JOURNAL OF VIROLOGY, Feb. 1980, p. 887-901
0022-538X/80/02-0887/15$02.00/0

Vol. 33, No. 2

Antigenic and Immunogenic Characteristics of Nuclear and Membrane-Associated Simian Virus 40 Tumor Antigen

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Antisera were prepared in syngeneic hosts against subcellular fractions of simian virus 40 (SV40)-transformed cells (MoαPM, MoαNuc), glutaraldehyde-fixed SV40-transformed cells (HaαH-50-G, MoαVLM-G), and electrophoretically purified denatured SV40 tumor antigen (T-ag) (RaαT). Immune sera were also collected from animals bearing tumors induced by SV40-transformed cells (HaαT, MoαT, HAF) and from SV40-immunized animals that had rejected a transplant of SV40-transformed cells (HaαS, MoαS). Immunological reagents prepared against cell surface (MoαPM, HaαS, MoαS, HaαH-50-G, MoαVLM-G) reacted exclusively with the surface of SV40-transformed cells by indirect immunofluorescence or protein A surface antigen radioimmunoassay. Immunological reagents prepared against the nuclear fraction (MoαNuc) or whole-cell determinants (HaαT, MoαT, HAF, RaαT) reacted with both the nuclei and surface of SV40-transformed or -infected cells. All reagents were capable of immunoprecipitating 96,000-molecular weight large T-ag from solubilized whole cell extracts of SV40-transformed cells. The exclusive surface reactivity of HaαS exhibited in immunofluorescence tests was abolished by solubilization of subcellular fractions, which then allowed immunoprecipitation of T-ag by HaαS from both nuclear and plasma membrane preparations. Specificity was established by the fact that all T-reactive reagents failed to react in serological tests against chemically transformed mouse cells, and sera from mice bearing transplants chemically transformed mouse cells (MoαDMBA-2) failed to react with SV40-transformed mouse or hamster cells. Reagents demonstrating positive surface immunofluorescence and protein A radioimmunoassay reactions against SV40-transformed cells were capable of blocking the surface binding of RaαT to SV40-transformed cells in a double-antibody surface antigen radioimmunoassay. This blocking ability demonstrated directly that a component specificity of each surface-reactive reagent is directed against SV40 T-ag. A model is presented which postulates that the differential detection of T-ag by the various serological reagents is a reflection of immunogenic and antigenic differences between T-ag polypeptides localized in nuclei and plasma membranes.

Expression of the early region of the simian virus 40 (SV40) genome, which spans from approximately 0.17 to 0.67 map units (13, 18, 36), results in the synthesis of a variety of immunologically defined antigens in SV40-infected and -transformed cells. These early antigens include large tumor antigen (T-ag) (molecular weight, 90,000 to 100,000; 35, 45) and small tumor antigen (t-ag) (molecular weight, ~20,000; 9, 43), which can be immunoprecipitated from labeled cell extracts utilizing serum from SV40 tumor-bearing animals. These same antigens are also immunoprecipitated from in vitro cell-free protein synthesis systems programmed with SV40 early mRNA, directly implicating virus specificity (30, 31). Another immunologically defined early antigen has been designated S-ag and is detected on the surface of SV40-transformed and -infected cells by immunofluorescence with serum from SV40-immunized hamsters that have rejected a syngeneic challenge of SV40-transformed cells (49). An additional early antigen is the tumor-specific transplantation antigen (TSTA), presumably a cell surface determinant, that is defined by the induction of specific rejection of a potentially tumorigenic transplant of SV40-transformed cells (6, 50). Finally, an early determinant designated U-ag can be detected in cells infected by the adenovirus-SV40 hybrid Ad2$^+$ND1 with serum from African green monkeys hyperimmunized with Ad2$^+$ND1-infected syngeneic kidney cells (23). U-ag induced by Ad2$^+$ND1 yields a perinuclear immunofluorescence reaction and is presumably located on the carboxyl end of T-ag since the SV40 insertion is between 0.11 and 0.28 map units in Ad2$^+$ND1 (28).

Recently, the antigenic relationship between

some of these SV40 early antigens has been clarified by the use of monospecific antiserum prepared against electrophoretically purified large T-ag (7, 20, 21). T-ag and t-ag were found to react with monospecific T-reactive antiserum by immunoprecipitation, establishing partial antigenic homology between the two species. Lanford and Butel (21) also demonstrated reactivity of the monospecific reagent with S-ag and U-ag by immunofluorescence, strongly suggesting antigenic relatedness of both these species with T-ag.

Soule and Butel (44) recently detailed the subcellular localization of 96,000- molecular weight T-ag. T-ag was localized in the nuclear and plasma membrane fractions of SV40-transformed and -infected cells by cell fractionation and immunoprecipitation. In the present study, the antigenic and immunogenic properties of the membrane-associated anti-T-reactive protein were investigated. Antisera were prepared in syngeneic hosts by various methods and were found to react either exclusively with the surface or with both the surface and nuclei of SV40-transformed cells. Immunological assays utilizing these reagents indicated that nuclear T-ag is a sufficient immunogen for the generation of surface T-reactivity. However, the surface anti-T-reactive protein, when present in native cell surface vesicles, is not a sufficient immunogen for the generation of antibody which will react with nuclear T-ag. A model which invokes the exposure of different antigenic sites on the T polypeptide is presented to explain these observations.

MATERIALS AND METHODS

Cell cultures and virus infection. Two SV40-transformed cell lines were utilized: VLM, a line of BALB/c mouse embryo cells transformed in vitro by SV40 (53) and H-50, derived from an SV40-induced tumor in a newborn Syrian hamster (1). Both lines are virus free and have been shown to contain SV40 nuclear T-ag and SV40 TSTA (27, 32, 47) and to exhibit transformed cell growth properties (3). Three additional cell lines were included: BHK-21 cells, a continuous line of Syrian baby hamster kidney cells (26), 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 submitted for publication), and SA7Tu21 cells, established from an SA7-induced Syrian hamster tumor (M. K. Estes and J. S. Butel, unpublished data). Cells were propagated as monolayer or roller-bottle cultures as previously described (44). Spinner cultures were utilized to produce large quantities of VLM cells. Siliconized spinner culture flasks were filled with spinner-modified Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N. Y.) supplemented with 5% fetal bovine serum (FBS; Irvine Scientific, Santa Ana, Calif.), 50 μg of gentamicin sulfate (Schering Corp., Bloomfield, N. J.) per ml, and 0.075% $NaHCO_3$. Cells were seeded at a density of 1×10^5 to 2×10^5 cells per ml and harvested or transferred when a density of 1×10^6 cells per ml was reached.

TC-7 cells, a clonal line derived from African green monkey kidney (33), were propagated as previously described (29). For infection with wild-type SV40, TC-7 cell monolayers were washed three times with Tris-buffered saline (TBS; 5) and inoculated with 5 to 10 plaque-forming units per cell. After adsorption for 120 min at 37°C, residual inoculum was removed and culture medium was added.

Cell fractionation and enzyme assays. For tumor antigen extraction from radiolabeled plasma membranes and nuclei, cells were fractionated by the two-phase polymer method of Brunnette and Till (4) with modifications as previously described by Soule and Butel (44). For production of purified plasma membranes and nuclei for animal immunization, the Mg-dextran gradient method of Schmidt-Ullrich et al. (41) was utilized with slight modification. The homogenization medium consisted of 0.05 M Tris-hydrochloride (pH 7.2), 0.2 mM $MgSO_4$, and 0.25 M sucrose (14). Trasylol (Mobay Chemical Co., New York, N. Y.) was included at a concentration of 1% (vol/vol) in all solutions to decrease proteolytic degradation.

Briefly, VLM cells were resuspended in homogenization medium at a concentration of 2×10^7 to 3×10^7 cells per ml and disrupted by N_2 cavitation at 4°C for 20 min at 800 lb/in^2. Nuclei were removed from the resulting homogenate by centrifugation at $450 \times g$ for 15 min and further purified as previously detailed (44). The postnuclear supernatant was centrifuged for 20 min at $20,000 \times g$, and the resulting pellet (large granule fraction) was applied to Mg-dextran step gradients (41) and centrifuged at 50,000 rpm at 4°C for 2 h in a Spinco SW50.1 rotor. The post-large granule supernatant was then centrifuged at $100,000 \times g$ for 60 min to isolate microsomal membranes. The lowest density band (1.05 g/cm^3) from the step gradient was mixed with microsomal membranes, subjected to hypoosmotic shock (41), and centrifuged to equilibrium on Mg-dextran cushion (1.09 g/cm^3) at 35,000 rpm and 4°C for 18 h in a Spinco SW50.1 rotor. Material banding on top of the cushion was removed, pelleted at $100,000 \times g$ for 60 min, and retained as the plasma membrane fraction for animal immunization for further analysis.

To determine enrichment and relative purity of subcellular fractions resulting from the Mg-dextran purification technique, marker enzyme activities were determined. Na^+-K^+-dependent adenosine triphosphatase (ATPase) activity indicated plasma membrane and was assayed as previously described (51). Glucose-6-phosphatase was utilized as a marker for endoplasmic reticulum (19), with D-[U-^{14}C]glucose-6-phosphate (241 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) serving as substrate. Monoamine oxidase, a marker for mitochondria, was determined by a modification of a previously described isotopic method (52). Instead of using tryptamine-2-[^{14}C]hydrochloride, [^{3}H]tryptamine (1.3 Ci/mmol; Amersham) was substituted at a concentration of 0.5 μCi per reaction. Protein was determined by the method of Lowry et al. (25).

Preparation of immunological reagents. A battery of immunological reagents was used throughout this study (summarized in Table 1); they were prepared as follows.

(i) Hamster anti-T, mouse anti-T, and mouse anti-DMBA-2/BALB Cl 4 tumor sera were produced in the syngeneic host after subcutaneous (s.c.) tumor production by H-50, VLM, or DMBA-2/BALB Cl 4 cells, respectively. Methodology for production of hamster anti-T serum has been described (3). For production of mouse anti-T and mouse anti-DMBA-2/BALB Cl 4 serum, adult male BALB/c mice were inoculated with 1×10^6 VLM or DMBA-2/BALB Cl 4 cells, respectively, by s.c. route, and the tumor-bearing mice were bled 3 to 5 weeks later.

(ii) Hamster anti-H-50-G and mouse anti-VLM-G sera were produced by hyperimmunizing adult Syrian hamsters and BALB/c mice with glutaraldehyde-fixed H-50 or VLM cells, respectively. The animals received 5 intraperitoneal injections of $0.5\text{-}1.5 \times 10^7$ glutaraldehyde-fixed cells (46) and were bled 10 days after the final injection.

(iii) Hamster anti-S and mouse anti-S sera were prepared essentially as described (48, 49). For production of hamster anti-S, Syrian hamsters were given 3 intraperitoneal inoculations at weekly intervals with 1×10^7 to 2×10^7 plaque-forming units of wild-type SV40. At day 7 after the last virus inoculation, animals were challenged with 2×10^5 H-50 cells s.c. After 60 days animals without tumors (44 out of 46) were rechallenged with 1×10^5 to 2×10^5 H-50 cells s.c. per week for 3 weeks and bled 1 week after the last challenge. Mouse anti-S was prepared in adult male BALB/c mice. Two weekly s.c. injections of 1×10^7 to 2×10^7 plaque-forming units of wild-type SV40 were administered. Beginning at week 1 after the second virus inoculation, animals were challenged weekly with 1×10^6 to 5×10^6 VLM cells by the intraperitoneal route, except for the final dose which was administered s.c., for a total of 6 weeks and were bled 1 week after the last challenge dose. Control experiments have demonstrated that mice immunized with 1×10^7 plaque-forming units of SV40 reject a VLM cell challenge of greater than 5×10^6 cells.

(iv) Mouse anti-plasma membrane and mouse anti-nuclei sera were prepared in adult male BALB/c mice. Cell fractions were derived from 6-liter suspension cultures of VLM cells isolated by the Mg-dextran gradient methodology described above. Mice were given three injections of VLM plasma membrane (500 to 700 μg of protein per injection) or three injections of VLM nuclei (500 to 1,000 μg of protein per injection) mixed in complete Freund adjuvant s.c. at weekly intervals and bled 8 days after the final injection.

(v) Monospecific rabbit anti-T serum was prepared in New Zealand white rabbits by immunization with T-ag excised from sodium dodecyl sulfate (SDS)-polyacrylamide gels which had been immunoprecipitated from SV40-transformed rabbit kidney cells. This antiserum was designated RTAgS in a previous report (21).

(vi) Hamster ascites fluid was obtained from adult male Syrian hamsters in which ascites was produced by the intraperitoneal injection of SV40-transformed hamster ascites cells, HaAsc (21).

(vii) Ascites fluid from rats bearing ascites induced by polyoma virus-transformed rat cells was generously provided by T. Benjamin. This reagent has been shown to react with polyoma virus T-ag (42).

(viii) Normal serum pools were obtained from hamsters, mice, and rabbits before immunization.

Antigen detection by immunofluorescence, protein A radioimmunoassay, and double-antibody radioimmunoassay. Detection of intranuclear T-ag by indirect immunofluorescence was carried out as previously detailed (3). Methodology for indirect surface immunofluorescence on live cells was as described (48), with the modification reported by Lanford and Butel (21). The immunofluorescence staining pattern of cell preparations stored in suspension in

TABLE 1. *Summary of immunological reagents utilized in this study*

Serum[a]	Abbreviation	Species of animal	Immunogen
Hamster anti-T	HaαT	Hamster[b]	H-50 cells[c]
Mouse anti-T	MoαT	Mouse[d]	VLM cells[c]
Mouse anti-DMBA-2/BALB Cl 4	MoαDMBA-2	Mouse	DMBA-2/BALB Cl 4 cells[c]
Hamster anti-H-50-G	HaαH-50-G	Hamster	Fixed H-50 cells
Mouse anti-VLM-G	MoαVLM-G	Mouse	Fixed VLM cells
Hamster anti-S	HaαS	Hamster	SV40 + live H-50 cells
Mouse anti-S	MoαS	Mouse	SV40 + live VLM cells
Mouse anti-plasma membranes	MoαPM	Mouse	Purified VLM plasma membranes
Mouse anti-nuclei	MoαNuc	Mouse	Purified VLM nuclei
Rabbit anti-T-ag	RaαT	Rabbit[e]	Purified T-ag
Hamster ascites fluid	HAF	Hamster	Hamster ascites cells
Normal hamster serum	NHS	Hamster	None
Normal mouse serum	NMS	Mouse	None
Normal rabbit serum	NRS	Rabbit	None
Rat anti-polyoma-T	RatαPy-T	Rat	Polyoma virus-transformed rat cells

[a] Serum designation in Materials and Methods.
[b] Hamster: adult male Syrian hamster.
[c] Tumor-bearing animal.
[d] Mouse: adult male BALB/c mouse.
[e] Rabbit: New Zealand white rabbit.

90% buffered glycerin at −30°C was found not to change during a 72-h period.

The ^{125}I-labeled protein A radioimmunoassays for the detection of cell surface antigens were performed essentially as described (12). Briefly, cells were dispersed with 0.05% Versene, and 1×10^6 to 2×10^6 cells were reacted with various amounts of serum in a total volume of 0.5 ml of TBS supplemented with 10% FBS. After 60 min of incubation of 0°C, the cells were washed four times with TBS and reacted with 0.5 μCi of ^{125}I-labeled protein A (30 mCi/mg; Amersham) in a total volume of 0.5 ml of TBS supplemented with 2 mg of human hemoglobin (Sigma Chemical Co., St. Louis, Mo.) per ml. After 60 min of incubation at 0°C, the cells were washed four times with TBS and counted in a Nuclear Chicago gamma counter.

A double-antibody surface antigen radioimmunoassay was developed for the demonstration of anti-T specificity in various immunological reagents. H-50 cells were dispersed with 0.05% Versene and 2.5×10^6 cells were reacted with 0.1 ml of a 1:5 dilution of a mouse, hamster, or rat blocking antiserum for 60 min at 0°C. Cells were then washed and reacted with 0.1 ml of a 1:160 dilution of NRS or RaαT for 60 min at 0°C. Cells were again washed and reacted with 0.5 μCi of immunoglobulin G (IgG) fraction of goat anti-rabbit IgG (Cappel Laboratories, Cochranville, Pa.) labeled with ^{125}I as previously described (15). Cells were washed again and transferred to a clean tube, and the final cell pellet was counted in a Nuclear Chicago gamma counter. The diluent for all antiserum dilutions and cell washings consisted of TBS supplemented with 25% FBS. NRS and RaαT used for this assay were absorbed twice at 1:10 dilution with BHK-21 and SA7Tu21 cells in suspension (each at a concentration of 5×10^7 cells) for 60 min at 0°C.

Radioactive labeling, immunoprecipitation, and PAGE. $^{32}P_i$ and [^{35}S]methionine labeling were performed by using either two roller-bottle cultures of transformed cells for cell fractionation or two to four 75-cm^2 monolayer cultures of transformed or infected cells for in situ extraction of T-ag. Carrier-free $^{32}P_i$ was obtained from Union Carbide (Tuxedo, N. Y.), and [^{35}S]methionine (>600 Ci/mmol) was obtained from Amersham. Conditions for radioactive labeling of infected or transformed cells have been described (44).

Solubilization of labeled extracts of subcellular fractions and immunoprecipitation with hamster anti-T serum and protein A bearing *Staphylococcus aureus* strain Cowan I (17) have been reported by us (44). For in situ antigen extraction of labeled cells, monolayers were solubilized with a solution of 20 mM Tris-hydrochloride (pH 8), 137 mM NaCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% (vol/vol) Nonidet P-40, 10% (vol/vol) glycerol, 1 mM phenylmethylsulfonylchloride, and 1% (vol/vol) Trasylol as previously described (35). Clarified extracts were immunoprecipitated with 25 to 50 μl of an appropriate serum, and resultant immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as previously reported (44).

RESULTS

Subcellular fractionation of SV40-transformed cells. Subcellular fractions from VLM and H-50 cells isolated by the two-phase polymer extraction methodology of Brunnette and Till (4) were analyzed for relative purity in a previous communication from this laboratory (44). Briefly, the plasma membrane fraction contained an undetectable level of acid-insoluble [^{3}H]thymidine and <10% of the total endoplasmic reticulum marker (reduced nicotinamide adenine dinucleotide diaphorase). The Na^+-K^+-ATPase specific activity increased about 10-fold in the plasma membrane fraction as compared with the initial cell homogenate. The credibility of that method for subcellular localization of T-ag was further substantiated by reconstruction experiments in which detergent-solubilized $^{32}P_i$-labeled cell extract was added to an excess of unlabeled cell homogenate before polymer partitioning. Undetectable amounts of labeled T-ag were immunoprecipitated from the plasma membrane fraction, indicating an insignificant level of nonspecific adsorption of T-ag to plasma membrane during fractionation.

Characterization of the plasma membrane fraction from VLM cells isolated by Mg-dextran centrifugation as detailed in Materials and Methods is presented in Table 2. Glucose-6-

TABLE 2. *Characterization of plasma membrane fraction from VLM cells isolated by Mg-dextran centrifugation*[a]

Fraction	Marker enzyme activity					
	Na^+-K^+-ATPase[b]		Glucose-6-phosphatase[c]		Monoamine oxidase[d]	
	Total activity	Sp act[e]	Total activity	Sp act	Total activity	Sp act
Homogenate	1,920	20	211	2.2	821	8.55
Plasma membrane	1,170 (60.9)[f]	260	0.235 (0.11)	5.23×10^{-2}	<1 (<0.12)	<1

[a] Approximately 5×10^6 VLM cells were homogenized by N_2 cavitation and fractionated by Mg-dextran gradient centrifugation as described in Materials and Methods. A portion of each fraction equivalent to 50 μg of protein was used for each enzyme assay. Total protein in homogenate and plasma membrane fractions was 96 and 4.5 mg, respectively.

[b] Na^+-K^+-ATPase activity expressed as nanomoles of P_i liberated per 0.5 h.

[c] Glucose-6-phosphatase activity expressed as nanomoles of [^{14}C]glucose liberated per 0.5 h.

[d] Monoamine oxidase activity expressed as nanomoles of ^{3}H-labeled indole acetic acid liberated per 0.5 h.

[e] Specific activity expressed as enzyme activity per microgram of protein.

[f] Numbers in parentheses represent percent activity in plasma membrane fraction as compared with homogenate.

phosphatase and monoamine oxidase, markers for endoplasmic reticulum and mitochondria, respectively, were reduced to extremely low levels in the plasma membrane fraction (≤0.1% of total activity in homogenate). The specific activity of Na^+-K^+-ATPase, a marker for plasma membranes, was increased 13-fold compared with the initial homogenate, whereas the recovery of the plasma membrane marker from the homogenate was 60.9%. The increase in plasma membrane marker activity and total yield of the marker closely approximate values obtained by Schmidt-Ullrich et al. (41) for Mg-dextran gradient fractionation of SV40-transformed hamster lymphocytes. Purified plasma membranes represented 4.7% of total cellular protein.

Detection of surface and intranuclear antigens by immunofluorescence. Indirect immunofluorescence assays were performed by using the sera described in Materials and Methods and Table 1. Surface (unfixed cells) and intranuclear (acetone-fixed cells) reactivities against H-50, VLM, and SV40-infected TC-7 cells were monitored. Results are summarized in Table 3. Examples of positive and negative surface immunofluorescence reactions are demonstrated by the photomicrographs in Fig. 1. Cells which reacted positively by surface immunofluorescence were observed microscopically to contain fluorescent globules distributed evenly over their surface. By focusing through the plane of the cell, the positive surface fluorescence was transformed into a fluorescent ring surrounding each cell. Cells negative for surface immunofluorescence were extremely dull and nearly undetectable microscopically.

Examples of nuclear T-ag immunofluorescence staining reactions against H-50 cells are shown in Fig. 2. Although the HaαS, MoαS, HaαH-50-G, and MoαVLM-G reagents exhibited almost undetectable reactivity with intranuclear T-ag (Fig. 2F, HaαS), occasionally a few faint nuclear flecks were observed, so the nuclear reactivity of those antisera is designated as ± in Table 3.

Data summarized in Table 3 may be used to divide these immunological reagents into two classes based upon reactivity with the surface or nuclei of SV40-transformed or -infected cells. The first class of reagents reacted strongly with both the nuclei and surface of transformed or infected cells and included HaαT, MoαT, MoαNuc, and RaαT. The second class of reagents reacted strongly with the surface of SV40-transformed cells but reacted weakly, if at all, with nuclei of transformed cells. These reagents included HaαS, HaαH-50-G, MoαS, MoαVLM-G, and MoαPM.

TABLE 3. *Immunofluorescence reactivity of immunological reagents against cell surface components and intranuclear T antigen in SV40-transformed and -infected cells*[a]

Serum	Cell surface reaction[b]			Nuclear reaction[c]		
	H-50	VLM	Infected TC-7[d]	H-50	VLM	Infected TC-7
NHS	0[e]	0	0	0	0	0
NMS	0	0	0	0	0	0
NRS	0	0	0	0	0	0
MoαDMBA-2	0	0	0	0	0	0
HaαT	+++	+++	+++	++++	++++	++++
HAF	++++	++++	++++	++++	++++	++++
HaαS	+++	+++	ND[f]	±	±	ND
HaαH-50-G	+++	+++	ND	±	±	ND
MoαT	+++	+++	ND	++++	++++	ND
MoαS	+++	+++	ND	±	±	ND
MoαVLM-G	+++	+++	ND	±	±	ND
MoαPM	+++	+++	ND	0	0	ND
MoαNuc	+++	+++	ND	+++	+++	ND
RaαT	++++	++++	++++	++++	++++	++++

[a] The methodology for preparation and designations for these immunological reagents is detailed in Materials and Methods and Table 1.

[b] For surface immunofluorescence tests, live cells were prepared and stained as described in Materials and Methods. All reagents were used at a 1:5 dilution in TBS except NRS and RaαT, which were diluted 1:20.

[c] For nuclear immunofluorescence tests, acetone-fixed cells on glass coverslips (15 mm) were processed as detailed in Materials and Methods. Antisera were diluted to the same levels as used in surface immunofluorescence tests.

[d] Infected TC-7, SV40-infected TC-7 cells.

[e] Relative intensity of reactions was scored on a scale of 0 to ++++, indicating a dull negative to a bright positive reaction, respectively.

[f] ND, Not determined.

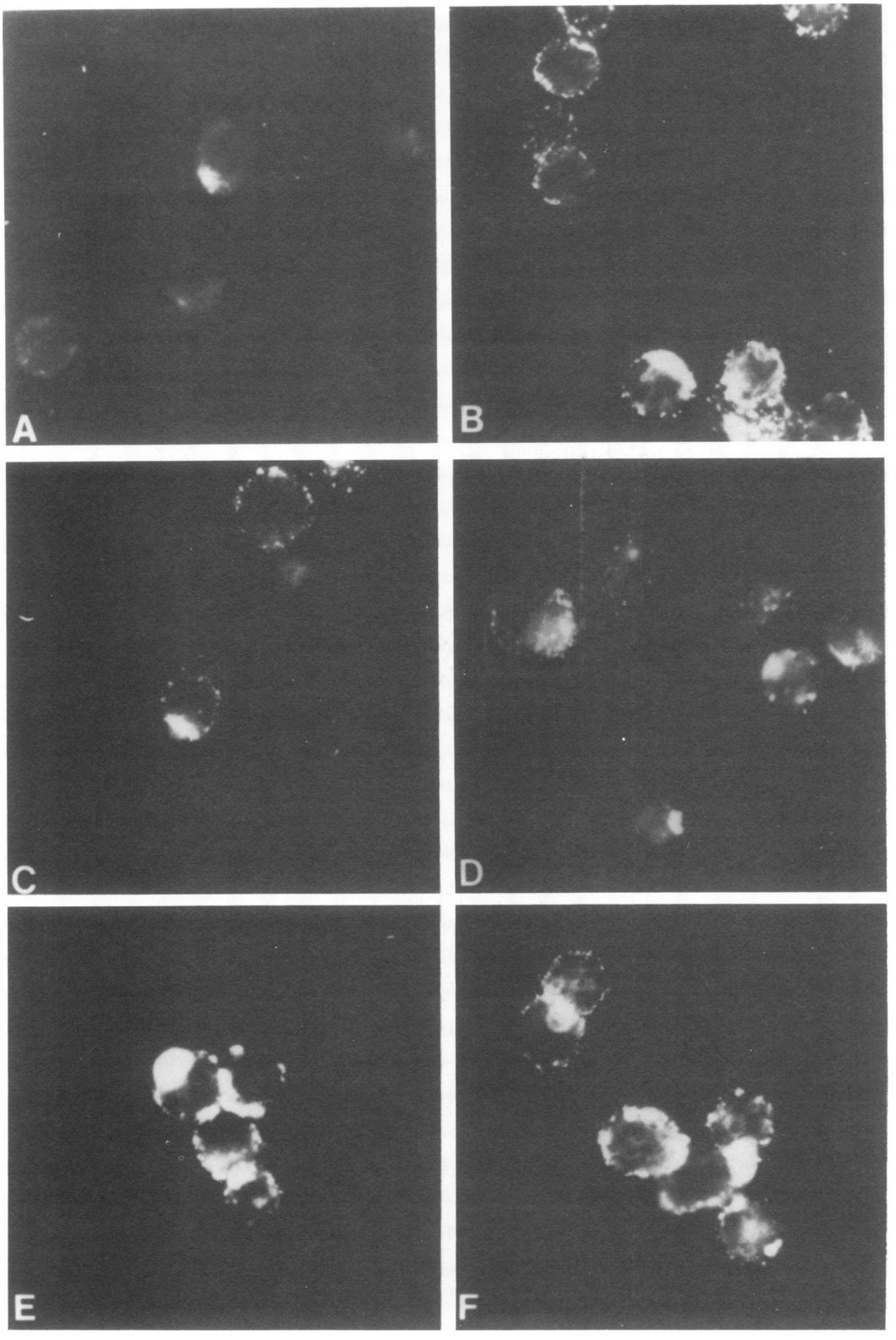

FIG. 1. *Reactivity of representative immunological reagents with the surface of SV40-transformed and -infected cells by indirect immunofluorescence. Monolayer cultures were dispersed with 0.05% Versene and were stained in suspension as detailed in Materials and Methods. Description of immunological reagents is presented in Materials and Methods and Table 1. VLM cells were used in (A), H-50 cells were used in (B–E), and SV40-infected TC-7 cells were used in (F). Cells were reacted with the following antisera: (A) NHS; (B) MoαT; (C) MoαS; (D) MoαPM; (E and F) RaαT. Fluorescent fields were photographed under identical conditions (×450).*

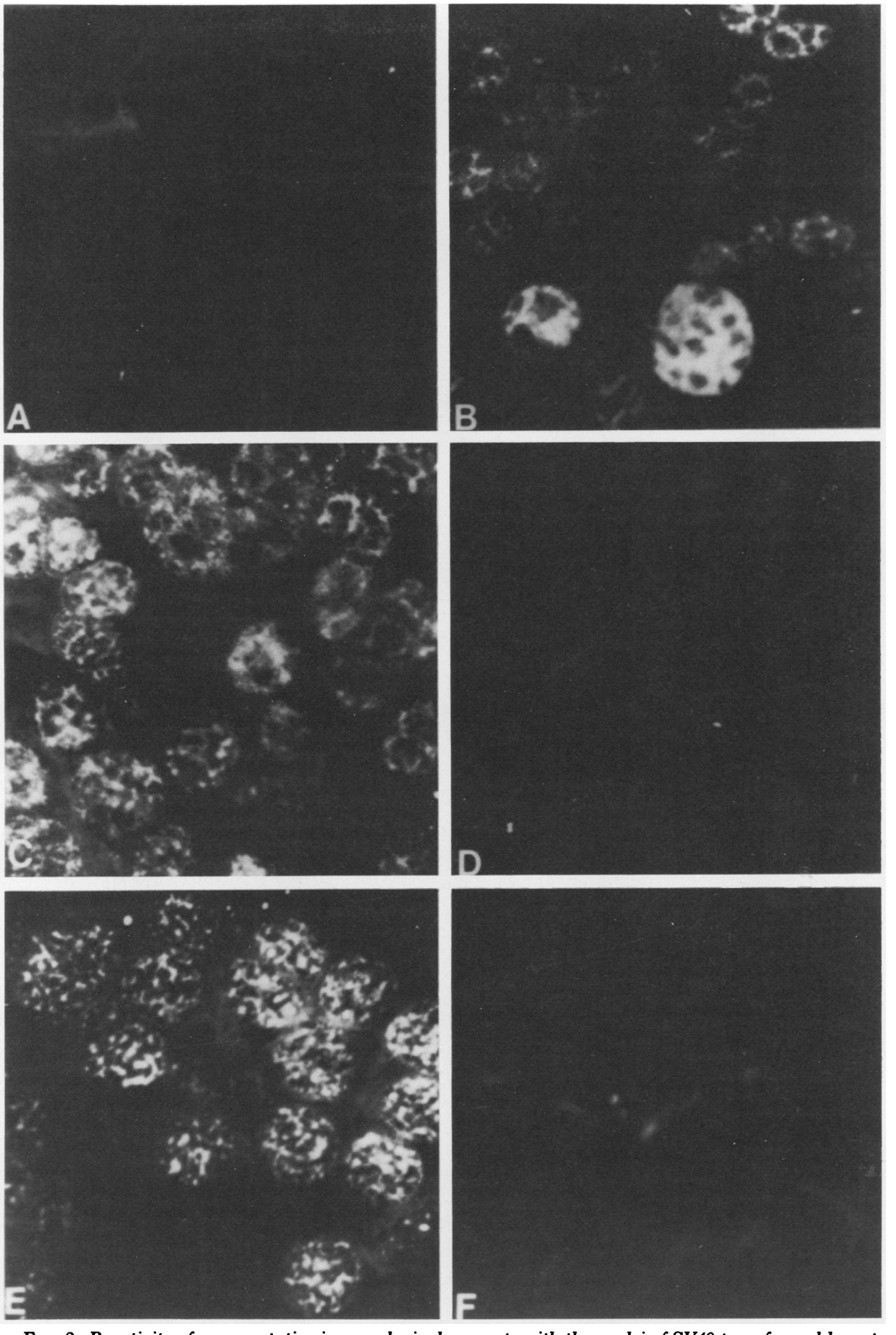

FIG. 2. *Reactivity of representative immunological reagents with the nuclei of SV40-transformed hamster (H-50) cells by indirect immunofluorescence. H-50 cells growing on glass coverslips were fixed with acetone and stained as detailed in Materials and Methods. Description of immunological reagents is presented in Materials and Methods and Table 1. The following antisera were used: (A) MoαPM; (B) MoαNuc; (C) MoαT; (D) NHS; (E) HaαT; and (F) HaαS. (×450).*

Since all of the reagents were prepared in syngeneic hosts, immunological reactivity was expected to be directed against transformation-specific antigens, including perhaps T-ag. The likelihood that the observed immunofluorescence reactions were due to derepressed host antigens was reduced by the failure of the MoαDMBA-2 tumor serum to react with SV40-transformed cells. The possibility that the reagents may be reacting with T-ag was derived from the positive reactions obtained with RaαT serum (prepared against purified, denatured, whole-cell T-ag). The differential reactivity of the two classes of immunological reagents raised the possibility that T-ag is a polyvalent antigen and that only a subset of the determinants exposed on the surface membrane is reactive when the molecule is located in the nucleus.

Detection of surface antigens by ^{125}I-labeled protein A radioimmunoassay. To increase the sensitivity and allow quantitation of the surface reaction, an ^{125}I-labeled protein A radioimmunoassay was employed. This assay provides an alternative approach to immunofluorescence for detection of anti-T-reactive surface antigens. Versene-dispersed H-50 or VLM cells (1×10^6 to 2×10^6) were reacted with various amounts of normal or T-reactive antiserum and ^{125}I-labeled protein A as detailed in Materials and Methods. Radioimmunoassay data obtained utilizing HaαT, HAF, MoαT, and RaαT, along with appropriate normal sera, are presented for H-50 (Table 4), and for VLM and DMBA-2/BALB Cl 4 cells (Table 5). The average P/N ratio for all T-reactive antisera at all concentrations tested was 3.25 with H-50 cells and 3.03 against VLM cells. It should be noted that the monospecific antiserum prepared in rabbits against purified large T-ag reacted well with the surface of both H-50 and VLM cells. Although normal rabbit serum exhibits higher background values in this assay, the RaαT achieved very positive P/N ratios of 3.83 (Table 4) and 2.44 (Table 5).

It is informative to compare the average P/N ratios obtained with the two hamster reagents, HaαT and HAF. Using H-50 as target cells, an average P/N ratio of 2.83 was obtained with HaαT, whereas HAF P/N ratios averaged 3.34. Similarly, by using VLM cells, P/N ratios averaged 2.11 for HaαT and 3.76 for HAF. The increased surface reactivity of HAF as compared with that of HaαT is of interest since the HAF reagent has a 3 $\log_2$ higher nuclear anti-T titer by immunofluorescence than does HaαT (21). This suggests a positive correlation between the level of reactivity with nuclear T-ag by immunofluorescence and the surface anti-T-reactive protein(s) detected in the ^{125}I-labeled protein A radioimmunoassay.

TABLE 4. *Demonstration of anti-T-reactive cell surface antigen on transformed hamster (H-50) cells using an ^{125}I-labeled protein A surface antigen radioimmunoassay*[a]

Serum[b]	Serum vol (μl)	^{125}I counts per minute ($\times 10^{-3}$)	P/N[c]
NHS	1	2.09	1.00
	5	2.08	1.00
	10	2.11	1.00
	20	2.04	1.00
HaαT	1	2.63	1.26
	5	5.78	2.78
	10	6.77	3.21
	20	8.28	4.06
HAF	1	4.53	2.17
	5	8.67	4.17
	10	6.52	3.09
	20	8.00	3.92
NMS	5	3.11	1.00
MoαT	5	12.60	4.05
NRS	5	14.94	1.00
RaαT	5	57.29	3.83

[a] Versene-dispersed H-50 cells were reacted with the indicated amount of serum for 60 min at 0°C, washed, and reacted with ^{125}I-labeled protein A as described in Materials and Methods.

[b] Designation of each type of serum is presented in Materials and Methods and Table 1.

[c] P/N, ^{125}I counts per minute for test serum divided by ^{125}I counts per minute for normal serum of the same species at the same concentration.

DMBA-2/BALB Cl 4 cells, a chemically transformed mouse line, was utilized in the ^{125}I-labeled protein A cellular surface antigen radioimmunoassay to establish a negative P/N ratio for the assay and to further document the SV40 specificity of the observed results. The average P/N ratio obtained with the high-titer HAF serum was 1.03, with a range of 0.83 to 1.13 (Table 5). As other controls, H-50 and VLM cells were reacted with 25 μl of undiluted MoαDMBA-2 serum or NMS in the ^{125}I-labeled protein A surface antigen radioimmunoassay. The P/N ratios obtained were 1.13 and 0.91 for H-50 and VLM cells, respectively (data not shown).

Therefore, the average P/N ratios obtained for T-reactive antisera (>2.0) were considered positive reactions and not merely reflections of nonspecific binding of tumor-bearing sera to transformed cells. The SV40-related specificity of the reaction was substantiated by the failure of the heterologous MoαDMBA-2 serum or a

TABLE 5. *Demonstration of anti-T-reactive cell surface antigen on transformed mouse cells using an ^{125}I-labeled protein A surface antigen radioimmunoassay*[a]

Cell line	Serum[b]	Serum vol (μl)	^{125}I counts per minute ($\times 10^{-3}$)	P/N[c]
VLM	NHS	1	0.90	1.00
		5	1.15	1.00
		10	1.23	1.00
		20	0.88	1.00
VLM	HaαT	5	1.84	1.60
		10	2.30	1.87
		20	2.51	2.85
VLM	HAF	1	1.50	1.67
		5	7.08	6.16
		10	3.80	3.09
		20	3.61	4.10
VLM	NMS	5	2.11	1.00
VLM	MoαT	5	7.42	3.52
VLM	NRS	5	4.25	1.00
VLM	RaαT	5	10.38	2.44
DMBA-2/BALB Cl 4	NHS	1	0.73	1.00
		5	0.82	1.00
		10	0.86	1.00
		20	0.84	1.00
DMBA-2/BALB Cl 4	HAF	1	0.80	1.10
		5	0.68	0.83
		10	0.97	1.13
		20	0.89	1.06

[a] Versene-dispersed mouse cells were reacted with the indicated amount of serum for 60 min at 0°C, washed, and reacted with ^{125}I-labeled protein A as described in Materials and Methods.

[b] Designation for each type of serum is presented in Materials and Methods and Table 1.

[c] P/N, ^{125}I counts per minute for test serum divided by ^{125}I counts per minute for normal serum of the same species at the same concentration for the homologous cell line.

variety of normal sera to react with the target cells.

Demonstration of anti-T specificity of immunological reagents by double-antibody surface antigen radioimmunoassay. Serological analyses by immunofluorescence and protein A radioimmunoassay had documented the SV40-specific immunoreactivity of the immunological reagents described in Table 1. However, those assays could not directly assign anti-T specificity to a given reagent, with the exception of RaαT. To determine whether the reagents contained reactivity directed against surface-associated T-ag, a double-antibody radioimmunoassay was established. In that assay, various reagents were tested for the ability to block the cell surface binding of RaαT. This serum was chosen as the test reagent because of its defined specificity against the T-polypeptide and its ability to react with all known determinants (T, S, and U) on the molecule (21). For this radioimmunoassay, H-50 cells were serially reacted with a blocking reagent, with NRS or RaαT, and with ^{125}I-labeled IgG from goat anti-rabbit IgG as described in Materials and Methods.

The results of three separate blocking radioimmunoassays are summarized in Table 6. Values for negative blocking were established by determining P/N ratios when no serum, NHS, NMS, MoαDMBA-2, or RatαPy-T was utilized as the blocking reagent before the addition of rabbit serum. For all those non-SV40-associated reagents, P/N values greater than 2 were consistently obtained. In contrast, differing degrees of positive blocking of RaαT surface binding were observed with the various hamster (Table 6, experiment 1: HAF, HaαT, HaαS, HaαH-50-G) and mouse (Table 6, experiment 2: MoαT, MoαS, MoαPM, MoαVLM-G; experiment 3: MoαPM, MoαNuc) reagents. Complete blocking was obtained with the reagent prepared against purified plasma membranes (MoαPM, P/N = 0.98 to 1.15); the lower blocking levels observed with some of the other surface reactive reagents (HaαH-50-G, MoαVLM-G, and HaαS) might be a reflection of a reduced anti-surface T-ag titer.

Blocking with the SV40-related positive reagents resulted in reductions of P/N values to levels ranging from 0.94 to 1.70. An average P/N ratio of 1.09 was calculated from all values obtained when SV40 surface-reactive reagents were present as blocking sera in the binding assay. The use of unabsorbed NRS or RaαT did not significantly alter the P/N values obtained in the absence of a blocking serum (data not shown).

These results directly demonstrate that reagents able to block the defined anti-T reactivity of RaαT must possess reactivity toward SV40 T-ag. Similarly, these data indicate that the positive reactions exhibited by these reagents in surface immunofluorescence and protein A radioimmunoassay experiments are due, at least in part, to an anti-T specificity manifest at the cell surface.

It is noteworthy that the ability of the monospecific T antibody prepared in rabbits to react specifically with the surface of SV40-transformed cells has been demonstrated by three different serological methods (indirect immu-

TABLE 6. *Demonstration of anti-SV40 T specificity of surface-reactive immunological reagents using a double-antibody radioimmunoassay*[a]

Expt no.	Blocking antibody	Test antibody	^{125}I counts per minute bound	P/N[b]
1	None	NRS	4,632	
	None	RaαT	10,400	2.25
	NHS[c]	NRS	5,006	
	NHS	RaαT	10,825	2.16
	HAF	NRS	7,705	
	HAF	RaαT	7,469	0.97
	HaαT	NRS	7,340	
	HaαT	RaαT	6,889	0.94
	HaαS	NRS	5,738	
	HaαS	RaαT	9,467	1.65
	HaαH-50-G	NRS	8,825	
	HaαH-50-G	RaαT	14,411	1.63
2	None	NRS	4,710	
	None	RaαT	12,120	2.57
	NMS	NRS	4,685	
	NMS	RaαT	10,695	2.25
	MoαT	NRS	7,870	
	MoαT	RaαT	6,725	0.85
	MoαS	NRS	8,480	
	MoαS	RaαT	11,370	1.34
	MoαPM	NRS	9,550	
	MoαPM	RaαT	9,385	0.98
	MoαVLM-G	NRS	9,180	
	MoαVLM-G	RaαT	15,575	1.70
3	None	NRS	6,415	
	None	RaαT	13,400	2.09
	MoαDMBA-2	NRS	5,605	
	MoαDMBA-2	RaαT	13,060	2.33
	MoαPM	NRS	5,480	
	MoαPM	RaαT	6,310	1.15
	MoαNuc	NRS	7,075	
	MoαNuc	RaαT	5,150	0.73
	RatαPy-T	NRS	6,395	
	RatαPy-T	RaαT	13,055	2.04

[a] Versene-dispersed H-50 cells were serially reacted with a blocking reagent, with NRS or RaαT, and with IgG fraction of goat anti-rabbit IgG labeled with ^{125}I as described in the text. The results from three independent experiments are reported.

[b] The P/N ratio was calculated as counts per minute obtained with RaαT divided by counts per minute obtained for NRS where cells had previously been reacted with the same blocking serum. The degree of blocking was significant ($P < 0.001$) when analyzed by Student's t test.

[c] Designations for immunological reagents are presented in Table 1 and in the text.

nofluorescence, ^{125}I-labeled protein A radioimmunoassay, and double-antibody radioimmunoassay).

Detection of surface and intranuclear polypeptides by immunoprecipitation. To identify the polypeptide target(s) of antigenic reactivity recognized by the various immunological test reagents, immunoprecipitation tests were performed. SV40-infected TC-7 cells were pulse-labeled with [^{35}S]methionine, and monolayers were extracted with buffered (pH 8.0) NP40 as described in Materials and Methods. These conditions solubilize T-ag from nuclei (35) and also solubilize plasma membrane-associated T-ag (unpublished data). Analysis of the immunoprecipitates by SDS-PAGE is presented in Fig. 3 and 4. All the immunological reagents tested, with the exception of the three normal sera and the MoαDMBA-2 serum, were capable of immunoprecipitating 96,000-molecular weight T-ag from whole cell extracts, demonstrating again that all those antisera possessed T-reactive specificity. In addition, all the reagents demonstrating anti-T reactivity against SV40-infected TC-7 cells also immunoprecipitated T-ag from extracts of VLM and H-50 cells (data not shown).

However, the use of whole-cell extracts did not prove that the immunoprecipitated polypeptide was in the same cell fraction as the antigen detected by immunofluorescence. To determine the subcellular localization of the antigen immunoprecipitated by surface T-reactive reagents from whole-cell extracts and to test the hypothesis generated from immunofluorescence data that a subset of antigenic determinants present on the surface anti-T-reactive antigen is exposed on nuclear T-ag, subcellular fractions were analyzed. HaαS was selected as the anti-

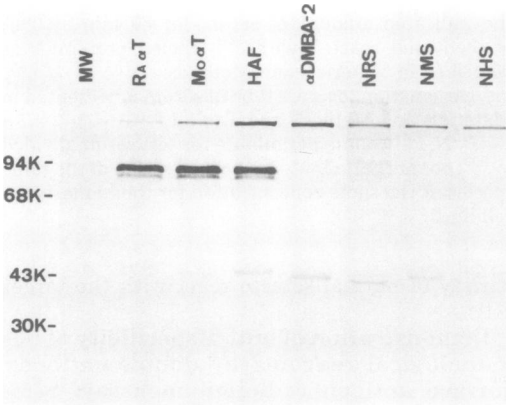

FIG. 3. *Reactivity of immunological reagents with SV40-infected TC-7 cell extracts analyzed by immunoprecipitation and SDS-PAGE. Infected cells were labeled with [^{35}S]methionine and solubilized, and portions were immunoprecipitated as detailed in Materials and Methods. Designation and description of immunological reagents is presented in Materials and Methods and Table 1. The position of molecular weight markers (MW) is indicated on the left.*

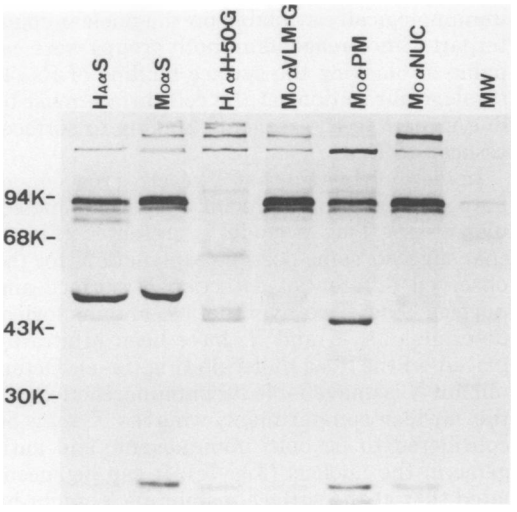

FIG. 4. *Reactivity of immunological reagents with SV40-infected TC-7 cell extracts analyzed by immunoprecipitation and SDS-PAGE. Infected cells were labeled with [^{35}S]methionine and analyzed as described in the legend to Fig. 3. The T-ag band precipitated by HaαH-50-G was clearly visible before photographing the autoradiogram.*

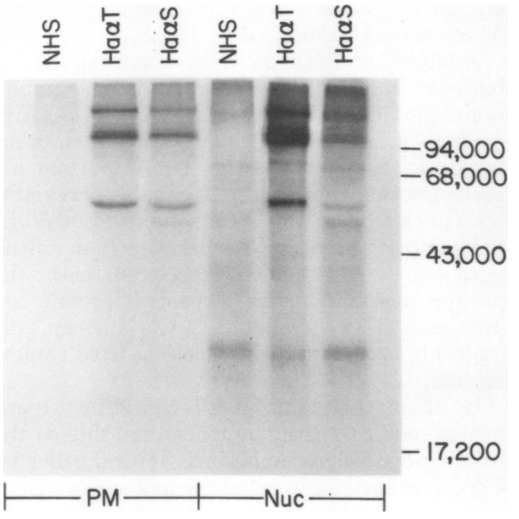

FIG. 5. *Reactivity of selected immunological reagents with subcellular fractions of SV40-transformed mouse (VLM) cells analyzed by immunoprecipitation and SDS-PAGE. VLM cells were labeled with $^{32}P_i$ and fractionated by two-phase polymer partitioning, and subcellular fractions were solubilized and immunoprecipitated as detailed in Materials and Methods. Designation and description of immunological reagents is presented in Materials and Methods and Table 1. Nuc, Nuclei; PM, plasma membrane. Positions of molecular weight markers are indicated on the right.*

serum for immunoprecipitation studies due to its exclusive surface reactivity.

VLM and H-50 cells were pulse-labeled with $^{32}P_i$ and fractionated by the two-phase polymer method, and the resulting nuclei and plasma membrane fractions were solubilized with a buffered detergent mixture as described previously (44). Under the solubilizing conditions used, the full complement of antigenic determinants present on nuclear and plasma membrane T-ag should be made available for immunoreactivity. Solubilized subcellular fractions were immunoprecipitated with HaαS serum (which was only surface-reactive by immunofluorescence) and HaαT serum (which contained both nuclear and surface reactivity). The analyses of these immunoprecipitates by SDS-PAGE is shown in Fig. 5 and 6 for VLM and H-50 cells, respectively. Both reagents, HaαS and HaαT, were capable of immunoprecipitating 96,000-molecular weight T-ag from both surface membranes and nuclei from both SV40-transformed cell systems. Quantitatively less T-ag was immunoprecipitated by HaαS serum than with HaαT serum, probably a reflection of a serum with a lower titer. HaαS appeared to immunoprecipitate T-ag more efficiently from extracts of plasma membranes than from nuclei when compared to the relative amounts of T-ag precipitated by HaαT from the subcellular fractions (Fig. 5 and 6). This fact might indicate that nuclear T-ag lacks a determinant(s), even after solubilization, that is pres-

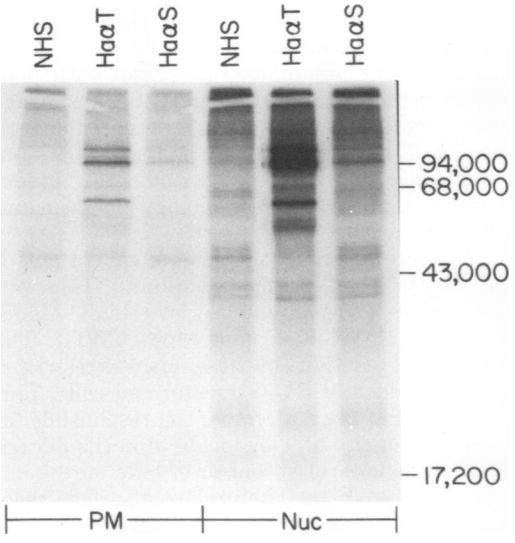

FIG. 6. *Reactivity of selected immunological reagents with subcellular fractions of SV40-transformed hamster (H-50) cells analyzed by immunoprecipitation and SDS-PAGE. H-50 cells were labeled with $^{32}P_i$ and fractionated by two-phase polymer partitioning, and subcellular fractions were analyzed as described in the legend to Fig. 5.*

ent on the surface-membrane counterpart and which is only recognized by HaαS.

Analysis of BHK-21 cells, a normal Syrian hamster line, by cell fractionation and immunoprecipitation did not reveal anti-T-reactive proteins in either nuclear or surface membrane fractions (data not shown). The important observation that HaαS serum is capable of reacting with nuclear T-ag only after solubilization of the polypeptide, supports the premise that not all antigenic determinants associated with the plasma membrane anti-T-reactive protein are immunologically available on the nuclear T-ag molecule in its native and acetone-fixed conformation.

In all of these immunoprecipitation experiments, small t-ag was not detected due to the labeling conditions employed. Several other labeled proteins of intermediate molecular weight are present on the autoradiograms in addition to the 96,000-molecular weight T-ag. The origin and specificity of these additional proteins are not known.

DISCUSSION

SV40 T-reactive immunological reagents characterized in this study may be categorized into two groups, based upon the presumed antigenic presentation of T-ag during the immunization process. The first group of antisera derived T-reactivity after a response against whole-cell T-ag; the group includes RaαT, HaαT, HAF, and MoαT. This group of reagents, when tested by immunofluorescence or surface antigen radioimmunoassay, reacted with both the nucleus and the surface of SV40-transformed or -infected cells. The reagent prepared against nuclear T-ag (MoαNuc) also contained both nuclear and surface reactivity, indicating immunogenic and antigenic homology between nuclear and surface T-ag.

The second group of reagents gained anti-T reactivity when surface-membrane T-ag was presented during immunization (HaαS, MoαS, MoαPM, HaαH-50-G, and MoαVLM-G). The serological reactivity of these sera was restricted to the surface of SV40-transformed cells. This suggests that the determinant(s) responsible for immunogenicity of T-ag localized in the plasma membrane is (are) absent or cryptic on the nuclear form of T-ag. The exclusive surface reactivity of HaαS exhibited in immunofluorescence tests was abolished after solubilization of transformed cell nuclei. The HaαS serum could immunoprecipitate both nuclear and membrane-bound 96,000-molecular weight T-ag. This observation strengthens the possibility that determinants exposed on the surface T-ag are not immunologically available on the nuclear counterpart. Since reagents in both groups were capable of blocking the surface binding of RaαT, serological reactions at the cell surface must be due in part to the reagents binding to surface-associated T-ag.

In view of the fact that T-reactive reagents in both groups immunoprecipitated 96,000-molecular weight T-ag, a model is presented (Fig. 7) that suggests some possible explanations for the observed differential detection of surface and nuclear T-ag. Two hypothetical immunological determinants, X and Y, have been arbitrarily placed on the T-ag molecule (Fig. 7a–e). Determinant Y is unavailable for immunoreactivity in the nuclear compartment, whereas X may be considered to be both immunogenic and antigenic in the nucleus (Fig. 7e). It can be speculated that at the surface membrane Y might be immunoreactive, whereas X may be antigenic but lack the immunogenic determinant (Fig. 7a–d). Hence, an immune response against nuclear (MoαNuc) or whole-cell T-ag (HaαT, MoαT, HAF, RaαT) would result in a reagent capable of recognizing the antigenic determinant X at the cell surface. Conversely, the immune response against plasma membrane T-ag (HaαS, MoαS, HaαH-50-G, MoαVLM-G, MoαPM)

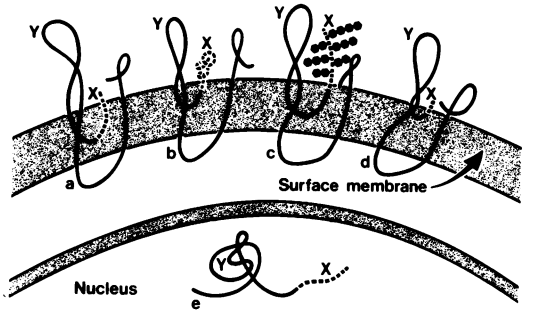

FIG. 7. *Model postulating possible mechanisms for differential antigenicity and immunogenicity of SV40 T-ag localized in the nucleus and plasma membrane. Two hypothetical determinants, X and Y, have been arbitrarily positioned on the T-ag polypeptide. Determinant Y is absent or cryptic (non-immunoreactive) on the molecular form contained in the nucleus (e) but is completely immunoreactive on polypeptides in the surface membrane (a–d). Determinant X is both immunogenic and antigenic on nuclear T-ag (e), whereas it lacks immunogenicity but retains antigenicity when localized in the surface membrane (a–d). Possible reasons for the loss of the immunogenicity of determinant X include burial in the bilayer (a), inactive tertiary configuration (b), modification such as glycosylation (c), and signal sequence processing (d). The antimembrane reagents react with nuclear T-ag only after prior solubilization to reveal determinant Y. See text for additional details.*

would result in a reagent capable of reacting only with determinant Y on the cell surface since Y is not reactive in nuclear T-ag. Such reagents would not react with X in the nucleus since X is not immunogenic in the plasma membrane. Solubilized nuclear T-ag would be immunoprecipitated by the surface-reactive reagents because Y would then be exposed and reactive.

There are a variety of possible reasons for the postulated antigenic and immunogenic differences between nuclear and plasma membrane-localized T-ag. The immunogenic portion of X could be buried in the bilayer (Fig. 7a), or the tertiary structure of the molecule could result in a nonimmunogenic configuration (Fig. 7b). In both cases an antigenic determinant on X remains reactive. Another possibility is that membrane-associated T-ag may be glycosylated or otherwise modified such that immunogenicity is destroyed while the antigenic determinant on X remains active (Fig. 7c). However, despite repeated efforts we have been unable to detect evidence of glycosylation of the membrane-associated T-ag.

An additional explanation for the loss of immunogenicity of determinant X is based on the signal sequence hypothesis of Blobel and Dobberstein (2) which involves the cleavage of a short peptide from the amino terminus of secretory proteins. In the context of this model for T-ag (Fig. 7d), the signal sequence could be the immunogenic portion of X, whereas the antigenic sequence would remain intact after cleavage. It is of interest that the *src* gene product specified by RSV appears to be modified by signal sequence processing following in vitro translation when heterologous membrane vesicles are added (16).

A series of studies reported by Schmidt-Ullrich and associates (24, 37, 38, 40) described the presence of two proteins in the plasma membranes of SV40-transformed hamster lymphocytes (GD248 cells). Both these proteins are glycosylated (39). The relationship between these proteins and T-ag is unclear due to the specificity of the antisera employed.

It has previously been reported that HaαT serum does not react with the surface of SV40-transformed or -infected cells (8, 49). The discrepancy with this report might be due to the relatively higher titers of the anti-T sera used in this study compared with those of reagents previously available. The reported failure of the hamster anti-T serum to react by ^{125}I-labeled protein A radioimmunoassay could also be due to the low affinity of protein A for hamster IgG. Langone (22) has recently demonstrated a higher affinity of protein A for rabbit IgG as compared with mouse IgG by a radioimmune competition binding assay. We observed that when HaαT was used in an indirect immunofluorescence experiment, no nuclear T reactivity was observed when protein A-FITC was substituted for antiglobulin FITC, whereas RaαT reacted well (unpublished data). Since the titers of both sera are comparable, the phenomenon might be due to differences in affinity of hamster and rabbit IgG for protein A. Therefore, a hamster anti-T reagent of low titer might not detect surface anti-T-reactive proteins.

Deppert and Pates (10, 11) recently analyzed Ad2$^+$ND1- and Ad2$^+$ND2-infected HeLa cells for anti-T-reactive protein by immunofluorescence. Antiserum prepared against SDS-PAGE-purified T-ag from SV40-transformed hamster cells detected nuclear, perinuclear, and surface-associated anti-T-reactive antigens. When the reagent was absorbed on acetone-fixed, SV40-transformed mouse cells, all three activities were abolished or significantly reduced in Ad2$^+$ND2-infected HeLa cells. Hamster anti-T serum from SV40 tumor-bearing hamsters was nonreactive against the surface of Ad2$^+$ND2-infected cells, possibly due to the Formalin fixation procedure.

The relationship, if any, of surface membrane-associated T-ag to virus-specific TSTA has not yet been established. Since considerable evidence supports the ability of SV40 large T-ag to induce TSTA activity (8, 34), preparative amounts of plasma membranes from SV40-transformed cells are being isolated. By analyzing the biological and biochemical properties of plasma membrane T-ag from these preparations, a more exact relationship of this molecule to nuclear T-ag and TSTA may then be elucidated.

ACKNOWLEDGMENTS

This investigation was supported in part by research grant CA 22555 from the National Cancer Institute.

We are especially grateful to Dan Medina for providing BALB/c mice and Gordon R. Dreesman for helpful discussions throughout the course of this study. The expert technical assistance of Elizabeth Torres is acknowledged.

ADDENDUM IN PROOF

D. F. Mark and P. Berg have recently suggested (Cold Spring Harbor Symp. Quant. Biol., in press) the existence of a third splice site in SV40 early mRNA which would result in a T-ag polypeptide that would be highly hydrophobic at the carboxy terminus, making it a candidate membrane protein. Such an altered molecule could explain the differential reactivity of nuclear and membrane-associated T-ag.

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