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Modification of Simian Virus 40 Large Tumor Antigen by Glycosylation

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The SV40-encoded transforming protein, large tumor antigen (T-ag), is multifunctional. Chemical modifications of the T-ag polypeptide may be important for its multifunctional capacity. T-ag is additionally modified by glycosylation. T-ag was metabolically labeled in SV40-infected cells with tritiated galactose or glucosamine, but not with mannose or fucose. The identity of glycosylated T-ag was established by immunoprecipitation with a variety of T-ag-specific antisera, including monoclonal antibodies. Incorporation of labeled sugar into T-ag was inhibited in the presence of excess unlabeled sugars, but not in the presence of excess unlabeled amino acids. Labeled monosaccharides could be preferentially removed from T-ag with a mixture of glycosidic enzymes. In addition, galactose was removed from purified T-ag by acid hydrolysis and identified as such by thin-layer chromatography. T-ag oligosaccharides were resistant to treatment with EndoH, and glycosylation was not inhibited by tunicamycin. Together, these data strongly suggest that T-ag is glycosylated. Several characteristics, including lack of mannose labeling, EndoH resistance, and tunicamycin resistance, suggest that T-ag is not an N-linked glycoprotein. Rather, these properties are more consistent with the identification of T-ag as an O-linked glycoprotein. © 1985 Academic Press, Inc.

INTRODUCTION

Simian virus 40 (SV40) encodes the synthesis of two early proteins, designated the small tumor antigen (t-ag) and the large tumor antigen (T-ag), that exhibit apparent molecular weights (MW) of about 20,000 and 90,000, respectively. Both are chemically modified polypeptides, and their entire amino acid sequences are known (Fiers *et al.*, 1978; Reddy *et al.*, 1978). It is thought that T-ag mediates most of the events culminating in cellular transformation by SV40. Accordingly, a vast array of biochemical and biological functions has been attributed to this polypeptide (reviewed by Martin, 1981; Tooze, 1981; Rigby and Lane, 1983). However, the molecular basis for its multifunctional capacity remains obscure.

One possible explanation invokes the known chemical modifications of the T-ag polypeptide. Theoretically, those modifi-

cations could generate distinct forms of T-ag that might perform different functions in the host cell. The modifications reported for T-ag include phosphorylation (Tegtmeyer *et al.*, 1977), N-terminal acetylation (Mellor and Smith, 1978), poly-ADP-ribosylation (Goldman *et al.*, 1981), fatty acid acylation (Klockmann and Depert, 1983), and glycosylation (Schmidt-Ullrich *et al.*, 1977, 1982). However, the evidence suggesting that T-ag possesses the latter modification (i.e., glycosylation) is both limited and indirect. Therefore, we have addressed this question by performing detailed biochemical studies. The results of these analyses establish that T-ag is glycosylated and that its oligosaccharide moiety (or moieties) contains galactose and glucosamine and/or their derivatives. The oligosaccharides probably do not contain mannose or fucose and are resistant to treatment with endo- β -N-acetylglucosaminidase H (EndoH). In addition, glycosylation of T-ag is not inhibited by tunicamycin (TM), suggesting that it is not an N-linked, but rather, is probably an O-linked glycoprotein.

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Glycosylation of viral transformation proteins has been recognized in only a few retrovirus systems and has not previously been conclusively demonstrated with any DNA tumor virus. All of the known retroviral transformation glycoproteins are N-glycosylated (Dresler *et al.*, 1979; Hayman *et al.*, 1983; Privalsky *et al.*, 1983); none has been shown to be O-glycosylated. Only a single viral glycoprotein, the E1 matrix glycoprotein of certain coronaviruses, is believed to be exclusively O-glycosylated (Holmes *et al.*, 1981; Niemann and Klenk, 1981). Thus, the demonstration of O-glycosylation of SV40 T-ag provides a novel basis for further investigation of T-ag multifunctionality, particularly with respect to the significance of this modification. In addition, T-ag may be a useful model for the study of O-linked glycoproteins in general, which remain relatively poorly characterized.

MATERIALS AND METHODS

Cells and virus. TC-7 cells (Robb and Huebner, 1973) were propagated in enriched Eagle's minimum essential medium (E-MEM; GIBCO, Grand Island, N. Y.; Noonan *et al.*, 1976). Mm5mt/c1 cells (Owens and Hackett, 1972), mammary tumor cells that produce mouse mammary tumor virus (MMTV), were propagated in Dulbecco's modified minimum essential medium (D-MEM, GIBCO; Slagle *et al.*, 1984). Wild-type SV40 was passaged at a low multiplicity of infection (m.o.i.) and plaque assayed in TC-7 cells (Noonan and Butel, 1978). Vesicular stomatitis virus (VSV; Indiana strain) was passaged and titrated as described for SV40, except that it was not subjected to freeze-thawing. C3H mouse mammary tumor virus [(C3H)-MMTV] concentrates were provided by the Biological Carcinogenesis Branch, Division of Cancer Cause and Prevention, National Cancer Institute.

Antisera. Normal hamster serum (NHS), hamster ascites fluid containing antibodies against T-ag (HAF), normal rabbit serum (NRS), and rabbit antiserum against purified T-ag (R α T) or disrupted (C3H)-MMTV (α MMTV) have been previously

described (Lanford and Butel, 1979; Slagle *et al.*, 1984). Rabbit antiserum against purified VSV (Rb α VSV) was generously provided by Dr. Trudy Morrison. T-ag or cellular protein p53-specific monoclonal antibodies used in this study included PAb 100, 101, and 122 (Gurney *et al.*, 1980), PAb 204 (Lane and Hoeffler, 1980), and PAb 402, 405, 414, 416, 419, 421, 423, and 430 (Harlow *et al.*, 1981). Hybridomas were cultured and antibodies were precipitated from culture supernatants as previously described (Harlow *et al.*, 1981; Santos and Butel, 1984).

Infections and metabolic labeling. TC-7 monolayers were mock infected with E-MEM or infected with SV40 or VSV at an m.o.i. of 50 plaque-forming units (PFU)/cell (Jarvis *et al.*, 1984). For VSV infections, 1.5 μ g/ml actinomycin D (Sigma Chemical Co., St. Louis, Mo.) was included with the inoculum and maintained throughout the infection. At appropriate times postinfection (p.i.), monolayers were washed with Tris-buffered saline (TBS) and starved in glucose-free E-MEM for 30 min (Martineau *et al.*, 1972). Cells were then labeled with [³⁵S]-methionine, D-[1-³H]galactose, D-[1-³H]-glucosamine, D-[2-³H]mannose, or L-[6-³H]-fucose (Amersham, Arlington Heights, Ill.). Labeling times and radioisotope concentrations are specified in the figure legends. Mm5mt/c1 cells were labeled as previously described (Slagle *et al.*, 1984).

Special labeling conditions. In some experiments, SV40-infected cells were labeled with [³⁵S]methionine or ³H-mono-saccharides in the presence of excess unlabeled amino acids, excess unlabeled sugars, or TM. Normal (1 \times) amino acid concentrations in E-MEM were approximately: Trp, 0.1 mM; Met, 0.3 mM; His, 0.4 mM; Gly and Phe, 0.5 mM; Ser and Arg, 0.6 mM; Leu, Ile, Thr, and Val, 1.0 mM; Lys, 1.4 mM; and Gln, 3.0 mM. E-MEM and glucose-free E-MEM containing 5 times (5 \times), 10 times (10 \times), or 50 times (50 \times) the normal concentration of amino acids were prepared. For labeling in the presence of excess amino acids, infected cells were treated with E-MEM containing different amino acid concentrations be-

ginning at 2 hr p.i. Cells were starved from 20.5 to 21 hr p.i. using glucose-free E-MEM supplemented with the same amino acid concentrations and were labeled for 3 hr using 25 $\mu\text{Ci/ml}$ [^{35}S]methionine or 100 $\mu\text{Ci/ml}$ [^3H]galactose in the same medium. E-MEM normally contained 1 mg/ml glucose. For labeling in the presence of excess unlabeled sugars, E-MEM also contained 1 mg/ml galactose for 1 \times sugar-enriched medium or higher concentrations of both sugars for 5 \times , 10 \times , and 50 \times sugar-enriched medium. Infected cells were starved in glucose-free E-MEM from 20.5 to 21 hr p.i., then labeled for 3 hr using 25 $\mu\text{Ci/ml}$ [^{35}S]methionine or 100 $\mu\text{Ci/ml}$ [^3H]galactose in E-MEM containing different sugar concentrations. Finally, for TM experiments, E-MEM and glucose-free E-MEM containing various concentrations of TM were prepared. Treatment was initiated at 15 hr p.i. and maintained throughout the 30-min starvation and 3-hr labeling periods (20.5–24 h p.i.).

Extraction, immunoprecipitation, and gel electrophoresis. Labeled monolayers were washed, detergent-extracted in the presence of leupeptin (Jarvis *et al.*, 1984), and extracts were clarified. After incubation with an appropriate antiserum, formalin-fixed *Staphylococcus aureus* strain Cowan I (SACI) (Kessler, 1975) was added to adsorb immune complexes. If monoclonal antibody was used, a goat anti-mouse IgG bridge was included. Immunoprecipitates were then washed three times with wash buffer (WB; 50 mM Tris, pH 8.0, 100 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS), disrupted, and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Soule and Butel, 1979). Gels were impregnated with Autofluor (National Diagnostics, Somerville, N. J.), dried, and exposed to X-ray film at -70° .

Treatment of T-ag or MMTV polypeptides with glycosidases. SV40-infected TC-7 cells or Mm5mt/c1 cells were glucose starved, doubly labeled with [^{35}S]methionine and [^3H]glucosamine, and extracts were immunoprecipitated with HAF or α MMTV and SACI. Immune complexes

were washed three times with WB, then twice with TBS. Pellets were resuspended in TBS supplemented with 200 μM leupeptin (Sigma) and 1000 kIU Trasylol (Mobay Chemical Co., New York, N. Y.), mixed, and triplicate samples were removed for determination of pretreatment acid-precipitable ^3H and ^{35}S radioactivity. TBS, EndoH (Miles Laboratories, Inc., Elkhart, Ind.), or *Charonia lampas* mixed glycosidases (Miles) were added, and mixtures were incubated with shaking at 37° for 15 hr. Triplicate samples were then removed for determination of post-treatment acid-precipitable ^3H and ^{35}S radioactivity, and the remainder was processed for SDS-PAGE analysis. Pre- and post-treatment samples were disrupted, clarified, and spotted onto glass microfiber filters (Whatman, Clifton, N. J.). Filters were dried, then washed once with 10% trichloroacetic acid (TCA), twice with 5% TCA, and once with 95% ethanol. Filters were redried, then assayed for ^3H and ^{35}S radioactivity by liquid scintillation spectroscopy.

Acid hydrolysis of T-ag and analysis of monosaccharides. Glucose-starved, SV40-infected TC-7 cells were doubly labeled with [^{35}S]methionine and [^3H]galactose, and T-ag was isolated by immunoprecipitation and SDS-PAGE. T-ag was excised, eluted from the gel, concentrated, and desalted on a Sephadex G25M column (Pharmacia Fine Chemicals, Uppsala, Sweden). The sample was then reconcentrated and SDS was extracted (Henderson *et al.*, 1979). Carrier galactose was added, and the mixture was suspended in 2.0 N H_2SO_4 and sealed under nitrogen in a glass ampule. After hydrolysis for 4 hr at 100° , the reaction mixture was diluted to 0.1 N H_2SO_4 and applied to a Dowex 50W-X8 cation-exchange column. The flow-through was neutralized, clarified, and lyophilized. This material was then applied to a silica gel G thin-layer plate (Fisher Scientific Co., Pittsburg, Pa.) and chromatographed with *n*-propanol:water (7:1, v/v) in a closed chamber (Gal, 1968). The plate was dried and stained with 30% aqueous ammonium bisulfate for visualization of the carrier and standard mono-

saccharides. After measurement of R_f values, the lanes in which hydrolysates had been chromatographed were fractionated, and fractions were assayed for radioactivity by liquid scintillation spectroscopy.

RESULTS

Metabolic Labeling of T-ag in SV40-Infected Cells with Radioactive Monosaccharides

In initial experiments designed to determine if T-ag is glycosylated, we utilized metabolic labeling of SV40-infected cells with [35 S]methionine or tritiated monosaccharides.

A polypeptide of about 88,000 (88K) in apparent MW was detected by immunoprecipitation of [35 S]methionine-labeled, SV40-infected cell extracts with HAF (Fig. 1A, lane 3). This polypeptide was identified as T-ag because it was not detected in mock-infected cell extracts immunoprecipitated with NHS or HAF or in infected cell extracts immunoprecipitated with NHS (lanes 1, 2, and 4). A comigrating

polypeptide, presumably T-ag, was also detected in [3 H]galactose- (lane 8) and [3 H]glucosamine-labeled (lane 16), infected cell extracts immunoprecipitated with HAF; it was absent in the corresponding mock-infected or NHS-treated controls (lanes 5-7 and 13-15). Labeled T-ag was not recovered from cells pulsed with [3 H]mannose (lanes 9-12) or [3 H]fucose (lanes 17-20), although unlabeled T-ag was immunoprecipitated from infected cells with HAF as evidenced by Coomassie blue staining. Incorporation of [3 H]galactose into T-ag was enhanced when infected cells were pretreated with glucose-free E-MEM and the isotope was added in this same medium. This can be seen by comparing lanes 1 and 2 in Fig. 1B. The T-ag shown in lane 1 was obtained by labeling unstarved, infected cells, while that in lane 2 was obtained by labeling cells after a 30-min starvation period in glucose-free E-MEM. These results suggest that at least a portion of the T-ag population synthesized in SV40-infected cells is modified by glycosylation. Only

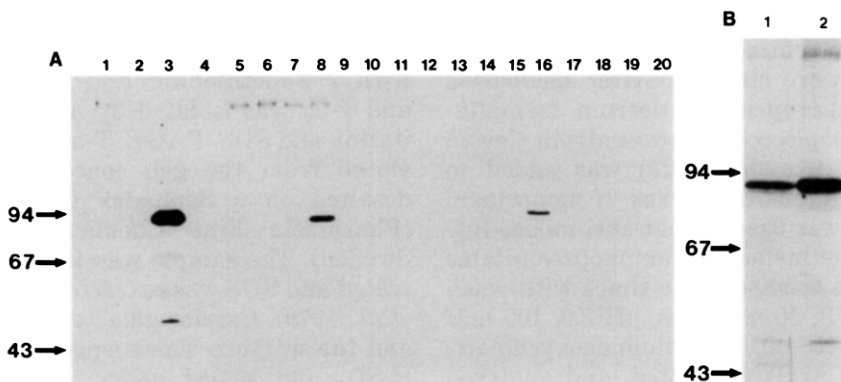


FIG. 1. Metabolic labeling of SV40 T-ag with various monosaccharides. Confluent TC-7 cell monolayers were washed with warm TBS, then mock infected with E-MEM (A: lanes 1-2, 5-6, 9-10, 13-14, and 17-18) or infected with 50 PFU/cell SV40 (A: lanes 3-4, 7-8, 11-12, 15-16, and 19-20; B: lanes 1 and 2). After adsorption for 2 hr at 37°, the cells were fed with E-MEM and returned to 37°. Except for the sample in B, lane 1, cells were starved prior to labeling for 30 min in glucose-free E-MEM. Cells were then labeled from 21 to 24 hr p.i. with 100 μ Ci/ml [35 S]methionine (A: lanes 1-4), D-[1- 3 H]galactose (A: lanes 5-8; B: lanes 1 and 2), D-[2- 3 H]mannose (A: lanes 9-12), D-[1- 3 H]glucosamine (A: lanes 13-16), or L-[6- 3 H]fucose (A: lanes 17-20). After labeling, cells were washed, extracted, and clarified extracts were immunoprecipitated with NHS (A: lanes 1, 4, 5, 7, 9, 11, 13, 15, 17, and 19) or HAF (A: lanes 2, 3, 6, 8, 10, 12, 14, 16, 18, and 20; B: lanes 1 and 2). Immune complexes were adsorbed using SACI, washed thoroughly, then disrupted and analyzed by SDS-PAGE on 8% acrylamide gels. Numbers to the left of the figure indicate the positions of molecular weight standards, designated by their molecular weights $\times 10^{-3}$.

galactose and glucosamine were incorporated into T-ag, suggesting that its putative oligosaccharide moiety(ies) contains those sugars and/or their derivatives.

It was possible that the relatively short (3 hr) labeling time used in the initial experiments had not permitted equilibration of intracellular sugar pools with each input labeled monosaccharide, producing false negative results. Therefore, SV40-infected cells were labeled for 3, 6, or 18 hr using tritiated galactose, glucosamine, or mannose, and samples were processed as before. Radioactive T-ag was detected in all of the galactose-labeled samples and in the 18-hr glucosamine-labeled sample, but was not detected in any of the mannose-labeled samples (Fig. 2). These observations support the interpretation that putative T-ag oligosaccharide(s) contain galactose and glucosamine and/or their derivatives but lack mannose. It was not firmly established that the oligosaccharide(s) lack fucose, since that sugar was

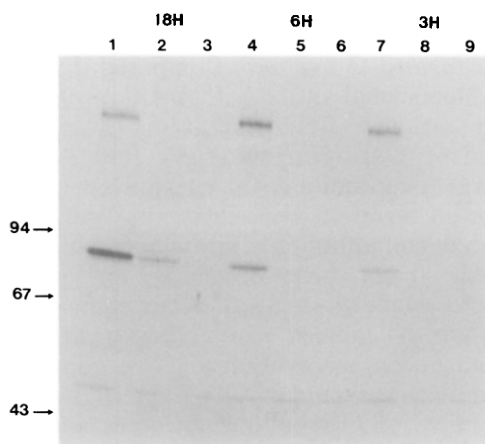


FIG. 2. Metabolic labeling of SV40 T-ag with various monosaccharides. Confluent TC-7 monolayers were infected with SV40 as described in the legend to Fig. 1. Cells were labeled without prior glucose starvation from 6 to 24 hr p.i. (18 hr; lanes 1-3), 18-24 hr p.i. (6 hr; lanes 4-6), or 21-24 hr p.i. (3 hr; lanes 7-9) with 20 μ Ci/ml D-[1- 3 H]galactose (lanes 1, 4, 7), D-[1- 3 H]glucosamine (lanes 2, 5, 8), or D-[2- 3 H]mannose (lanes 3, 6, 9). Samples were then processed and analyzed as described in the legend to Fig. 1, using HAF for immunoprecipitation and 8% acrylamide gels for analysis. Molecular weight markers are shown on the left.

not included in this experiment. However, except for the 18-hr galactose-labeled sample, T-ag was not as intensely labeled in this experiment as in the experiment shown in Fig. 1A. Thus, shorter pulses with higher concentrations of radioactive sugars generally provided better incorporation than longer pulses with lower concentrations, suggesting that adequate uptake of the input sugars had occurred under those labeling conditions.

Preliminary experiments using [35 S]-methionine labeling had established the maximal time of T-ag synthesis under our conditions of infection to be about 20-24 hr p.i. (data not shown). This time period was chosen for the experiments described above since a large amount of T-ag would be available for labeling and the detection of monosaccharide-labeled T-ag would be optimized. However, if glycosylated T-ag represents a discrete subpopulation of T-ag, then its maximal time of synthesis might differ from that of overall T-ag synthesis. Therefore, we examined the absolute and relative incorporation of [35 S]methionine and [3 H]galactose into T-ag at various times p.i. The absolute incorporation of both isotopes was greatest at 21-24 hr p.i., suggesting that maximal glycosylation occurred at the same time as maximal overall T-ag synthesis (Table 1). However, the relative incorporation of [3 H]galactose compared to [35 S]methionine was highest very early in infection, as shown by $^3\text{H}:$ ^{35}S ratios (Table 1). The ratio decreased steadily until about 12 hr p.i., then remained approximately constant until 48 hr p.i. This result might indicate that relatively more T-ag molecules or more sites on T-ag are glycosylated early in infection. Although a large degree of variability in the $^3\text{H}:$ ^{35}S ratios was observed among different experiments, the ratio repeatedly decreased with time after infection.

Positive Identification of the 88K Glycoprotein as T-ag

In the experiments described above, an 88K glycoprotein was identified as T-ag because it was immunoprecipitated from

TABLE 1

INCORPORATION OF [³⁵S]METHIONINE AND [³H]GALACTOSE INTO T-ag AT VARIOUS TIMES POSTINFECTION^a

Hours p.i.	³⁵ S cpm ^b	³ H cpm ^b	Ratio ³ H cpm: ³⁵ S cpm
Experiment 1			
24	16796	2154	0.13
36	12891	1466	0.11
48	8884	862	0.10
Experiment 2			
12	1529	376	0.25
15	2695	503	0.19
18	4007	704	0.18
21	4302	814	0.19
24	3971	725	0.18
36	2356	458	0.19
48	1776	315	0.18
Experiment 3			
6	240	320	1.33
9	1376	732	0.53
12	3320	974	0.29
24	7188	1741	0.24

^a SV40-infected cells were glucose starved for 30 min, then doubly labeled with [³⁵S]methionine (25 μ Ci/ml) and [³H]galactose (100 μ Ci/ml) for 3 hr, ending at the time points indicated in the table. T-ag was immunoprecipitated from clarified cell extracts, gel purified, and eluted from the gel. Concentrated eluents were then quantitated for ³⁵S and ³H content by double-label liquid scintillation spectroscopy.

^b Raw counts per minute (cpm) were determined using two channels on a Beckman model LS-250 spectrometer, preadjusted to detect ³⁵S or ³H. Raw cpm were corrected for relative efficiency of counting on those channels as compared to an open channel and, in the case of ³H cpm, for a small amount of contamination with ³⁵S cpm. Correction factors were determined by counting singly labeled samples under conditions identical to those used for the experimental samples.

infected cell lysates with HAF and was absent in the corresponding mock-infected and NHS controls. However, since HAF is a polyclonal antiserum, exhibiting multiple immunologic reactivities, it was necessary to more conclusively identify this 88K polypeptide.

Several different T-ag-specific and control antisera were used for immunopre-

cipitation of galactose-labeled, infected cell extracts (Fig. 3). HAF, R α T, and all 10 of 10 different monoclonal antibodies against T-ag immunoprecipitated the galactose-labeled 88K polypeptide. This polypeptide was not immunoprecipitated with NHS, NRS, or a control monoclonal antibody directed against human IgG. These results identify the 88K glycoprotein as T-ag, since it is extremely unlikely that another protein would possess that many cross-reactive antigenic determinants.

Evidence against Randomization of Input Monosaccharide Label

An important consideration in the interpretation of metabolic labeling experiments, particularly those involving monosaccharides, is the possibility that randomization of label may occur. The input monosaccharide might become metabolically converted to another form in the cell, such as amino acids, which could then be incorporated into the protein of interest. The following experiments were performed to evaluate this possibility.

Monoclonal antibody PAb 419 recognizes an antigenic determinant on the amino end of T-ag (Harlow *et al.*, 1981). Since large T-ag and small t-ag share common amino terminal sequences, that antibody would coimmunoprecipitate both polypeptides from [³⁵S]methionine- or [³H]galactose-labeled infected cell extracts. Both should be labeled with galactose if randomization were occurring. In contrast, small t-ag would not be labeled if randomization of label did not occur and the glycosylation site(s) on large T-ag were located on a portion of the polypeptide distal to those sequences shared with small t-ag. As expected, PAb 419 immunoprecipitated both polypeptides from [³⁵S]methionine-labeled infected cell extracts (Fig. 4). Coomassie blue staining revealed that both large T-ag and small t-ag were also immunoprecipitated from galactose-labeled infected cell extracts (data not shown). However, only large T-ag was detectably labeled with [³H]galactose. This observation, although in-

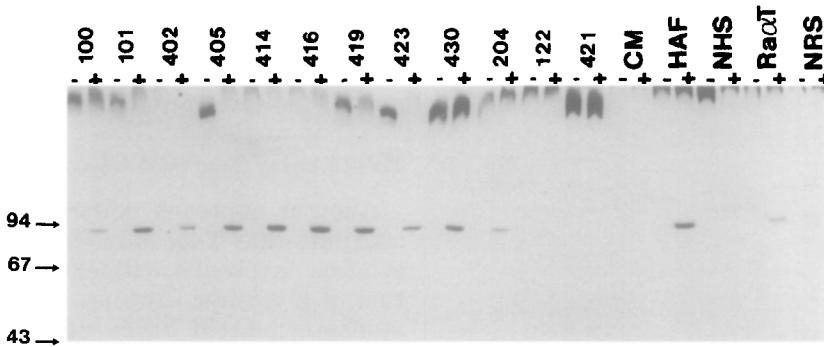


FIG. 3. Immunoprecipitation of galactose-labeled SV40 T-ag with various antisera. TC-7 cells were mock infected (–) or SV40 infected (+), glucose starved, and labeled with D-[1-³H]galactose as described in the legend to Fig. 1. Cells were extracted, then clarified extracts were immunoprecipitated with different antisera. Immune complexes were adsorbed, washed, disrupted, and analyzed on 8% acrylamide gels. The headings at the top of the figure indicate the antisera used; numbers refer to PAb designations for T-ag and p53-specific monoclonal antibodies, CM refers to a control monoclonal antibody directed against human IgG. Other antisera abbreviations are described under Materials and Methods. Molecular weight markers are shown on the left.

direct, suggests that randomization of the input sugar label had not occurred to a significant degree. In addition, it is likely that the glycosylation sites on large T-ag are not located within the region of the polypeptide encoded from nucleotides 5163 to 4917, since that is the region in common with small t-ag.

A more direct approach to the potential problem of randomization involved metabolic labeling of T-ag with [³⁵S]methionine or [³H]galactose in the presence of excess unlabeled amino acids or sugars. The incorporation of [³⁵S]methionine should be inhibited by the presence of unlabeled amino acids but unaffected by the presence of unlabeled sugars. Conversely, if [³H]galactose were incorporated into T-ag in the form of sugar, that incorporation should be unaffected by unlabeled amino acids but inhibited by unlabeled sugars. The results with [³⁵S]methionine labeling were as predicted; 63 and 82% inhibitions were observed when labeling was performed in the presence of 5× and 10× amino acids, respectively (Fig. 5A, Table 2). No inhibition was observed with up to 10× sugars; instead, [³⁵S]methionine incorporation was slightly enhanced with increasing sugar concentration (Fig. 5B, Table 2). At 50× sugars, methionine incorporation was strongly inhibited, for

unknown reasons. Perhaps uptake of metabolites from the growth medium was inhibited at this sugar concentration. Coomassie blue staining revealed little difference in the amount of T-ag synthesized in cells treated with various sugar concentrations (data not shown). For [³H]galactose incorporation, excess unlabeled amino acids had little effect, except for a slight inhibition at the 10× level (Fig. 5A, Table 2). Coomassie blue staining revealed a slight reduction in the amount of T-ag immunoprecipitated from cells treated with 10× amino acids (data not shown). In addition, cells treated with higher (50×) levels of amino acids were killed. Thus, it is likely that the slight inhibition of [³H]galactose incorporation observed in the presence of 10× amino acids was due to minor toxicity and reflected a decrease in overall T-ag synthesis. Incorporation of [³H]galactose into T-ag was significantly inhibited in the presence of unlabeled sugars (Fig. 5B); 90, 95, 96, and 96% inhibitions were observed for 1×, 5×, 10×, and 50× sugars, respectively (Table 2). These results strongly suggest that T-ag was specifically labeled with [³H]galactose due to glycosylation events, rather than simply due to randomization of the input sugar label.

Another possibility not addressed in

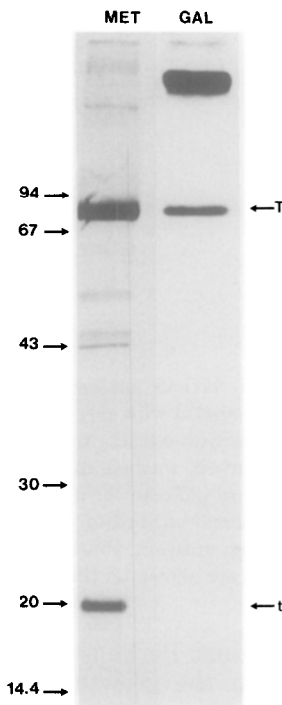


FIG. 4. Differential labeling of SV40 tumor antigens. SV40-infected TC-7 cells were glucose-starved, then labeled from 21 to 24 hr p.i. with 25 $\mu\text{Ci/ml}$ [^{35}S]methionine (MET) or 100 $\mu\text{Ci/ml}$ D-[1- ^3H]galactose (GAL). After extraction and clarification, PAb 419 was used for immunoprecipitation. Immune complexes were harvested, washed, disrupted and analyzed on 12% acrylamide gels. T and t on the right-hand side mark the positions of large and small tumor antigens, respectively. Molecular weight markers are shown on the left.

the randomization control experiments described above is that [^3H]galactose could have been metabolically converted to labeled nucleotides. In fact, galactose can be converted to ribose through the phosphogluconate pathway and could then be incorporated into T-ag as poly-ADP-ribose or RNA oligonucleotides. These products are found in covalent or tight association, respectively, with T-ag (Goldman *et al.*, 1981; Khandjian *et al.*, 1982). However, this possibility was precluded by using galactose tritiated in the number 1 position as the input sugar. That carbon and its hydrogens are lost in the early steps of the phosphogluconate pathway before ribose is produced. Thus, nucleotides con-

taining ribose generated from D-[1- ^3H]galactose would not be radioactive, and labeled T-ag would not be detected as a result of such a conversion.

Treatment of T-ag with Glycosidic Enzymes

Another approach undertaken to substantiate that T-ag is modified by glycosylation involved treatment with a mixture of glycosidic enzymes. T-ag was immunoprecipitated from doubly labeled,

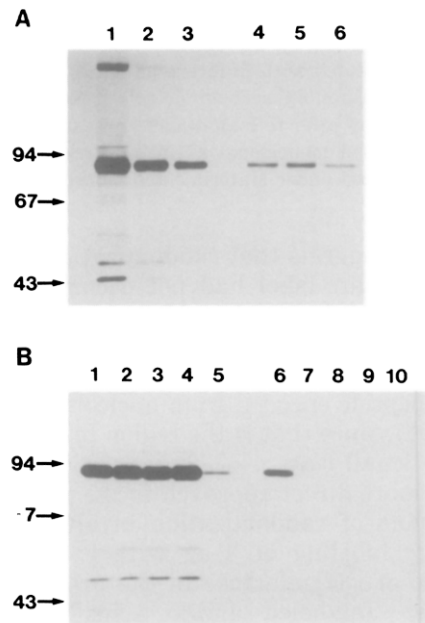


FIG. 5. Ability to label SV40 T-ag with [^{35}S]methionine or [^3H]galactose in the presence of excess unlabeled amino acids or sugars. (A) SV40-infected TC-7 cells were glucose starved and labeled in the presence of 1 \times (lanes 1 and 4), 5 \times (lanes 2 and 5), or 10 \times (lanes 3 and 6) amino acids, as described under Materials and Methods. Cells were labeled with 25 $\mu\text{Ci/ml}$ [^{35}S]methionine (lanes 1-3) or 100 $\mu\text{Ci/ml}$ [^3H]galactose (lanes 4-6), extracted, and T-ag was immunoprecipitated from clarified extracts with HAF. Disrupted immunoprecipitates were analyzed on 8% acrylamide gels. (B) Glucose-starved, SV40-infected TC-7 cells were labeled in the presence of 0 \times (lanes 1 and 6), 1 \times (lanes 2 and 7), 5 \times (lanes 3 and 8), 10 \times (lanes 4 and 9), or 50 \times (lanes 5 and 10) glucose- and galactose-containing E-MEM. Cells were labeled with 25 $\mu\text{Ci/ml}$ [^{35}S]methionine (lanes 1-5) or 100 $\mu\text{Ci/ml}$ [^3H]galactose (lanes 6-10), then processed and analyzed as described for A. Molecular weight markers are shown on the left.

TABLE 2

INCORPORATION OF [³⁵S]METHIONINE OR [³H]GALACTOSE INTO T-ag IN THE PRESENCE OF EXCESS UNLABELED AMINO ACIDS OR SUGARS^a

Labeling conditions	³⁵ S cpm	% Control		
		³⁵ S cpm	% Control	
Amino acids				
1×	15419	100	6080	100
5×	5744	37	6712	110
10×	2831	18	4609	76
50×	Toxic ^b	—	Toxic	—
Sugars				
0×	21620	100	8914	100
1×	22267	103	863	10
5×	23361	108	448	5
10×	28691	133	396	4
50×	2352	11	362	4

^a T-ag bands were excised from each of the gel lanes shown in Fig. 5. Gel slices were solubilized by the method of Mahin and Lofberg (1966) and incorporation of [³⁵S]methionine or [³H]galactose was quantitated by liquid scintillation spectroscopy.

^b TC-7 cells were killed when treated with this concentration of amino acids.

infected cell extracts, and the immune complexes were adsorbed to SACI, washed, and treated with TBS or various amounts of mixed glycosidases, as described under Materials and Methods. Aliquots of the

reaction mixture were removed before and after treatment and assayed for acid-precipitable ³H and ³⁵S radioactivity (Table 3).

A small decrease in acid-precipitable ³H and ³⁵S radioactivity was observed in the control samples after incubation with TBS for 15 hr at 37°. This was probably due to nonspecific degradation or some autoproteolytic activity on the part of T-ag, as proposed by Seif (1982). Incubation in the presence of increasing amounts of mixed glycosidases resulted in a small loss of acid-precipitable ³⁵S radioactivity, probably reflecting contamination of the enzyme preparation with proteases. However, we observed a much more marked reduction in the recovery of acid-precipitable ³H radioactivity from the same samples; the decrease was greater than 60% relative to the TBS control at the lowest concentration of enzyme.

This result suggests that the input ³H label was incorporated into T-ag as a monosaccharide, since it was preferentially removed from T-ag with glycosidic enzymes, and supports the interpretation that T-ag is glycosylated.

Removal and Recovery of Galactose from T-ag

The most direct evidence for glycosylation would require removal of sugar from

TABLE 3

REMOVAL OF LABELED CARBOHYDRATE FROM SV40 LARGE T-ag WITH A MIXTURE OF GLYCOSIDASES^a

Enzyme (mg)	Protein (³⁵ S cpm) ^b			Carbohydrate (³ H cpm) ^b		
	Pre	Post	Post/Pre (%)	Pre	Post	Post/Pre (%)
None	17,633	15,775	90	3448	2691	78
0.5	12,461	12,080	97	2649	964	36
1.0	13,555	11,866	88	2620	909	35
1.5	15,614	12,460	80	4732	659	14
2.0	9,235	6,952	75	1668	322	19

^a SV40-infected TC-7 cells were glucose starved, then labeled from 21 to 24 hr p.i. with 100 μCi/ml each of [³⁵S]methionine and D-[1-³H]glucosamine. T-ag was immunoprecipitated with HAF and SACI, and immunoprecipitates were washed with WB and TBS. SACI-bound immune complexes were then resuspended in TBS and treated with 0, 0.5, 1.0, 1.5, or 2.0 mg of mixed glycosidases for 15 hr at 37°. Aliquots of each sample were removed before and after treatment, TCA precipitated, and analyzed for ³⁵S and ³H radioactivity.

^b ³⁵S and ³H cpm were determined as described in the legend to Table 1.

purified T-ag, followed by recovery and identification of that sugar. Again, T-ag was doubly labeled, then purified as described under Materials and Methods and hydrolyzed in 2.0 N H₂SO₄ for 4 hr at 100° in the presence of carrier galactose. This treatment was expected to preferentially release neutral monosaccharides from T-ag with minimal hydrolysis of the polypeptide chain. The hydrolysate was then subjected to cation-exchange chromatography, and the flow-through was neutralized and lyophilized. Analysis of the sample before and after cation exchange revealed that most of the ³⁵S-labeled material had been removed from the hydrolysate (Table 4). This was expected, since positively charged ³⁵S-labeled protein, peptides, and amino acids would bind to the column matrix in the presence of acid. The flow-through was comprised primarily of ³H-labeled material, assumed to be galactose since it was the input ³H label and would not be expected to bind to the column matrix.

This assumption was verified by thin-layer chromatography of the flow-through material. Standard galactose reproducibly migrated to a position corresponding to fraction 28 in this thin-layer system (Fig. 6). The carrier galactose present in the hydrolysate also migrated to fraction 28, as revealed by ammonium bisulfate staining. The distribution of ³H radioactivity after chromatography of the hydrolysate is shown in Fig. 6. Two peaks were observed: a smaller peak at the point of sample application, and a larger peak which coincided with the position of galactose (fraction 28). The small amount of ³⁵S-labeled material remaining in the hydrolysate was found at the origin; no ³⁵S radioactivity comigrated with the galactose carrier. These results suggested that the majority of the ³H-labeled material recovered from purified T-ag was galactose and provided direct evidence that T-ag is glycosylated. We noted, however, that different neutral monosaccharides were not particularly well-resolved in this TLC

TABLE 4
SEPARATION OF PROTEIN AND CARBOHYDRATE BY CATION EXCHANGE AFTER ACID
HYDROLYSIS OF DOUBLE-LABELED SV40 T-ag^a

Sample	Protein (³⁵ S cpm) ^b	% of total cpm in sample	Carbohydrate (³ H cpm) ^b	% of total cpm in sample
Expt. 1				
Posthydrolysis	20,140	60	13,280	40
Post-ion exchange	1,568	21	5,776	79
Expt. 2				
Posthydrolysis	45,200	81	10,800	19
Post-ion exchange	380	11	2,930	89
Expt. 3				
Posthydrolysis	37,264	88	4,856	12
Post-ion exchange	270	5	5,490	95

^a SV40-infected TC-7 cells were starved in glucose-free E-MEM for 30 min, then labeled from 21 to 24 hr p.i. with 25 μCi/ml [³⁵S]methionine and 100 μCi/ml D-[1-³H]galactose. T-ag was isolated by immunoprecipitation and SDS-PAGE, then excised and eluted from the gel. After concentration, the sample was desalted by gel filtration, SDS was extracted, and acid hydrolysis was performed in the presence of carrier galactose, as described under Materials and Methods. The sample was diluted, aliquots were removed for liquid scintillation spectroscopy, and the remainder was applied to a small column (about 5 ml packed volume) of Dowex 50W-X8. The flow-through was collected, and aliquots were again removed for liquid scintillation spectroscopy.

^b ³⁵S and ³H cpm were determined as described in the legend to Table 1. Posthydrolysis = samples taken before application to the Dowex column; post-ion exchange = samples taken from column flow-through.

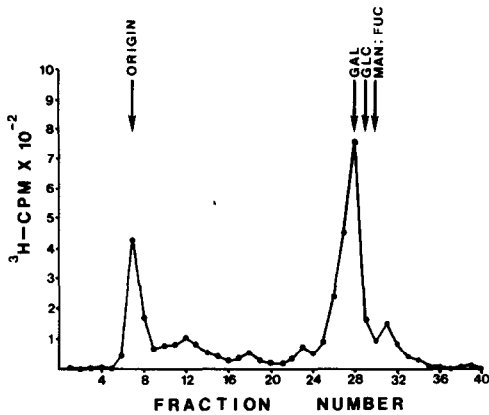


FIG. 6. Thin-layer chromatography of acid hydrolysate of SV40 T-ag. T-ag doubly labeled with [35 S]methionine and D-[1- 3 H]galactose was purified, acid hydrolyzed, and subjected to cation-exchange chromatography as described in the legend to Table 4. The flow-through was neutralized, concentrated, and analyzed by thin-layer chromatography as described under Materials and Methods. The carrier and standard sugars (denoted by arrows in the figure) were visualized by staining with 30% aqueous ammonium bisulfate. The radioactivity profile was determined by dividing the lane containing the hydrolysate into 0.5-cm fractions and analyzing each fraction by liquid scintillation spectroscopy.

system. Since the peak of radioactivity in that area was somewhat broad, we cannot discount completely the possibility that

galactose may have been converted to other sugars that were then incorporated into T-ag.

Effects of TM and EndoH

Experiments were designed to attempt to generate nonglycosylated T-ag that could be used to assess the role of glycosylation in T-ag function. TM and EndoH treatments were chosen because their effects are well-characterized and both reagents are readily available in purified form. The success of such treatments depended, of course, upon the structural characteristics of the glycoprotein in question.

To analyze the effect of EndoH, SV40-infected cells were labeled with [35 S]methionine and T-ag was immunoprecipitated with HAF and SACI. Immune complexes were washed, treated with TBS or various amounts of EndoH, disrupted, and analyzed by SDS-PAGE (Fig. 7A). No change in the electrophoretic mobility of T-ag was observed after EndoH treatment, suggesting that it was resistant to the effects of this endoglycosidase. Control experiments utilizing a known EndoH-sensitive glycoprotein, gp52 of MMTV (Dickson and Atterwill, 1980), established

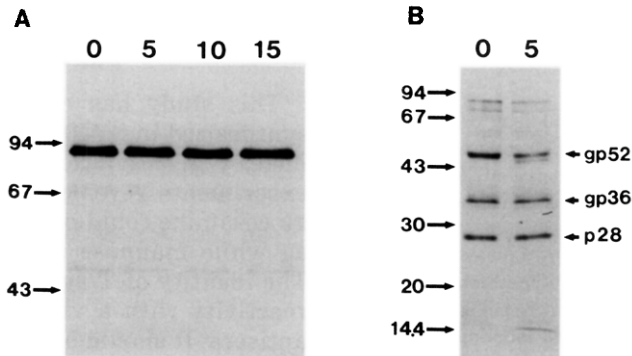


FIG. 7. Effect of EndoH on SV40 T-ag or MMTV polypeptides. T-ag samples were labeled with [35 S]methionine and harvested as immune complexes adsorbed to SACI as described in the legend to Table 3. MMTV polypeptides were labeled with [35 S]methionine in Mm5mt/c1 cells and immunoprecipitated from clarified cell extracts using α MMTV serum and SACI. After washing, the samples were treated with 0, 5, 10, or 15 mU EndoH for 15 hr at 37°. Samples were then washed, disrupted, and analyzed by SDS-PAGE on 8% acrylamide gels. (A) T-ag samples. (B) MMTV polypeptide samples.

that the treatment protocol was effective, even at the lowest concentration of enzyme used (Fig. 7B). It should be noted that EndoH treatment did not alter the electrophoretic mobility of the EndoH-resistant MMTV glycoprotein, gp36, or the nonglycosylated MMTV protein, p28.

To determine the effect of TM treatment, SV40-infected cells were treated with E-MEM or various concentrations of TM starting at 15 hr p.i., then were glucose-starved and labeled with [³⁵S]methionine or [³H]glucosamine from 21 to 24 hr p.i. After extraction, T-ag was immunoprecipitated and analyzed by SDS-PAGE (Fig. 8A). The mobility and intensity of labeling of T-ag with either isotope was unchanged in the presence of TM, suggesting that the glycosylation events

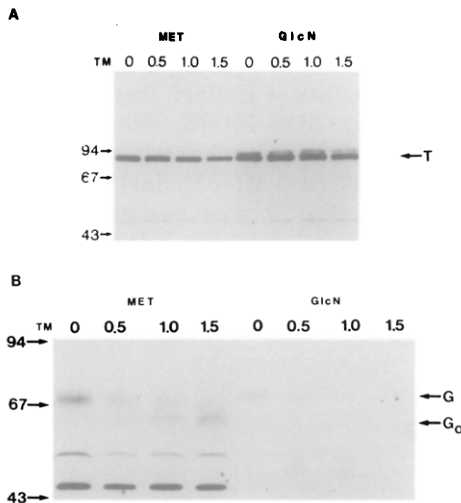


FIG. 8. Effect of TM on SV40 T-ag or VSV glycoprotein. (A) SV40-infected TC-7 cells were washed with warm TBS at 15 hr p.i., then E-MEM containing 0, 0.5, 1.0, or 1.5 $\mu\text{g/ml}$ TM was added, and the cells were returned to 37°. After 30 min starvation in glucose-free E-MEM, cells were labeled from 21 to 24 hr p.i. with 100 $\mu\text{Ci/ml}$ [³⁵S]methionine or D-[1-³H]glucosamine. All solutions were supplemented with TM during starvation and labeling. Cells were then extracted, and extracts were clarified, immunoprecipitated with HAF and SACL, disrupted, and analyzed by SDS-PAGE on 8% acrylamide gels. (B) VSV-infected TC-7 cells were TM-treated, glucose-starved, and labeled as in A, except that treatment began at 1 hr p.i., labeling was performed from 7 to 10 hr p.i., and VSV-infected cell lysates were immunoprecipitated using Rb α VSV.

TABLE 5

EFFECT OF TM ON INCORPORATION OF [³⁵S]METHIONINE OR [³H]GLUCOSAMINE INTO TOTAL CELLULAR PROTEIN^a

TM ($\mu\text{g/ml}$)	% Control ³⁵ S incorporation		% Control ³ H incorporation	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
0.5	105	106	46	44
1.0	96	101	35	42
1.5	ND ^b	82	ND	39

^a SV40-infected cells were treated with various concentrations of TM and labeled with 100 $\mu\text{Ci/ml}$ [³⁵S]methionine or D-[1-³H]glucosamine, as described under Materials and Methods. Cells were extracted, and triplicate samples were TCA precipitated as described under Materials and Methods. Values given in the table represent percentages of control, untreated samples processed in the same way.

^b ND = not determined.

were resistant to this inhibitor. Control experiments utilizing the TM-sensitive G glycoprotein of VSV (Leavitt *et al.*, 1977) established that TM treatment was effective in TC-7 cells even at the lowest concentration of inhibitor used (Fig. 8B). In addition, incorporation of [³H]glucosamine into total cellular protein was effectively inhibited by TM treatment (54–61%), with minimal effect evident upon overall protein synthesis (0–18%; Table 5).

DISCUSSION

This study has established that T-ag synthesized in SV40-infected cells is modified by glycosylation. Metabolic-labeling experiments revealed that galactose and glucosamine could be incorporated into T-ag while mannose and fucose could not. The identity of T-ag was confirmed by its reactivity with a variety of T-ag-specific antisera. It should be noted, however, that T-ag was not strongly labeled with either galactose or glucosamine. Many of our fluorograms required exposure times of 1–4 weeks, and those were obtained only after we had optimized the conditions used for infections and labeling. It was crucial to infect cells at a high m.o.i., to

prestarve cells with glucose-free medium, and to label with a high concentration of tritiated sugar added in the same medium at the maximal time of T-ag synthesis. In view of these limitations and of the resistance of glycosylated T-ag to treatments with EndoH and TM (see below), the possibility of randomization of input labeled sugar posed a difficult problem in the interpretation of these results. We therefore rigorously explored that possibility.

Several different lines of evidence substantiated that randomization alone could not account for the monosaccharide labeling of T-ag. First, only two of the four monosaccharides tested were incorporated into T-ag. Mannose, which was not detectably incorporated, can enter glycolysis after conversion to fructose-6-phosphate, and could have generated labeled amino acids through the tricarboxylic acid cycle (Lehninger, 1982). Second, only large T-ag was labeled with monosaccharides; small t-ag synthesized in the same cells was not. More direct evidence was obtained by labeling T-ag with [³H]galactose in the presence of excess unlabeled amino acids or sugars. Incorporation of the label was clearly inhibited by the presence of unlabeled sugars but was unaffected by the presence of unlabeled amino acids. In addition, treatment of doubly labeled T-ag with a mixture of glycosidases resulted in the preferential removal of ³H radioactivity, suggesting that ³H had been incorporated in the original input form of sugar. Finally, we were able to remove and identify the sugar from purified T-ag. After acid hydrolysis of [³H]galactose-labeled T-ag, the major peak of ³H radioactivity cochromatographed with carrier galactose. Taken together, these results strongly refute the possibility that T-ag was labeled with monosaccharides only after their metabolic conversion to some other form.

Thus, other explanations must be entertained for the relatively low level of incorporation of galactose or glucosamine into T-ag. One is probably the low specific activity of the tritiated monosaccharides we used for labeling. Those were commer-

cially available at only 2.4-25 Ci/mmol, as compared to 1000-1500 Ci/mmol for [³⁵S]methionine. Another possible explanation could be that only a specific subpopulation of T-ag is glycosylated. However, preliminary experiments from our laboratory suggest that this is not the case; rather, glycosylation appears to be a characteristic of T-ag in general (Jarvis and Butel, unpublished observations). We are currently investigating other possible explanations. For example, the number of oligosaccharides attached to T-ag, or the number of sugar residues per oligosaccharide, or both could be quite small.

The effects of EndoH and TM on glycosylation of T-ag were analyzed for preliminary characterization of the glycosylation events involved. EndoH cleaves high-mannose N-linked oligosaccharides distal to the linkage sugar, *N*-acetylglucosamine (Tarentino and Maley, 1974). TM is known to inhibit the initial glycosylation event in N-linked glycoproteins (Takatsuki *et al.*, 1975). Thus, these agents would exert their effects on T-ag only if it were an N-linked glycoprotein. This was possible, since there is a consensus N-glycosylation site (Asn-Arg-Thr; Hunt and Dayhoff, 1970) at amino acid residues 156-158 of T-ag. However, treatment with neither agent changed the electrophoretic mobility of [³⁵S]methionine-labeled T-ag, even though control experiments verified the efficacy of both treatment protocols. Because inhibition of glycosylation or removal of oligosaccharides might not be accompanied by a detectable change in electrophoretic mobility, TM experiments were also performed using [³H]glucosamine-labeled T-ag. No change in the intensity of labeling of T-ag was observed. These results suggest that T-ag is not an N-linked glycoprotein. This is consistent with our inability to label T-ag using mannose, since that sugar is a common constituent of N-linked glycoproteins. Thus, T-ag provides another example of a protein in which a consensus N-glycosylation site is not used, suggesting that the site is necessary, but not sufficient, for N-glycosylation (Hunt and Dayhoff, 1970).

T-ag is more probably an O-linked gly-

coprotein. This interpretation is compatible with the apparent absence of mannose, with EndoH resistance and with TM resistance. It is also compatible with the low level of monosaccharide incorporation, as discussed above, as the oligosaccharide moieties of many O-linked glycoproteins are small. For example, one of the oligosaccharides of the E1 glycoprotein of mouse hepatitis virus contains only a single residue each of sialic acid, galactose, and *N*-acetylgalactosamine; the other has only an additional sialic acid residue (Niemann *et al.*, 1984). We have attempted to verify the identification of T-ag as an O-linked glycoprotein by mild alkaline β -elimination (Spiro, 1966). However, this approach has not been successful because the T-ag polypeptide appears to be degraded under those conditions (0.05 *M* NaOH, 1.0 *M* NaBH₄, 15 hr at 45°; Jarvis and Butel, unpublished observations). In any case, some controversy exists over the use of this method to distinguish between N- and O-linked glycoproteins (Rasilo and Renkonen, 1981; Ogata and Lloyd, 1982). A better method would involve treatment of T-ag with acetylgalactosamine oligosaccharidase, which should cleave O-linked oligosaccharides. Unfortunately, this enzyme is no longer commercially available.

One of the most interesting characteristics of T-ag is its ability to mediate a large number of different functions in infected and transformed cells (Tooze, 1980; Martin, 1981; Rigby and Lane, 1983). While it remains difficult to explain its multifunctional nature at a molecular level, the structural characteristics of T-ag reveal some intriguing possibilities. One explanation involves the presence of several unique structural domains on the polypeptide, with each domain performing one or more distinct functions. In fact, different T-ag functions have been localized to specific portions of the polypeptide (Rigby and Lane, 1983). Another possibility emphasizes the localization of T-ag to distinct subcellular compartments in the host cell. Although the majority of T-ag is localized within the nucleus (Pope and Rowe, 1964; Rapp *et al.*, 1964), small

amounts are associated with the plasma membrane (Tevethia *et al.*, 1965; Soule and Butel, 1979; Deppert *et al.*, 1980; Chandrasekaran *et al.*, 1981; Santos and Butel, 1982; Soule *et al.*, 1982) and mitochondria (Schmidt-Ullrich *et al.*, 1977). T-ag species localized within different subcellular compartments might exhibit unique activities, due to putative structural differences and/or the influence of their local environments. A third model invokes the ability of T-ag to form different supramolecular complexes (Prives *et al.*, 1979; McCormick and Harlow, 1980; Fanning *et al.*, 1981). These complexes could represent distinct subpopulations of T-ag capable of mediating different functions in the host cell. It is clear that some supramolecular forms exhibit distinct DNA-binding and ATPase properties (Bradley *et al.*, 1982; Fanning *et al.*, 1982; Gidoni *et al.*, 1982). The existence of discrete T-ag subpopulations is also suggested by the finding that certain monoclonal antibodies react with only a subset of the total T-ag present in host cells (Gurney *et al.*, 1980; Scheller *et al.*, 1982).

Thus, the functional diversity of T-ag might be explained by its structural diversity. Structural variability might, in turn, be mediated by combinations of different chemical modifications. Glycosylation could play an important role in generating structurally and functionally discrete forms of T-ag. The study of glycoproteins from other systems has established that glycosylation influences their structure and/or function. For example, N-glycosylation of fibronectin and acetylcholine receptor is crucial to their structural integrity (Olden *et al.*, 1982). In the absence of glycosylation, those proteins were more susceptible to proteolytic degradation. In another example, the specific nuclear matrix association of certain high mobility group proteins was precluded in the absence of glycosylation (Weintraub *et al.*, 1983). This finding is particularly interesting, since some T-ag normally resides in association with the nuclear matrix of host cells (Staufenbiel and Deppert, 1983). Further, it has been shown that glycosylation is required for

the proper supramolecular assembly of thyroid-stimulating hormone (Reeves and Chang, 1983), suggesting that glycosylation might be important for the supramolecular assembly of T-ag subpopulations.

It should be noted that O-linked glycoproteins remain relatively poorly characterized. In contrast to N-linked glycoproteins, little is known of their biosynthesis or function. It is possible that T-ag will provide a good model for the study of this class of macromolecules.

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