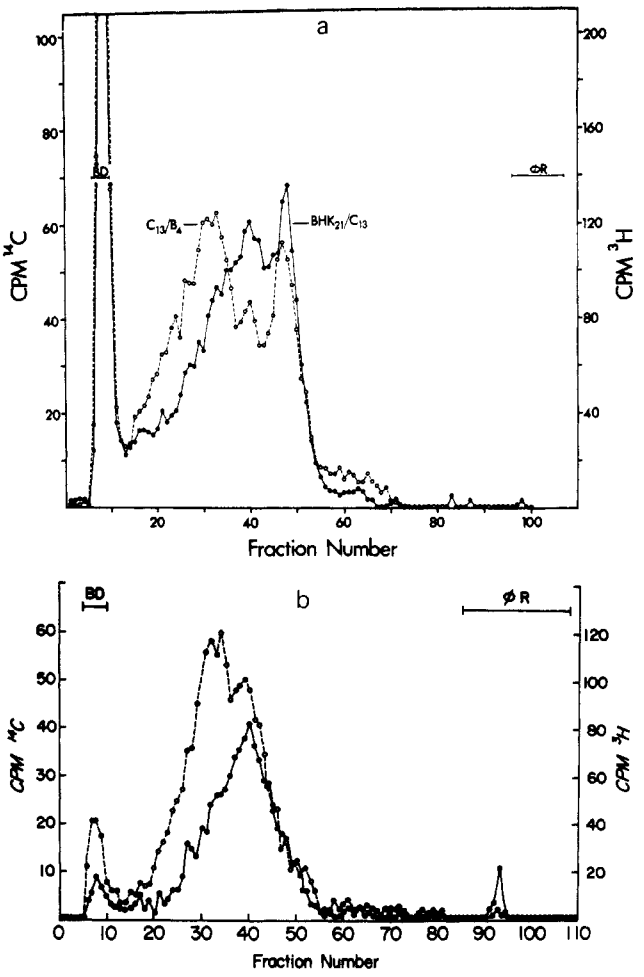

FIGURE 3: Chromatography on Sephadex G-50 of Pronase digests of glycopeptides extracted by Triton X-100 from nuclei. The Triton extracts of nuclei from BHK_{21}/C_{13} (●) and C_{13}/B_4 (○) cells grown in the presence of (a) D-[^{3}H]- or [^{14}C]glucosamine and (b) L-[^{3}H]- or [^{14}C]fucose were compared. All procedures are described in Materials and Methods. BD, fractions in which Blue Dextran was eluted. φR, fractions in which Phenol Red was eluted.

somes from the BHK_{21}/C_{13} cells was recovered as slow eluting material (fractions 60–75) from Sephadex G-50 column. Fucose-containing glycopeptides recovered from the ribosomal washes showed patterns similar to those obtained from the trypsinates or nuclei (Figure 5b).

The material which was precipitated with trichloroacetic acid from the soluble fractions of both cell lines showed a distribution of fucose-containing glycopeptides similar to the corresponding trypsinates or nuclei (Figure 5c). That is, the glycopeptides which eluted more rapidly from Sephadex G-50 were more abundant in the transformed cells than in the nontransformed cells. Only <1% of the total radioactivity of either cell line was recovered in this fraction. Table II gives a summary of the distribution of radioactivity found in the cell fractions of both cell lines.

Neuraminidase-Susceptible Material in the Nuclei. Incubation with neuraminidase of the nuclei isolated from BHK_{21}/C_{13} and C_{13}/B_4 cells labeled with radioactive D-glucosamine released 8 and 9% of the total radioactivity into the supernatant solutions. Under the same conditions, 42 and 24% of the radioactivity was released from the surface membranes. To obtain these percentages, the amount of radioactivity released into the supernatant solutions of control fractions

TABLE II: Distribution of Radioactivity in the Cell Fractions.

Fractions[a]	L-Fucose (^{3}H or ^{14}C),[b] Percentage of the Whole Cells	
	BHK_{21}/C_{13}	C_{13}/B_4
Surface membranes	19.2	20.5
Trypsinates	26.2	22.0
Nuclei	4.6	5.5
Ribosomal	0.3	0.5
Soluble material[c]	1.5	1.7

[a] Preparation of the fractions is described in Materials and Methods. [b] Average of four determinations. [c] Precipitated with trichloroacetic acid.

without neuraminidase but incubated in a similar manner, was subtracted from the amount released in the presence of neuraminidase.

The amount of sialic acid per nucleus can be calculated if an assumption is made that the radioactivity released in the presence of added neuraminidase represented sialic acid. BHK_{21}/C_{13} and C_{13}/B_4 cells contain 1.5×10^{-9} and 3.5×10^{-9} μmol of sialic acid per cell, respectively (Buck *et al.*, 1971). The percentage of the radioactivity of the total cell which is found in the nucleus from BHK_{21}/C_{13} and C_{13}/B_4 cells is 8 and 3%, respectively; 8 and 9% of this radioactivity is released by neuraminidase representing 9.6×10^{-12} μmol of sialic acid per BHK_{21}/C_{13} nucleus and 9.0×10^{-12} μmol of sialic acid per C_{13}/B_4 nucleus.

Discussion

This comparison of the nuclei isolated from transformed cells with their normal counterpart is part of a study defining the events occurring in a cell as the result of virus transformation. The comparisons show that after virus transformation the glycopeptides associated with the nuclei change in a manner similar to those changes observed previously (Buck *et al.*, 1970), in the glycopeptides of the surface membranes. That is, the distribution on Sephadex G-50 of glycopeptides from virus-transformed cells differs from that of the glycopeptides from control cells with the appearance of glycopeptides which eluted more rapidly from the gel (Figures 1 and 2). Most of the fucose- and glucosamine-containing glycoproteins of the nuclei were extracted with Triton X-100 under conditions where the nuclei appear intact (Table I). These results suggest that the glycoproteins are associated with the outer nuclear membrane which is probably removed by this extraction procedure.

The distribution on Sephadex G-50 of the glucosamine-containing glycopeptides from the nuclei of both cell lines was more similar to the distribution observed previously for the surface membranes of the same cell lines (M. C. Glick and C. A. Buck, unpublished observations) than described for the trypsinates (Figures 1 and 4). The patterns obtained for the fucose-containing glycopeptides (Figure 2) were less complex and more similar to the respective surface membranes or trypsinates (Buck *et al.*, 1970). Whether or not these glycopeptides are similar chemically will be defined by further separation (Glick, 1971) and chemical analysis.

Radioactive material in the nuclei which was eluted with

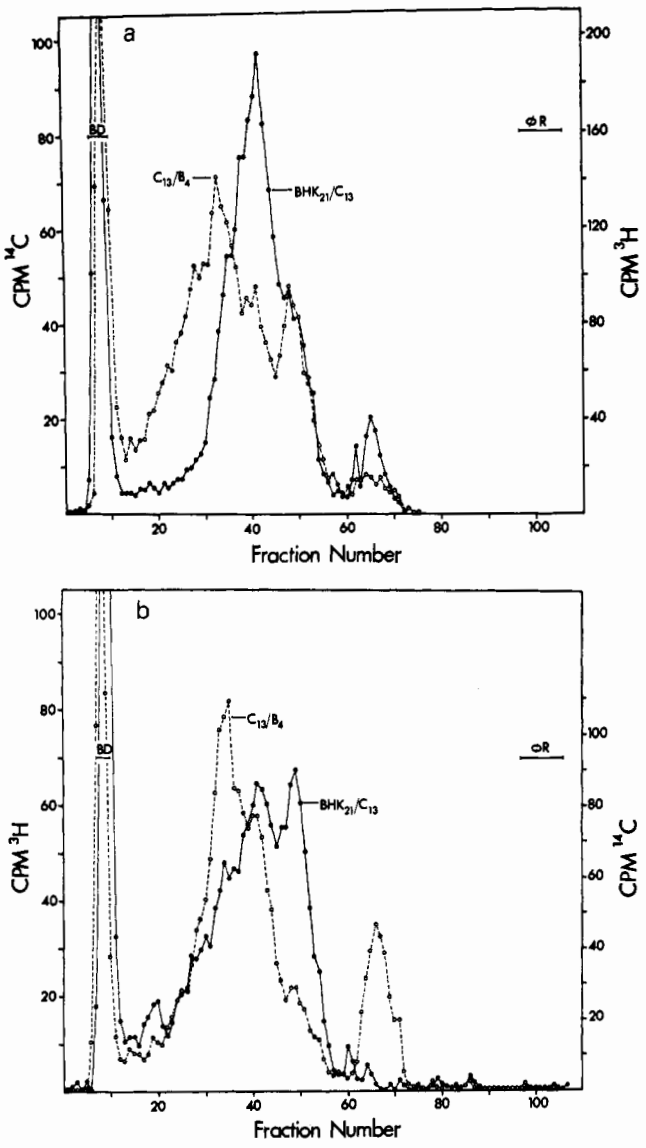

FIGURE 4: Chromatography on Sephadex G-50 of Pronase-digested glycopeptides extracted from nuclei with Triton X-100 and trypsinates. Cochromatography of (a) Triton extract of C_{13}/B_4 nuclei compared with the trypsinates from BHK_{21}/C_{13} cells and (b) Triton extract of BHK_{21}/C_{13} nuclei compared with the trypsinate of C_{13}/B_4 cells. The fractions were made radioactive by growth of the cells in the presence of D-[^{3}H]glucosamine (●) or D-[^{14}C]glucosamine (○). All procedures are described in Materials and Methods. BD, fractions in which Blue Dextran was eluted. ϕR, fractions in which Phenol Red was eluted.

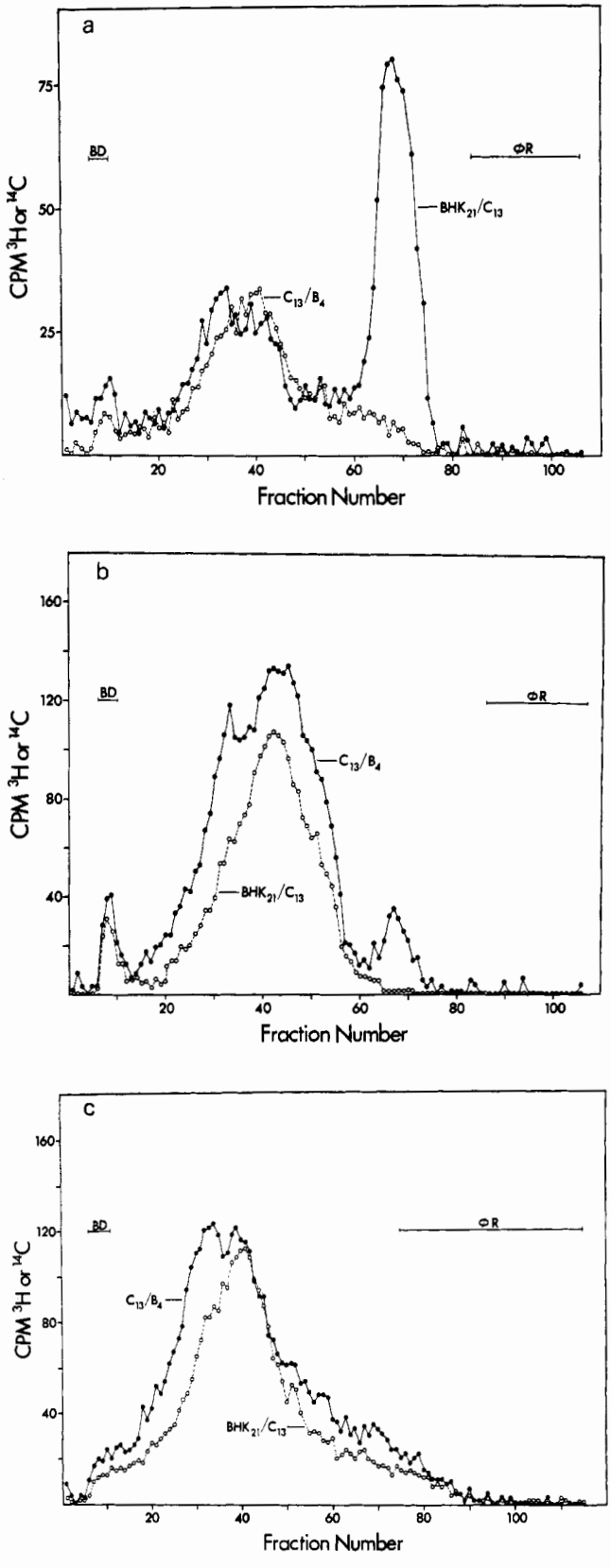

FIGURE 5: Chromatography on Sephadex G-50 of Pronase digested cell fractions. Ribosomal (a) and soluble (b) fractions prepared from BHK_{21}/C_{13} cells grown in the presence of L-[^{3}H]fucose or L-[^{14}C]-fucose (●) were compared to similar fractions prepared from C_{13}/B_4 cells. (c) Material, which was removed during the preparation of the ribosomes by washing with buffer and subsequently precipitated with 5% trichloroacetic acid. All procedures are described in Materials and Methods. BD, fractions in which Blue Dextran was eluted. ϕR, fractions in which Phenol Red was eluted.

the void volume of the gel (Figures 1 and 2) was present in both cell lines regardless of the radioactive precursor, D-glucosamine or L-fucose. Since this material was also observed in the Triton extract from the nuclei (Figure 3), it cannot represent aggregates formed in the presence of large amounts of DNA. Material in the void volume was observed previously in the trypsinates and surface membranes of BHK_{21}/C_{13} and C_{13}/B_4 cells labeled with radioactive D-glucosamine and was susceptible to degradation by testicular hyaluronidase (Buck *et al.*, 1970). However the surface membranes and trypsinates from these cells labeled with radioactive L-fucose contained only negligible amounts of radioactivity in the void volume of the gel (Buck *et al.*, 1970). Robbins and Pederson (1970) have reported the presence of fucose-containing glycopeptides

in nuclei from HeLa cells. They suggest that some of these glycopeptides aggregate in presence of 0.1% sodium dodecyl sulfate. The buffer which was used here for elution of the Sephadex gel contained 0.1% sodium dodecyl sulfate. It is possible that we are observing similar glycopeptides which are unique to the nuclei.

Examination of the fucose-containing glycopeptides from the ribosomal preparations showed a small amount of a heterogeneous population of glycopeptides (Figure 5a). The glycoproteins were digested with Pronase before they were analyzed so the heterogeneity could represent differences in the number of oligosaccharides attached to the Pronase-digested fragments. This however would imply that some synthesis of the oligosaccharide portion of the glycoprotein is occurring on the ribosomal-bound proteins. An alternate and more likely explanation would be the presence of small amounts of Golgi material contaminating the ribosomal preparation. The presence of radioactivity in the ribosomes of BHK_{21}/C_{13} cells which eluted from the gel in the fractions were free fucose is found is not understood (Figure 5a). The possibility that this radioactivity is the result of lysosomal contamination of the ribosomal preparation with the release of free fucose by a fucosidase is not likely since the fractions from both cell lines were incubated together with Pronase before chromatography. This material was seen in large amounts only in the ribosomal fraction of BHK_{21}/C_{13} cells.

Warren *et al.* (1972) have recently described the presence of a specific sialyl transferase which appears to be found in greater amounts in transformed cells than in their normal counterpart. The authors interpret the results to suggest that this transferase is responsible for the differences in the distribution of glycopeptides on Sephadex G-50 which were observed previously (Buck *et al.*, 1970, 1971). Since chemical analyses of these glycopeptides from BHK_{21}/C_3 and C_{13}/B_4 cells have shown increases in all monosaccharides relative to sialic acid (Glick, 1971) it is likely that other monosaccharide transferases could be increased also.

The amounts of sialic acid ($9–10 \times 10^{-12}$ μmol/nucleus) calculated from the results of the neuraminidase-digested nuclei of BHK_{21}/C_{13} and C_{13}/B_4 cells are subject to much reservation. Chemical analyses of nuclei derived from L cells showed less than 5×10^{-12} μmol of sialic acid/nucleus (Glick *et al.*, 1971). These two values for the sialic acid levels are comparable and indicate that less than 0.1% of the sialic acid of the cell is present in the nucleus at least for these two cell lines. Others (Marcus *et al.*, 1965; Molnar, 1967; Wu *et al.*, 1969) have reported the presence of sialic acid in nuclear preparations. On the other hand, nuclear fractions from rat liver have been reported to contain only negligible amounts of sialic acid (Kashnig and Kasper, 1969). Comparisons made of results obtained with the use of different techniques for cell fractionation should be considered with caution, especially when the components to be compared could be derived from the contamination with surface membrane fragments.

A number of attempts were made to compare the glycopeptides from the nuclei or the Triton extracts of nuclei with their respective trypsinates. In every case the resulting elution pattern from Sephadex G-50 was a heterogeneous distribution of fast-eluting material. A comparison of the Triton extracts of nuclei from the transformed cells with the trypsinate derived from the BHK_{21}/C_{13} cells and the reverse comparison were possible (Figure 4a,b). This would suggest that technical artifacts are not responsible for the heterogeneity. Since a number of observations suggest that glycoproteins from the surface membrane may be related ultimately to cell division (Cox and Gesner, 1968; Burger and Noonan, 1970; Shoham *et al.*, 1970; Glick and Buck, 1972), the possibility that this heterogeneous population of glycoproteins which we observed is due to a specific interaction between the nucleus and surface membrane glycoproteins will be the subject of further investigations.

Acknowledgments

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