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Subcellular Localization of Simian Virus 40 Large Tumor Antigen

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The distribution of simian virus 40 large tumor antigen in subcellular fractions from simian virus 40-transformed hamster (H-50) and mouse (VLM) cells and from simian virus 40-infected monkey cells was determined. Solubilized [^{35}S]-methionine- or $^{32}P_i$-labeled surface membrane and nuclear fractions were prepared, immunoprecipitated with hamster anti-T serum, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Tumor antigen with an apparent molecular weight of ~96,000 was detected in both subcellular fractions. Minor components of ~68,000 and ~56,000 with anti-T reactivity which labeled with [^{35}S]methionine were also detected in both fractions from H-50 cells, as were components of ~140,000 and ~56,000 from VLM cells. The 56,000 component appeared to be greatly reduced in $^{32}P_i$-labeled surface membrane fractions. Normal cells or cells transformed with a heterologous agent, such as polyoma virus or a chemical carcinogen, lacked immunoprecipitable tumor antigen. Cell fractionation was monitored by [^{3}H]thymidine labeling, NADH-diaphorase activity, and Na^+-K^+-dependent ATPase activity. These analyses revealed only trace contamination of surface membranes by nuclei, extremely low levels of nuclear rupture during homogenization, and an approximate 10-fold enrichment of surface membrane. Reconstruction experiments demonstrated that soluble tumor antigen failed to associate or copurify with surface membranes during fractionation procedures. These results indicate the presence of a protein in the plasma membrane of cells transformed or infected by simian virus 40 that is immunologically indistinguishable from nuclear tumor antigen.

Simian virus 40 (SV40) tumor antigen (T-ag), first detected by complement fixation with serum from hamsters bearing SV40-induced tumors (anti-T serum) (6), was localized to the nucleus by indirect immunofluorescence (44). More recently, T-ag has been identified as a virus-specified phosphoprotein of molecular weight ~90,000 to 100,000 (53, 54). T-ag extracted and purified from isolated nuclei after sonic treatment (43) or by solubilization of SV40-transformed or -infected cells with buffered (pH 8) Nonidet P-40 (47) will react with anti-T serum in immunoprecipitation tests. A lower-molecular-weight SV40 tumor antigen (small t-ag; molecular weight, 15,000 to 20,000) has also been detected in similar cell systems, although the subcellular localization of t-ag is still unclear (15, 41, 52). Early SV40 mRNA directs the synthesis of both large T- and small t-ag in in vitro protein synthesis systems (41, 42), establishing both antigens unequivocally as products of the early region of the viral genome.

SV40 early genetic expression is pleiotropic in that it gives rise not only to the two tumor antigens described above, but also to a perinuclear antigen (U-antigen) and a tumor-specific transplantation antigen (TSTA) (12, 60, 61). Evidence suggests that the virus-specified TSTA is inserted into surface membrane of transformed and infected cells. This antigen has the ability to induce cell-mediated rejection of a syngeneic transplant of SV40-transformed cells in animals previously immunized with live SV40 virus (17, 24, 28, 29, 57), SV40-infected cells (23, 55, 56), SV40-transformed cells (22, 28), or solubilized total extranuclear membrane preparations from SV40-transformed cells (2, 3, 13, 14, 20, 25, 34, 46, 59).

The exact relationship between nuclear T-ag and cell surface-associated TSTA is unclear. Anderson et al. (4) demonstrated that purified T-ag isolated from nuclei of human cells transformed by SV40 possessed high levels of TSTA activity. Rogers et al. (46) localized the majority of TSTA activity in nuclear preparations from SV40-transformed mouse cells; reduced levels of TSTA activity were associated with a crude membrane fraction. Both experimental approaches led to the conclusion that a virus-specified product localized in the cell nucleus pos-

sessed TSTA activity. The antigenic and structural relationships between the nuclear and surface membrane components remain unanswered.

In view of the functional similarities between purified SV40 nuclear T-ag and cell surface preparations from SV40-transformed cells, studies were initiated to examine surface membranes for determinants immunologically related to the virus-specified nuclear T-ag protein. Molecules immunologically indistinguishable from large T-ag were found to be localized in the plasma membranes of SV40-infected and -transformed cells.

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MATERIALS AND METHODS

Cell cultures and virus. Two SV40-transformed cell lines were utilized: (i) VLM cells, derived by transformation in vitro of BALB/c mouse embryo cells by SV40 (63) (kindly provided by S. S. Tevethia), and (ii) H-50 cells, derived from a tumor induced in a newborn Syrian hamster by SV40 (5). Both cell lines are virus-free and have been shown to contain SV40 nuclear T-ag and SV40 TSTA (28, 37, 44, 57, 63) and to exhibit transformed cell growth properties (8). Three additional cell lines were included: (i) BHK-21 cells, a continuous line of Syrian baby hamster kidney cells (35); (ii) $T100_2$ cells, a line of polyoma virus-transformed rat cells (obtained from S. S. Tevethia); and (iii) DMBA-2/BALB Cl 4 cells, established from a chemically induced BALB/c mouse mammary tumor (J. P. Dudley, D. Medina, and J. S. Butel, manuscript in preparation). Cells were routinely cultured in plastic flasks (Corning Glass Works, Corning, N.Y.), using Dulbecco-modified Eagle minimal essential medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; Flow Laboratories, Rockville, Md.), 50 μg of gentamicin sulfate (Shering Corp., Bloomfield, N.J.) per ml, and 0.3% sodium bicarbonate, in a humidified atmosphere of 10% CO_2 at 37°C. For growth in roller bottles (0.5 gallon [ca. 1.9 liters]; Bellco, Vineland, N.J.) in closed vessels at 37°C, Auto-Pow minimal essential medium (Flow Laboratories) supplemented with 10% FBS, 50 μg of gentamicin sulfate per ml, and 0.075% sodium bicarbonate was used.

TC-7 African green monkey kidney cells (45) were propagated as previously described (38). For infection with SV40, TC-7 cell monolayers were washed three times with Tris-buffered saline (11) and inoculated with 5 PFU of wild-type SV40 per cell. After adsorption for 90 min at 37°C, monolayers were washed three times with Tris-buffered saline and culture medium was added.

Radioactive labeling. $^{32}P_i$ and [^{35}S]methionine labeling were performed using either three roller bottle cultures of infected TC-7 cells 48 h postinoculation or two roller bottle cultures of transformed or normal cells 48 h after seeding. Such roller bottle cultures yield approximately 1×10^8 to 2×10^8 cells per bottle.

Before labeling with [^{35}S]methionine (Amersham, Arlington Heights, Ill.; 400 to 900 Ci/mmol), cells were first depleted of methionine by incubation for 2 h at 37°C in Eagle minimal essential medium (EMEM) without methionine supplemented with 2% dialyzed FBS. Cells were then labeled for 2 h with EMEM without methionine supplemented with 2% dialyzed FBS and 50 μCi of [^{35}S]methionine per ml (5 ml/roller bottle).

Before labeling with carrier-free $^{32}P_i$ (Union Carbide, Tuxedo, N.Y.), cells were depleted of phosphate by incubation for 16 h with EMEM without phosphate supplemented with 5% dialyzed FBS. Cells were then labeled for 2.5 h with EMEM without phosphate supplemented with 5% dialyzed FBS and 250 μCi of $^{32}P_i$ per ml (10 ml/roller bottle culture).

Subcellular fractionation and solubilization. The aqueous two-phase polymer method of Brunette and Till (10) was used to isolate radiolabeled nuclear and surface membrane-enriched fractions. The hypotonic $ZnCl_2$ swelling solution was supplemented with 1% Trasylol (Mobay Chemical Co., New York, N.Y.) to inhibit proteolytic degradation.

Isolated surface membranes were solubilized with 5 ml of a buffered detergent mixture (BDM) (modified from Brugge and Erikson [9]), consisting of 50 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid), pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, and 1% Trasylol at 0°C for 30 min. Nuclei obtained after the first polymer partitioning were purified by two washes with 30 ml of BDM per wash followed by centrifugation at $1,000 \times g$ for 10 min. Washed nuclei were resuspended in 5 ml of BDM and sonically treated as previously described (43). Solubilized nuclei and plasma membranes were then centrifuged at $100,000 \times g$ for 60 min at 4°C.

Immunoprecipitation and gel electrophoresis. The supernatants, after centrifugation at $100,000 \times g$ of solubilized surface membranes or nuclei, were divided into aliquots of 2.5 ml each. Normal hamster serum (50 μl) was added to one aliquot and an equal volume of hamster anti-T serum (8) was added to the other. The reactions were incubated on ice for 16 h. *Staphylococcus aureus* strain Cowan I, prepared and fixed as described by Kessler (27), was equilibrated with BDM for 15 min at 20°C and centrifuged at $2,400 \times g$ at 4°C for 10 min. Pelleted bacteria were resuspended in BDM as a 10% (wt/vol) solution, and 200 μl was added to each antigen-antibody reaction. After incubation for 5 min at 0°C, the immune complexes were washed three times with BDM by centrifugation at $2,400 \times g$ for 10 min. Antigen was eluted from the immune complex by resuspending the final bacterial pellet in 50 μl of electrophoresis sample buffer (0.0625 M Tris [pH 6.8], 2% SDS, 2% β-mercaptoethanol, 5% glycerin, and 0.005% bromophenol blue) and boiling for 3 min. Bacteria were removed by centrifugation at $17,000 \times g$ for 10 min at 4°C. The supernatant was carefully removed and subjected to SDS-gel electrophoresis on discontinuous 12.5% SDS-polyacrylamide slab gels (14 by 12 by 0.15 cm) as previously described (16, 19, 39, 40), with acrylamide–methylene bisacryl-

amide adjusted to 100:1. Samples were subjected to electrophoresis at 30 mA/gel until the bromophenol blue tracking dye reached the bottom of the gel. All electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, Calif).

Gels were stained for 60 min at 20°C in 50% methanol, 7% acetic acid, and 0.1% Coomassie brilliant blue R-250 and destained overnight with 25% methanol and 7% acetic acid. Destained gels were dried with heat under vacuum and were subjected to autoradiography on Kodak NS5T X-ray film.

Phosphorylase A (molecular weight, 94,000), bovine serum albumin (68,000), ovalbumin (43,000), and myoglobin (17,200) were used as molecular weight markers and were purchased from Schwarz/Mann (Orangeburg, N.Y.) or Sigma Chemical Co. (St. Louis, Mo.).

[^{3}H]TdR labeling and enzyme assays. To monitor whole nuclear contamination of subcellular fractions, cells were labeled with [^{3}H]thymidine (TdR) (Schwarz/Mann; 55 Ci/mmol) for 60 min with EMEM supplemented with 2% dialyzed FBS and 2.5 μCi of [^{3}H]TdR per ml. After the labeling period, cells were fractionated as previously described, and an aliquot of each subcellular fraction was precipitated with 10% trichloroacetic acid. Precipitates were collected on glass fiber filters (Whatman, Clifton, N.J.), washed, and dried. Radioactivity was determined by counting the filters in a toluene-Fluoralloy cocktail (Beckman Instruments, Fullerton, Calif.), using a Beckman LS250 spectrometer.

Purification of subcellular fractions was analyzed by monitoring marker enzyme activities: NADH-diaphorase (62) indicated endoplasmic reticulum, and Na^+-K^+-dependent ATPase (62) served as a marker of plasma membrane. Inorganic phosphate was analyzed as previously described (21). Before enzyme analysis, $ZnCl_2$ was removed from surface membranes and nuclei by saline washes. Protein was quantitated by the method of Lowry et al. (33), using bovine serum albumin as a standard.

Reconstruction experiments. To assess the potential of soluble T-ag to non-specifically adsorb to surface membranes, a reconstruction experiment was performed. One 75-cm^2 flask of VLM cells was pulse-labeled with $^{32}P_i$ as previously described. The monolayer was extracted with 0.5 ml of buffered (pH 8) 0.5% Nonidet P-40 as described by Rundell et al. (47) to solubilize T-ag. After removal of nuclei by low-speed centrifugation, the labeled extract was rapidly mixed with two unlabeled roller bottle cultures of homogenized VLM cells in 40 ml of hypotonic $ZnCl_2$ swelling solution. The reconstructed homogenate was partitioned into subcellular fractions, and each fraction was solubilized, immunoprecipitated, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as previously described. The supernatant from the 450 × *g* centrifugation of the initial homogenate (fraction S) was analyzed by concentrating the fraction by lyophilization and resuspension of the residue in 4 ml of BDM before immunoprecipitation.

RESULTS

Localization of anti-T-reactive proteins in SV40-transformed cells.

SV40-transformed hamster (H-50) and mouse (VLM) cells were labeled with [^{35}S]methionine and fractionated as described in Materials and Methods. Solubilized nuclei and cell surface-enriched fractions were immunoprecipitated, and the precipitates were analyzed on 12.5% SDS-polyacrylamide gels (SDS-PAGE). With H-50 cells (Fig. 1), the major polypeptide immunoprecipitated with anti-T serum from solubilized nuclei or from surface membranes migrated at ~96,000 molecular weight; minor components which migrated at ~68,000 and ~56,000 molecular weights were also present. Similar results were obtained with VLM cells labeled with [^{35}S]methionine (Fig. 2). Anti-T serum specifically immunopre-

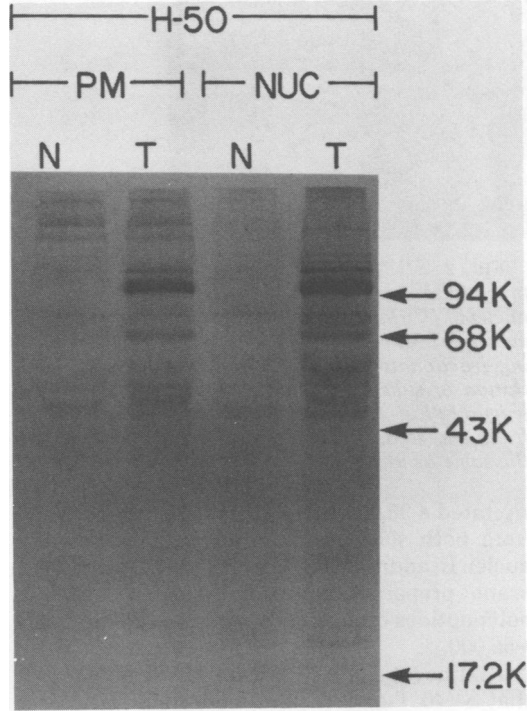

FIG. 1. *Analysis on 12.5% SDS-PAGE of immunoprecipitates from solubilized plasma membranes and nuclei of [^{35}S]methionine-labeled, SV40-transformed hamster (H-50) cells. Two roller bottle cultures of methionine-depleted H-50 cells were labeled for 2 h with methionine-free medium supplemented with 50 μCi of [^{35}S]methionine per ml. Labeled cells were partitioned into nuclei (Nuc) and plasma membranes (PM), solubilized, and immunoprecipitated as described in the text. Radioactivity applied to each lane of the gel represents the total radioactivity immunoprecipitated from the fraction with a specified serum (N, normal hamster serum; T, hamster anti-T serum). Standard proteins used as molecular weight markers were: 94,000, phosphorylase A; 68,000, bovine serum albumin; 43,000, ovalbumin; 17,200, myoglobin.*

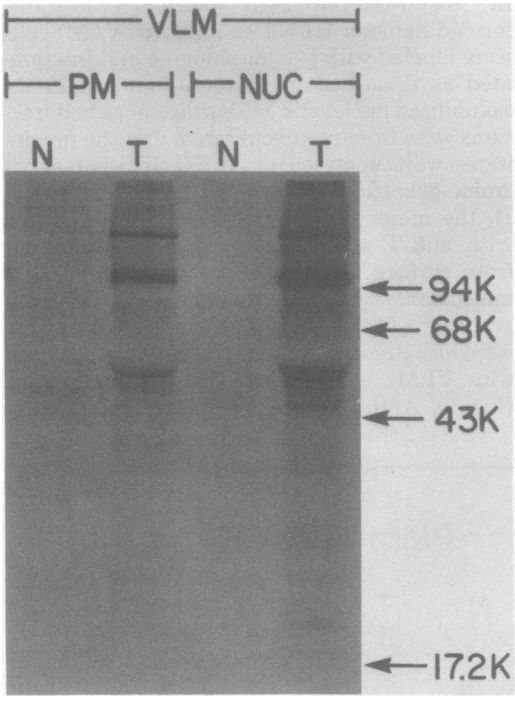

FIG. 2. *SDS-PAGE analysis (12.5%) of immunoprecipitates from solubilized plasma membranes and nuclei of [^{35}S]methionine-labeled, SV40-transformed mouse (VLM) cells. Conditions for radioactive labeling, fractionation, solubilization, and immunoprecipitation of subcellular fractions are detailed in the legend to Fig. 1 and in the text. Designations for cell fractions, sera, and molecular weight markers are the same as in Fig. 1.*

cipitated a 96,000-molecular-weight polypeptide from both solubilized surface membranes and nuclei. In addition, the nuclear and surface membrane preparations contained anti-T-reactive polypeptides of molecular weights ~140,000 and ~56,000.

Tegtmeyer et al. (53) previously demonstrated that SV40 T-ag is a phosphoprotein. To detect a possible difference in modification between the anti-T-reactive nuclear and surface determinants, H-50 and VLM cells were labeled with $^{32}P_i$ and analyzed for T-ag distribution. A 96,000-molecular-weight anti-T-reactive phosphoprotein was present in immunoprecipitates of both surface and nuclear fractions from H-50 cells (Fig. 3). Nuclear extracts of $^{32}P_i$-labeled H-50 cells contained an additional anti-T-specific phosphoprotein of molecular weight ~56,000 which appeared to be absent or significantly reduced in the surface-enriched fraction. SDS-PAGE analysis of $^{32}P_i$-labeled immunoprecipitates of solubilized surface membrane and nuclear fractions from VLM cells is presented in Fig. 4. Phosphorylated anti-T-reactive polypeptides of ~140,000, ~96,000, and ~56,000 molecular weight were present in both subcellular fractions, with the 96,000 component being the major species and the 56,000 component being somewhat diminished in the surface fraction.

To further establish the viral origin of the 96,000-molecular-weight anti-T-reactive protein, normal cells and cell lines transformed by heterologous agents were examined by subcellular fractionation, immunoprecipitation, and SDS-PAGE. Results obtained with the mouse mammary tumor cell line DMBA-2/BALB Cl 4, labeled with $^{32}P_i$, are presented in Fig. 5. No anti-T-reactive proteins were present in immunoprecipitates of solubilized surface membranes or nuclei. Immunoprecipitates of SV40 T-ag extracted from nuclei of infected TC-7 cells are included in lane 5 as a 96,000-molecular-weight marker. Similar experiments using $^{32}P_i$- or [^{35}S]methionine-labeled normal hamster

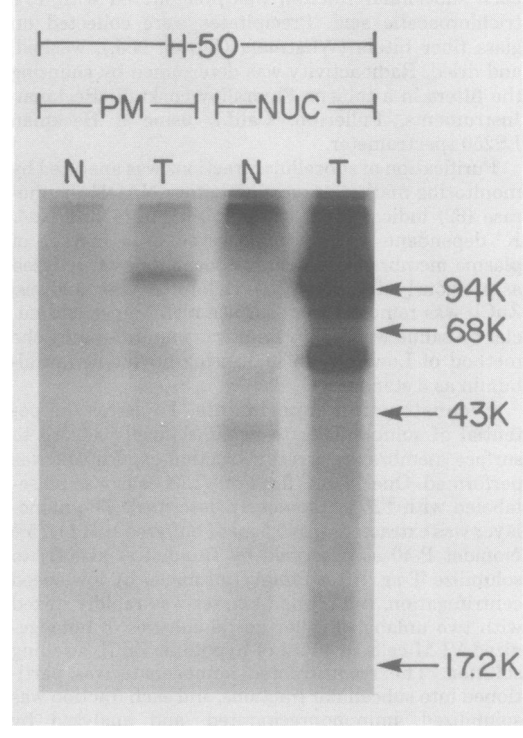

FIG. 3. *SDS-PAGE analysis (12.5%) of immunoprecipitates from solubilized plasma membranes and nuclei of $^{32}P_i$-labeled H-50 cells. Two roller bottle cultures of phosphate-depleted H-50 cells were labeled for 2.5 h with phosphate-free medium supplemented with 250 μCi of $^{32}P_i$ per ml. Conditions for fractionation, solubilization, and immunoprecipitation of subcellular fractions are described in the text. Designations for cell fractions, sera, and molecular weight markers are the same as in Fig. 1.*

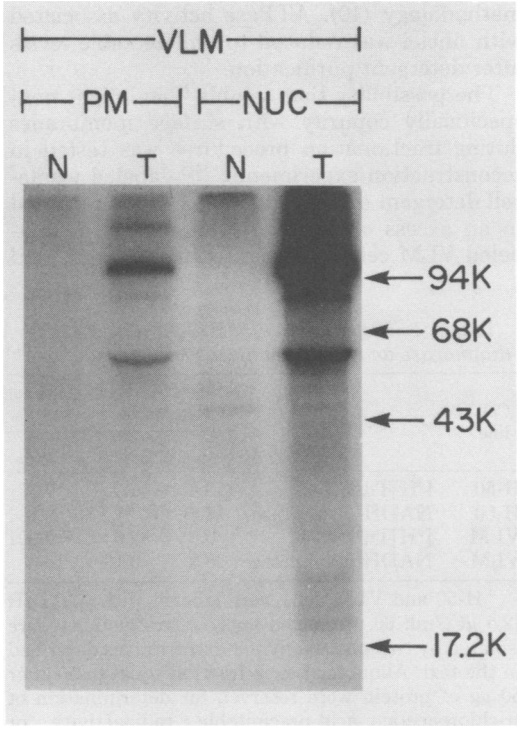

FIG. 4. *SDS-PAGE analysis (12.5%) of immunoprecipitates from solubilized plasma membranes and nuclei of $^{32}P_i$-labeled VLM cells. Conditions for radioactive labeling, fractionation, solubilization, and immunoprecipitation are indicated in the legend to Fig. 3. Designations for cell fractions, sera, and molecular weight markers are the same as in Fig. 1.*

(BHK-21) cells or $^{32}P_i$-labeled polyoma virus-transformed rat ($T100_2$) cells also demonstrated the absence of the 96,000-molecular-weight anti-T-reactive protein in immunoprecipitates of surface and nuclear fractions (data not shown).

Localization of anti-T-reactive proteins in SV40-infected TC-7 cells. SV40-infected TC-7 cells were labeled between 46 and 48 h postinoculation with $^{32}P_i$ and were fractionated and immunoprecipitated as described in Materials and Methods. SDS-PAGE of immunoprecipitates of solubilized surface-enriched and nuclear fractions is presented in Fig. 6. The major phosphoprotein immunoprecipitated from surface membrane and nuclei migrated at 96,000 molecular weight. A minor anti-T-reactive phosphoprotein of molecular weight 56,000 was observed in the immunoprecipitate of the nuclear extract but appeared to be absent from the immunoprecipitate of solubilized surface membrane. Anti-T-reactive phosphoproteins immunoprecipitated from a nuclear extract of H-50 cells are included in lane 5 for purposes of comparison.

Purity of subcellular fractions. Nuclear contamination of subcellular fractions was determined by labeling cells with [^{3}H]TdR, fractionating, and quantitating trichloroacetic acid-precipitable ^{3}H counts per minute for each subcellular fraction as described in Materials and Methods. The subcellular distribution of trichloroacetic acid-insoluble [^{3}H]TdR during fractionation of H-50 and VLM cells is itemized in Table 1. The label was associated almost exclusively with the purified nuclear fraction, with only trace amounts of radioactivity present in the 450 $\times$ *g* supernatant (S) of the initial cell homogenate and in the surface membrane (PM) fractions. Similar results were obtained with a 16-h labeling period (data not shown). These data clearly demonstrate the absence of gross nuclear contamination of the cell surface-enriched fraction. In addition, the possibility of nuclear rupture during homogenization is precluded by the low levels of label associated with the 450 $\times$ *g* supernatant (S) fraction of the homogenate. The sum of activity in the three fractions is less than 100% due to losses during the purification procedure.

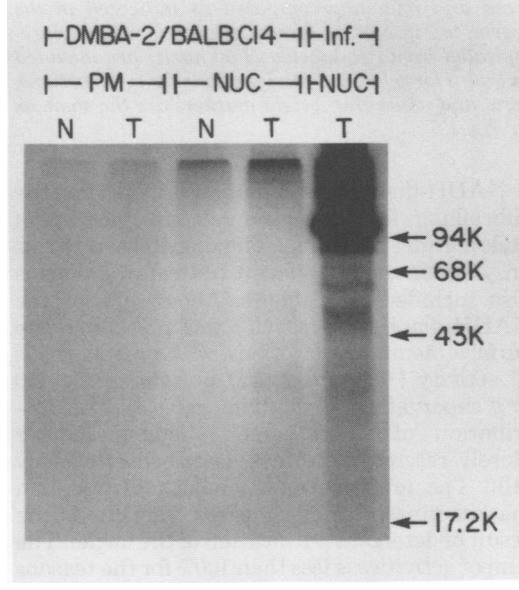

FIG. 5. *SDS-PAGE analysis (12.5%) of immunoprecipitates from solubilized plasma membranes and nuclei of $^{32}P_i$-labeled DMBA-2/BALB Cl 4 cells. Conditions for radioactive labeling, fractionation, solubilization, and immunoprecipitation of subcellular fractions are indicated in the legend to Fig. 3. Anti-T-reactive proteins immunoprecipitated from $^{32}P_i$-labeled, SV40-infected TC-7 cells are included in lane 5 for reference. Designations for cell fractions, sera, and molecular weight markers are the same as in Fig. 1.*

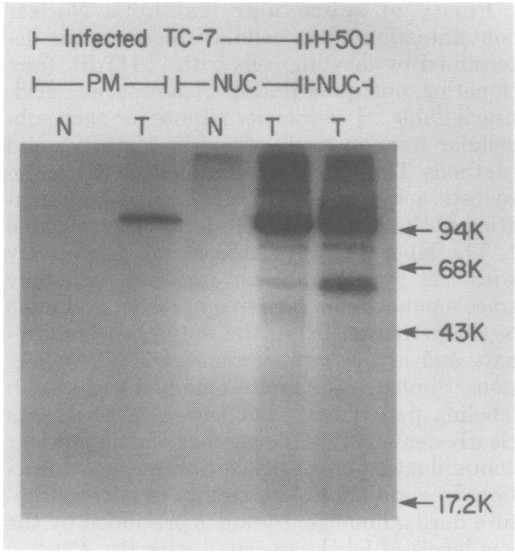

FIG. 6. *SDS-PAGE analysis (12.5%) of immunoprecipitates from solubilized plasma membranes and nuclei of $^{32}P_i$-labeled, SV40-infected TC-7 cells. Cells were labeled between 46 and 48 h postinoculation (multiplicity of infection = 5 PFU/cell) and were fractionated, and subcellular fractions were solubilized and immunoprecipitated as indicated in the legend to Fig. 3. Anti-T-reactive proteins immunoprecipitated from $^{32}P_i$-labeled H-50 nuclei are included in lane 5 for reference. Designations for cell fractions, sera, and molecular weight markers are the same as in Fig. 1.*

NADH-diaphorase activity was assayed in the subcellular fractions as a relative measure of endoplasmic reticulum. The distribution of this enzyme among H-50 and VLM cell fractions is also included in Table 1. About 10% of the NADH-diaphorase activity was present in the surface membrane fractions, with greater levels of activity (14.8 and 28.3%) present in the 450 × *g* supernatant of the homogenates. This distribution of endoplasmic reticulum marker closely resembles profiles described previously (10). The low level of endoplasmic reticulum marker present in the nuclear fractions is the result of detergent purification of the nuclei. The sum of activities is less than 100% for the reasons stated above for TdR labeling.

The specific activity of Na^+-K^+-ATPase was utilized to assess relative enrichment of surface membrane during fractionation (Table 2). For H-50 and VLM cells, the ATPase specific activity was increased 9.3 and 12.9 times, respectively, in the surface membrane fraction as compared to the initial homogenate. These values are in close agreement with overall enrichment previously observed utilizing similar purification methodology (10). ATPase activity associated with nuclei was reduced to undetectable levels after detergent purification.

The possibility that soluble T-ag might nonspecifically copurify with surface membranes during fractionation procedures was tested in reconstruction experiments. $^{32}P_i$-labeled whole-cell detergent extracts of VLM cells were added to an excess of previously homogenized unlabeled VLM cells as described in Materials and

TABLE 1. *Distribution of [^{3}H]TdR and NADH-diaphorase activities among subcellular fractions*[a]

Cell line	Assay	Relative activity (% of activity in homogenate)[b]		
		S	N	PM
H-50	[^{3}H]TdR	0.4	65.0	0.1
H-50	NADH-diaphorase	14.8	<0.1	8.1
VLM	[^{3}H]TdR	0.6	82.0	<0.001
VLM	NADH-diaphorase	28.3	0.13	10.0

[a] H-50 and VLM cells were labeled with [^{3}H]TdR (2.5 μCi/ml; 60 min), and subcellular fractions were isolated by two-phase polymer extraction as described in the text. Aliquots of each fraction equal to 50 μl or 50 μg of protein were reserved for determination of trichloroacetic acid-precipitable radioactivity or NADH-diaphorase activity, respectively.

[b] Trichloroacetic acid-precipitable [^{3}H]TdR in the homogenate of H-50 and VLM cells was 1×10^7 to 3×10^7 cpm. NADH-diaphorase activity was determined as relative enzyme units per milligram of protein and more conveniently expressed as percent activity in homogenate. S, Supernatant from 450 × *g* centrifugation of the homogenate; N, detergent-washed nuclei; PM, plasma membrane.

TABLE 2. *Distribution of Na^+-K^+-ATPase activity among subcellular fractions*[a]

Cell line	Activity[b]				Enrichment factor[c]
	H	S	N	PM	
H-50	0.41	0.4	<0.01	3.8	9.3
VLM	0.37	<0.01	<0.01	4.8	12.9

[a] H-50 and VLM cells were fractionated by the aqueous two-phase polymer method described in the text. An aliquot of each subcellular fraction equivalent to 50 μg of protein was analyzed for Na^+-K^+ATPase activity.

[b] Activities are expressed as micromoles of P_i liberated per milligram of protein per hour. Reported values represent the observed increase in P_i liberation when the reaction contained Na^+ and K^+ as compared to a reaction containing Na^+ alone. H, Initial cell homogenate. Other fraction designations are defined in Table 1.

[c] Enrichment factor was calculated by dividing the specific activity in membrane fraction by the specific activity in homogenate.

Methods. Purified nuclei and surface membranes, in addition to fraction S (supernatant from 450 × *g* centrifugation of homogenate), were solubilized, immunoprecipitated, and analyzed by SDS-PAGE. Electrophoretic analysis of the immunoprecipitates revealed the presence of 96,000-molecular-weight T-ag only in the S fraction (data not shown). Anti-T immunoprecipitates of solubilized nuclei and plasma membranes resulted in trace amounts of radioactivity which did not contain detectable amounts of T-ag. In another experiment, the amount of labeled VLM cell extract added to the unlabeled homogenate was increased to three 75-cm^2 flasks. The use of larger amounts of labeled extract increased proportionately the quantity of T-ag immunoprecipitated from the S fraction but did not reveal any anti-T-reactive polypeptides in the membrane or nuclear fractions (data not shown).

DISCUSSION

A 96,000-molecular-weight protein with immunological properties indistinguishable from nuclear SV40 T-ag has been localized in surface membrane-enriched fractions of SV40-transformed and -infected cells. The surface membrane-associated polypeptide resembles T-ag in that it is specifically immunoprecipitated with anti-T serum, it comigrates with T-ag at molecular weight 96,000 in SDS-polyacrylamide gels, and it is phosphorylated. Although the apparent molecular weights of T-ag from surface membrane and nuclei appear to be identical, the possibility of minor differences between the two species should be recognized. Minor components with approximate molecular weights of 68,000 and 56,000 were also specifically immunoprecipitated by anti-T serum from surface membrane preparations of H-50 cells, as were components of 140,000 and 56,000 from VLM cells.

Control experiments were designed to rule out the possibility that the 96,000-molecular-weight protein immunoprecipitated from solubilized surface membrane was either a cryptic cell-specified determinant exposed as a consequence of phenotypic transformation or clonal selection or a derepressed fetal antigen. The 96,000-molecular-weight anti-T-reactive surface protein was present in SV40-transformed mouse and hamster cells, as well as in productively infected monkey cells, but was not detected in immunoprecipitates from solubilized surface-enriched fractions from normal hamster cells, polyoma virus-transformed rat cells, or chemically transformed mouse mammary cells. The exclusive presence of the anti-T-reactive 96,000-molecular-weight surface antigen in SV0-transformed and -infected cells suggests that the determinant is a specific SV40 gene product. The absence of the antigen in normal cells and in cells transformed by heterologous agents argues strongly against the possibility that the surface antigen originates as a cryptic or derepressed normal cell determinant.

The Brunette and Till (10) methodology utilized for cell fractionation was chosen because of the limited number of cellular manipulations required and the rapidity with which the homogenate could be processed. Although the limitations of the aqueous two-phase polymer partitioning system have recently been reviewed (49), the method did allow discrete fractionation of nuclei from enriched surface membrane. Approximately 10-fold enrichment of surface membrane was achieved with both VLM and H-50 cells (Table 2), based on Na^+-K^+-ATPase specific activities. The vast majority of the acid-insoluble [^{3}H]TdR activity was associated with the nuclear fraction relative to the surface membrane fraction, precluding gross contamination of plasma membranes by nuclei.

Several lines of indirect evidence strongly reduce the possibility that nuclear T-ag adventitiously associated with surface membrane during extraction. Nuclei observed by phase-contrast microscopy appeared intact in the initial homogenate. The virtual absence of acid-insoluble [^{3}H]TdR from the supernatant of a 450 × *g* centrifugation of the homogenate indicates extremely low levels of gross nuclear damage during cell rupture. Furthermore, the pH of the cell homogenate was never greater than 6.8 and polymer partitioning was buffered to pH 6.5. Wild-type T-ag is not eluted from nuclei below pH 8.0 (54). The reconstruction experiments directly demonstrated the failure of soluble T-ag to associate or copurify with surface membranes during cell fractionation procedures. Therefore, the combination of low pH of extraction conditions, absence of [^{3}H]TdR leakage from nuclei into the homogenate, and the failure of exogenously added T-ag to adsorb to surface membranes greatly diminish the possibility that surface-associated T-ag originated from ruptured cell nuclei.

SV40 T-ag appears to be either extremely unstable in the cell cytoplasm or transported into another cellular compartment very rapidly. Only very small quantities of the antigen were immunoprecipitated from concentrated and solubilized supernatants of a 450 × *g* centrifugation of the initial homogenate (data not shown), reducing the possibility that cytoplasmic T-ag non-specifically adhered to surface membrane during extraction procedures. In addition, we

have adapted the dextran gradient cell fractionation procedure of Schmidt-Ullrich et al. (50) to VLM cells. The distribution of T-ag in nuclei and plasma membranes was identical to that observed when cells were fractionated by the Brunette and Till (10) method. Using this procedure, endoplasmic reticulum contamination in surface membrane preparations was reduced to <1% of total activity (data not shown). These preliminary data demonstrate that T-ag is isolated from the surface membrane fraction when cells are partitioned on the basis of either charge or density. These observations, coupled with the preceding biochemical assays and reconstruction experiments, document the relative purity of the subcellular fractions analyzed in this study and serve to validate the observation of the presence of SV40 large T-ag in the surface membrane of infected and transformed cells.

Localization of polyoma virus anti-T-reactive proteins appears to be different than the distribution of T-ag in SV40-transformed and -infected cells. Ito et al. (26) immunoprecipitated a 100,000-molecular-weight anti-T-reactive protein from nuclear and cytoplasmic extracts of polyoma virus-infected 3T6 cells. Another anti-T-reactive component with an apparent molecular weight of 55,000 was exclusively associated with the surface membrane fraction. Pulse-chase experiments suggested that the 55,000-molecular-weight protein was a cleavage product of the 100,000-molecular-weight species. Hence, it appears that polyoma virus T-ag might require proteolytic processing before insertion into surface membrane, whereas SV40 T-ag does not.

The subcellular localization of SV40-specific proteins has also been approached by Deppert et al. (18) by the use of nondefective adenovirus-SV40 hybrid-infected HeLa cells. For each hybrid virus examined, SV40-specific determinants were observed in surface membrane-enriched subcellular fractions. Of special interest was the stable localization of 42,000- and 56,000-molecular-weight proteins of $Ad2^{+}ND2$ in nuclei and plasma membranes, since that virus induces the synthesis of both SV40 U-antigen and SV40 TSTA (30, 31). Unfortunately, the hybrid viruses may direct the synthesis of functional proteins carying both SV40 and adenovirus 2 determinants (1), making definitive correlation to SV40-induced proteins difficult (49).

Schmidt-Ullrich and colleagues (32, 48, 50, 51) reported a comprehensive antigenic comparison of plasma membrane from normal hamster lymphocytes and a hamster lymphoma (GD248) induced by SV40. Bidimensional immunoelectrophoresis and bidirectional isoelectric focusing-immunoelectrophoresis utilizing antiserum prepared against purified GD248 plasma membranes was performed (48). Proteins of pI 4.5 and 4.7 present in mitochondria and surface membrane from GD248 cells were absent in the normal cell counterpart. It was hypothesized that those components might represent T-ag or TSTA. In another study (32), immunofluorescence was used to detect SV40-specific surface and perinuclear determinants in SV40-transformed cells utilizing anti-T serum, antiserum directed against GD248 plasma membranes, or antiserum prepared against intact GD248 tumor cells. Both antisera directed against surface determinants reacted by indirect immunofluorescence with surface and perinuclear regions of SV40-transformed cells. Only the anti-T serum reacted with nuclei.

Recent results obtained in this laboratory (manuscript in preparation) demonstrate that sera from SV40-immunized rodents which have rejected SV40-transformed cells ("S" antibody) react with 96,000-molecular-weight T-ag solubilized from nuclei and surface membranes of SV40-infected or -transformed cells. Such sera have been reported not to react with nuclei of acetone-fixed SV40-transformed cells by indirect immunofluorescence, but, rather, to yield a specific surface immunofluorescence reaction against living SV40-transformed cells (58). Although a precise explanation of the mechanisms responsible for these observations is not available, it may be that different antigenic determinants on the same polypeptide are exposed at the cell surface and in the nucleus. During detergent solubilization, all antigenic determinants could be exposed for antibody interaction. Such a phenomenon could explain the differential detection of T-ag by the methods of immunoprecipitation of solubilized extracts and in situ immunofluorescence by antisera prepared in different ways.

Determination of the relationships, if any, among the 96,000-molecular-weight T-ag present in purified plasma membranes, the minor components immunoprecipitated from the membranes, and SV40-specific TSTA await functional studies. Factors regulating the subcellular localization of SV40 early gene products and their role(s) in regulation of DNA synthesis in transformed cells (7, 11, 36) remain to be defined. Such studies will help clarify the basis for the pleiotropic nature of the SV40 gene *A* function.

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