Proc. Nat. Acad. Sci. USA
Vol. 71, No. 6, pp. 2342–2346, June 1974

Isolation of the Major Viral Glycoprotein and a Putative Precursor from Cells Transformed by Avian Sarcoma Viruses

(immune precipitation/polyacrylamide gel electrophoresis)

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Communicated by Robley C. Williams, March 22, 1974

ABSTRACT **Immune precipitation with a monospecific antiserum was employed to study the synthesis of the major viral glycoprotein gp85. Labeled gp85 was detectable by polyacrylamide gel electrophoresis of immune precipitates prepared from lysates of transformed cells which had been labeled for long term with radioactive amino acid or fucose. When immune precipitates were prepared from lysates of cells pulse-labeled with radioactive amino acid, the bulk of the precipitated counts did not appear in gp85 but in a heterogeneous protein fraction with a mean molecular weight of approximately 70,000; this fraction has been designated p70. If, however, the pulse label was followed by incubation of the cells in medium containing excess unlabeled amino acid, the bulk of the precipitated counts comigrated with gp85. Similar pulse-labeling experiments with radioactive fucose and glucosamine suggested that p70 represents incompletely glycosylated precursor to gp85.**

The viruses of the avian leukosis and sarcoma complex contain seven major structural proteins (reviewed in ref. 1). These proteins comprise four polypeptides, p27, p19, p15, and p12, which define group specific antigenicity; a component, p10, which has not been antigenically characterized; and two glycoproteins, gp85 and gp37, which define type-specific antigenicity and determine properties of viral host range, neutralization, and interference. Quantitative studies on the biosynthesis of these proteins have been limited to the group-specific polypeptides. Vogt and Eisenman (2), using immune precipitation of intracellular viral structural protein in conjunction with polyacrylamide gel electrophoresis, have detected both a high- and low-molecular-weight precursor to group-specific polypeptide. The same method, which was first utilized to study the synthesis of oncornavirus structural protein by Shanmugam *et al.* (3) and Fleissner and Tress (4), has also been used to quantitate the level of p27, the major group-specific polypeptide, in cells infected with three temperature-sensitive mutants of avian sarcoma viruses (5).

In experiments reported here, we have undertaken to study the synthesis of gp85, the major viral glycoprotein, by means of immune precipitation with a monospecific rabbit antiserum. Heretofore, information on the biosynthesis of the viral glycoproteins has been limited to studies employing immunofluorescence with neutralizing chicken sera (5–7). These sera recognize the viral type-specific or envelope antigen which is composed of the two glycoproteins (8, 9). As it is presently unclear whether such sera recognize only antigenic determinants containing carbohydrate, the possibility exists that they may not detect intermediates in the synthesis of gp85 which lack carbohydrate. This problem should be obviated by the use of an antiserum prepared against the purified glycoprotein in an animal of a species that is not the natural host for the virus.

Abbreviations: B_{77}, avian sarcoma virus B_{77} subgroup C; C/E, chicken-embryo cells resistant to subgroup E virus; PR RSV-C, Prague strain of Rous sarcoma virus, subgroup C; NP40, Nonidet P-40; TCA, trichloroacetic acid; SDS, sodium dodecyl-sulfate.

MATERIALS AND METHODS

Viruses and Cells. Clone-purified stocks of avain sarcoma virus B_{77} subgroup C (B_{77}) and the Prague strain of Rous sarcoma virus subgroup C (PR RSV-C) were used for infection of chick-embryo cells. C/E chick-embryos that were gs (group specific) negative were obtained from SPAFAS, Inc., Storrs, Conn. Primary cultures of 10- or 11-day-old chick embryos were prepared as described by Vogt (10). Infection and maintenance of secondary cultures were as described by Duesberg *et al.* (8).

Labeling of Intracellular Protein. For long term labeling experiments, the labeling medium consisted of medium 199 which was 95% deficient in amino acids and contained 2% calf serum. Confluent secondary cultures of noninfected or transformed chick embryo cells (cultured on 100-mm Falcon tissue culture dishes) were incubated for 10 hr in 5 ml of labeling medium supplemented with 50 μCi/ml of [^{3}H]amino acids or 25 μCi/ml of [^{3}H]fucose (15 Ci/mmol) (both from New Eng. Nuclear Corp.). After removal of this medium, the cells were washed three times with phosphate-buffered saline and the tissue culture dish was then frozen at −70°.

For pulse-labeling experiments, confluent cultures of noninfected or transformed cells were incubated for 15 min in Hanks balanced salt solution supplemented with either 100 μCi/ml of [^{3}H]amino acids or with 100 μCi/ml of [^{3}H]fucose, or 100 μCi/ml of [^{3}H]glucosamine (7.3 Ci/mmol) (New England Nuclear Corp.). Both sugars are known to be present in the glycoproteins of avian sarcoma viruses (8). After removal of this medium, the cells were washed three times with phosphate-buffered saline and the tissue culture dish was then frozen at −70°. In certain experiments, prior to the washing step, the cells were incubated for 4 hr in medium containing complete medium 199 supplemented with 2% calf serum.

Immune Precipitation of Cell Associated gp85. A cellular lysate was prepared by adding 2 ml of lysing buffer [0.15 M

NaCl, 0.01 M Tris·HCl (pH 8.1), and 1% (v/v) Nonidet P-40 (NP-40)] to the unfrozen culture dish which in each experiment had contained approximately 2×10^7 cells. The lysing buffer was left on the dish for 15 min at 4° and removed following vigorous aspiration. Nuclei and large membranous fragments were removed from the sample by centrifugation for 40 min at 100,000 × *g*. The resultant supernatant (hereafter referred to as the NP-40 lysate) was saved and the trichloroacetic-acid (TCA) precipitable radioactivity in this fraction was determined.

An indirect immune precipitation was then made with the NP-40 lysate. A given volume of the lysate, adjusted to contain a preselected number of TCA-precipitable counts per minute (cpm) as noted in each figure legend, was brought to 1 ml with lysing buffer. Then, 20 μl of monospecific rabbit anti-gp85 serum prepared against the gp85 of PR RSV-C was added to the lysate. The method of preparation of the purified gp85 used to elicit the antisera has been described (11). The antiserum was prepared by an initial footpad injection of 0.5 mg of the purified gp85 in Freund's adjuvant followed by an intravenous injection of 0.2 mg 6 weeks later. Following incubation at 37° for 1 hr, an excess (0.2 ml) of goat anti-rabbit γ-globulin sera (Antibodies Inc., Davis, Calif.) was added. After incubation for another hour at 37° and overnight at 4°, the resultant precipitate was collected by centrifugation and washed three times with 1.5 ml of lysing buffer which lacked NP-40. Control precipitates were made by substituting normal rabbit sera for the immune rabbit serum.

Sodium Dodecylsulfate (SDS) Gel Electrophoresis. In order to solubilize labeled protein, immune precipitates were boiled for several minutes in 0.2 ml of 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol. Samples of 0.1 ml were then electrophoresed on 5% SDS polyacrylamide gels prepared as described by Duesberg *et al.* (12). In all cases, [^{14}C]amino acid-labeled B_{77} or PR RSV-C viral protein was coelectrophoresed to serve as a marker in the gel. The preparation of labeled viral protein and the method for slicing and counting gels has been described (13).

RESULTS

Figs. 1 and 2 show the gel patterns of immune precipitates made with rabbit anti-gp85 of NP-40 lysates of cells which had been labeled for long term with [^{3}H]amino acids or fucose. Peaks a_1, a_2, and a_3 (Fig. 1) are invariably detected in immune precipitates of amino acid-labeled lysates. As originally characterized by Fleissner and Tress (4), such peaks represent actinlike proteins which spontaneously aggregate out of solution. Their absence from electropherograms of immune precipitates made with fucose-labeled cells (Fig. 2) indicates that they are not fucosylated.

In addition, a peak was present in electropherograms of immune precipitates of transformed cells which comigrated with the gp85 of purified virus (Figs. 1*A* and 2*A*). This peak represented approximately 0.2% in the case of amino-acid label and 3.0% in the case of fucose label of the total TCA-precipitable cpm present in the lysate prior to immune precipitation. This peak was absent from the precipitates of NP-40 lysates prepared from noninfected cells (Figs. 1*B* and 2*B*) as well as from precipitates of lysates of infected cells made with normal rabbit sera (Figs. 1*C* and 2*C*). We, therefore, conclude that this peak represents gp85. Although the antiserum employed for these experiments was prepared against the gp85

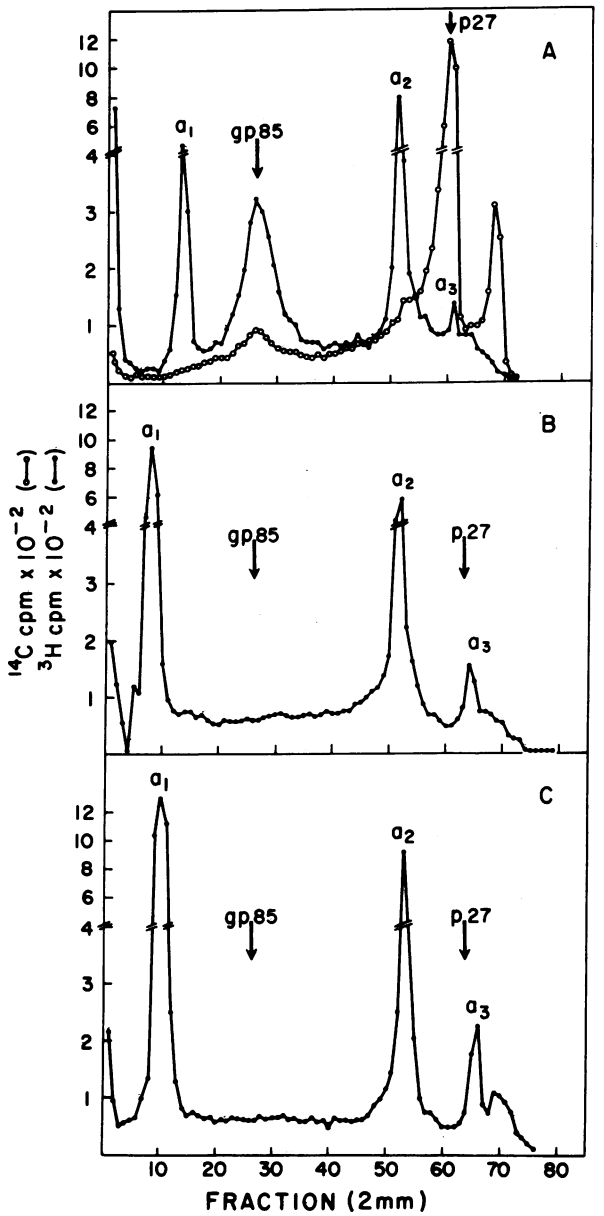

FIG. 1. Electrophoreses on polyacrylamide gels of (*A*) an anti-gp85 immune precipitate prepared with 0.10 ml of an NP-40 lysate of B_{77} transformed cells labeled for long term with [^{3}H]-amino acid; (*B*) the same as *A*, except 0.15 ml of an NP-40 lysate of noninfected cells was analyzed; and (*C*) the same as *A*, except normal rabbit sera was substituted for the anti-gp85 serum. Each sample of lysate contained 6×10^5 TCA-precipitable cpm. [^{14}C]Amino acid-labeled viral protein was coelectrophoresed on each gel to serve as a marker (the pattern is shown in *A*) and the positions of viral gp85 and p27 are indicated. (The numbers on the ordinates of Figs. 1–4 have been multiplied by 10^{-2} as indicated.)

of PR RSV-C, gp85 was detected in NP-40 lysates of cells transformed by B_{77} as well as by PR RSV-C. As both viruses belong to the same subgroup and can each be neutralized with chicken sera prepared against either one (14), this result reflects the presence of common antigenic determinants on the gp85 envelope glycoprotein of both viruses.

A minor peak was also detected in immune precipitates of fucose-labeled cells (fraction 55 of Fig. 2*A*). This peak possibly

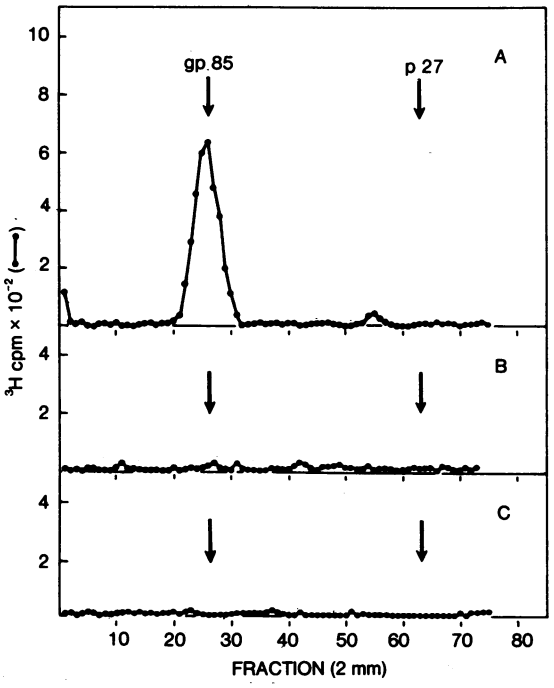

FIG. 2. Electrophoreses on polyacrylamide gels of (*A*) an anti-gp85 immune precipitate prepared with 0.25 ml of an NP-40 lysate of PR RSV-C transformed cells labeled for long term with [^{3}H]fucose; (*B*) the same as *A*, except 0.90 ml of an NP-40 lysate of noninfected cells was analyzed; and (*C*) the same as *A*, except normal rabbit sera was substituted for the anti-gp85 serum. Each sample of lysate contained 10^5 TCA-precipitable cpm. [^{14}C]Amino acid-labeled viral protein was coelectrophoresed on each gel to serve as marker, and the positions of viral gp85 and p27 are indicated.

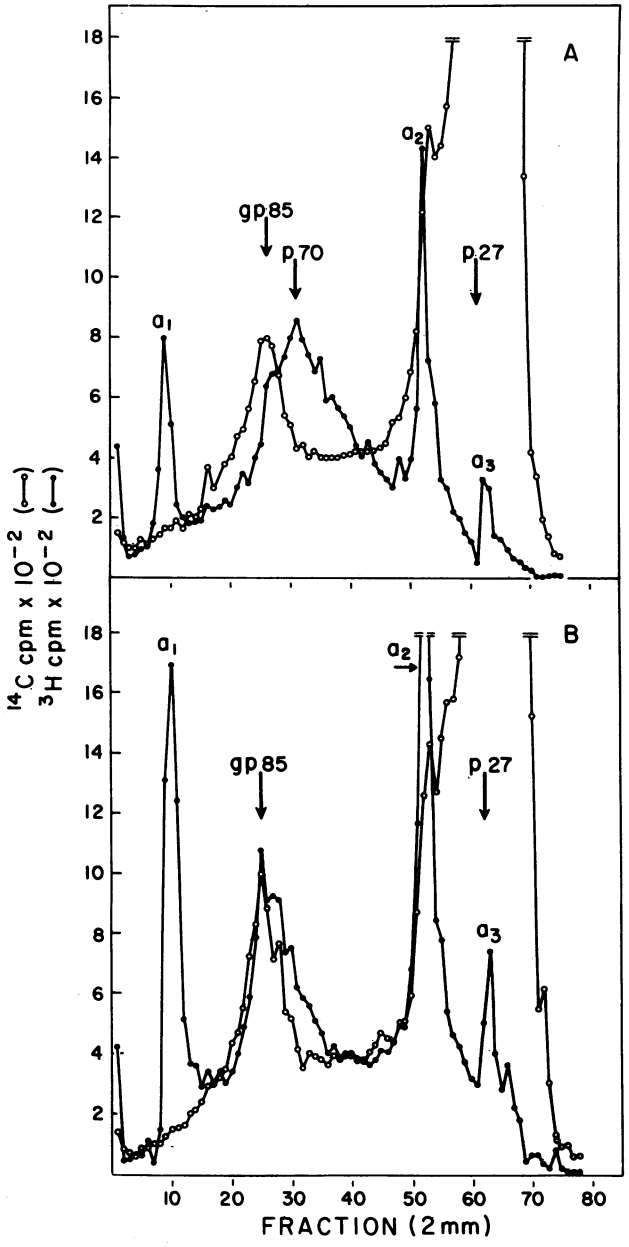

FIG. 3. Electrophoreses on polyacrylamide gels of (*A*) an anti-gp85 immune precipitate prepared with 0.75 ml of an NP-40 lysate of B_{77} transformed cells pulse-labeled with [^{3}H]amino acid and (*B*) same as *A*, except the pulse was followed by incubation of the cells in medium containing excess unlabeled amino acid and the immune precipitate was prepared with 1 ml of the NP-40 lysate. Each sample of lysate contained 1.5 × 10^6 TCA-precipitable cpm. [^{14}C]Amino acid-labeled viral protein was coelectrophoresed on each gel to serve as marker, and the positions of viral gp85 and p27 are indicated.

represents gp37, the minor glycoprotein of avian sarcoma viruses. gp37 has a similar electrophoretic mobility to the peak detected here and, in addition, is known to complex with gp85, even in the presence of mild detergent (1, 8), so that immune precipitation of gp85 might also remove some gp37 from the lysate.

It should be noted that our results do not define the exact state of the gp85 which we are detecting. The possibility that the gp85 was not cytoplasmic, but existed as part of labeled virus which was either in the process of budding or had readsorbed after maturation, was tested by treating the cells with 1% trypsin in 10^{-3} M EDTA prior to NP-40 lysis. This procedure has been reported with the murine oncornavirus system (15) to remove much adsorbed virus which has not yet penetrated the cell surface. Immune precipitation of the resultant lysate yielded 80–90% of the total gp85 counts compared to precipitates of lysates from nontrypsinized transformed cells. This result suggests that much of the labeled gp85 specifically precipitated was probably not contained in virus susceptible to removal by trypsin treatment.

Long-term labeling would not be expected to reveal intermediates in the synthesis of gp85 and, therefore, pulse-labeling conditions were employed for this purpose. As shown in Fig. 3*A*, a rather broad peak which migrated with a greater mobility than gp85 was observed with anti-gp85 immune precipitates of lysates of amino acid pulse-labeled transformed cells. Using the nonglycosylated group-specific polypeptides as molecular weight markers, the position on the gel of the peak fraction would indicate an approximate molecular weight of 70,000 and so this fraction has been designated p70. Though not shown because the electropherograms were indistinguishable from those in Fig. 1*B* and *C*, p70 was absent from immune precipitates of NP-40 lysates of uninfected cells made with the immune serum as well as from immune precipitates of lysates of transformed cells made with normal rabbit sera. As shown in Fig. 3*B*, when cells were pulsed with radioactive amino acids and then incubated with excess unlabeled amino

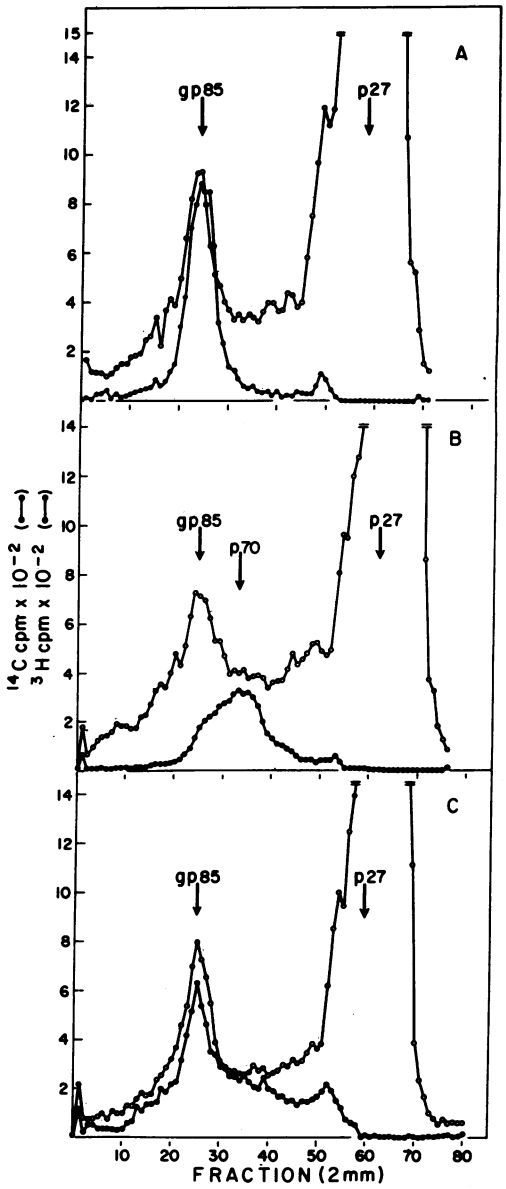

FIG. 4. Electrophoreses on polyacrylamide gels of (*A*) an anti-gp85 immune precipitate prepared with 1 ml (4 × 10^6 TCA-precipitable cpm) of an NP-40 lysate of transformed cells pulse-labeled with [^{3}H]fucose; (*B*) same as *A*, except [^{3}H]glucosamine was used in the pulse and the immune precipitate was prepared with 0.2 ml of the NP-40 lysate; and (*C*) same as *B*, except the pulse was followed by incubation in medium lacking labeled glucosamine and the immune precipitate was prepared with 0.5 ml of the NP-40 lysate. Each sample in *B* and *C* contained 2 × 10^6 TCA-precipitable cpm. [^{14}C]Amino acid-labeled viral protein was coelectrophoresed on each gel to serve as marker, and the positions of viral gp85 and p27 are indicated.

acids, there was a decrease in the number of counts in p70 concomitant with a near equivalent increase of counts in gp85.

The above results are consistent with the hypothesis that p70 represents precursor to gp85. In particular, the greater mobility and heterogeneity of p70 compared to gp85 suggested that p70 lacked the full complement of carbohydrate present in gp85. Labeling experiments with radioactive fucose and glucosamine were therefore carried out to test this possibility. Fucose is the terminal sugar residue in a number of glycoproteins (16) and might therefore be expected to be added after glucosamine. As shown in Fig. 4*A*, when cells were pulsed with labeled fucose, the radioactive counts specifically precipated with anti-gp85 serum migrated mainly with gp85 with a slight skew to p70. By contrast, in a pulse with labeled glucosamine, although label is detectable migrating with marker gp85, a majority of the counts specifically precipated with anti-gp85 serum appeared in the p70 region of the gel (Fig. 4*B*). Following an additional incubation for 4 hr, however, in medium which lacked labeled glucosamine, labeled gp85 was the major component detectable (Fig. 4*C*).

DISCUSSION

The isolation of cell-associated gp85 has been accomplished by the use of a method similar to one employed previously to isolate group-specific polypeptide (2, 4, 5). In contrast to earlier studies, however, a monospecific antiserum rather than polyspecific antiserum prepared against whole disrupted virus was utilized for immune precipitation. In the present study a gel system was also employed which allowed the clear separation of gp85 from the actinlike proteins which aggregate out of cellular lysates (4). Since, however, these proteins are not fucosylated, their presence was only detected in lysates of cells which had been labeled with amino acids. The possibility that we were scoring another protein as gp85 can be ruled out since the component isolated here had an identical mobility on polyacrylamide gel to the gp85 from purified virus and, in addition, was not present in uninfected cells or detected with nonimmune rabbit sera.

A component designated p70 was also precipitable with the anti-gp85 serum. p70 was readily detectable in lysates of transformed cells which had been pulse-labeled with radioactive amino acid or glucosamine. Upon removal of isotope and further incubation of the cells, label disappeared from p70 and appeared in gp85. These data are most easily reconciled with the notion that p70 represents precursor to gp85 in various stages of glycosylation. The observation that with a 15 min pulse-label radioactive fucose was found almost exclusively in gp85, whereas radioactive glucosamine was clearly present in both gp85 and p70, is consistent with the hypothesis that most of the fucose residues are added later than the glucosamine residues to the carbohydrate sidechains of the glycoprotein. Since gp85 contains 20–30% carbohydrate (unpublished results), the mobility on SDS gel of the fastest moving components in the heterogeneous p70 peak is of the order expected for precursor that is largely free of carbohydrate. A final assessment as to whether the molecules in p70 have a polypeptide moiety equivalent to that of gp85 must, however, await comparative chemical analyses of gp85 and p70.

If p70 is in fact precursor to gp85, it may be possible by use of detailed kinetic experiments in conjunction with immune precipitation of protein present in different subcellular fractions to deduce the pathway of carbohydrate addition whereby gp85 becomes the glycosylated form of p70. Such an approach has previously been used to define the pathway of carbohydrate addition to the light chain of immunoglobulin (17). Its application to a comparative study of gp85 biosynthesis in transformed and nontransformed cells replicating avian leukosis virus would be of particular interest in view of the observation that the glycoproteins of virus grown in transformed cells are more highly glycosylated than those of virus grown in nontransformed cells (18).

We thank Ellis Rucker and Carla Brooks for excellent technical assistance, Dr. Robert Friis for provision of the virus stocks used for infection, and Drs. Ralph Smith and Robert Green for provision of materials used in preparation of the sera. This investigation was supported by USPHS Research Grants AI-11806 and CA-15464 from the National Institutes of Health and Contract NIH-No. 1 CP 33308 from the Virus Cancer Program of the National Cancer Institute.

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