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Transformation by simian virus 40 induces virus-specific, related antigens in the surface membrane and nuclear envelope

(immunofluorescence/antisera against membrane/U-antigen/isolated nuclei/T-antigen)

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ABSTRACT **Nucleus- and mitochondrion-free membranes from hamster lymphocytes transformed by simian virus 40 (SV40), GD248 cells, cause guinea pigs to produce immune sera that reveal the presence in GD248 plasma membranes and mitochondria of two types of glycoprotein that are not detected in membranes of normal lymphocytes [Schmidt-Ullrich, R., Thompson, W. S. & Wallach, D. F. H. (1977) *Proc. Natl. Acad. Sci. USA* 74, 643–647]. Indirect immune fluorescence of living, SV40-transformed T19 hamster reticulum cells, Balb/c 3T3 mouse fibroblasts, and W18 VA2 human fibroblasts, using the antisera against GD248 membrane, at 4° produced a distinct cell surface fluorescence; however, above 20°, staining at the nuclear perimeter, the SV40 U-antigen reaction, becomes equally prominent. In SV40-transformed cells that had been fixed in cold acetone, as well as in purified GD248 nuclei, thermostable U-antigen staining is dramatic, but there is no reaction for nuclear T-antigen. Rabbit antisera against T19 cells gave immunofluorescence reactions equivalent to those obtained with the antisera against GD248 cells. Normal guinea pig or rabbit sera and cells that had not been transformed by SV40 gave no reaction. Our sera from tumor-bearing hamsters gave only nuclear T-antigen fluorescence. The results indicate the presence of related, SV40-specific antigens in the surface membranes, nuclear envelope, and possibly other intracellular organelles of SV40-transformed cells.**

The U-antigen of simian virus 40 (SV40) was discovered during work on cells infected by Ad2$^+$ND1, an adenovirus 2–SV40 hybrid virus (1, 2). Indirect immune fluorescence tests, using sera of hamsters bearing SV40-induced tumors, define the U-antigen of the infected cells by its perinuclear location and its stability to heating at 50° (1, 2). However, Robb (3), using an immunoprecipitation approach, has recently documented the existence of U-antigenic, 94,000-dalton proteins in SV40-transformed cells.

Immunofluorescence has hitherto not succeeded in localizing the U-antigen of SV40-transformed cells, whether the sera of hamsters bearing tumors or monkeys injected with Ad2$^+$ND1-infected cells are used (2). However, we now observe that hyperimmune guinea pig sera raised against purified, nucleus-free membranes from SV40-transformed hamster lymphocytes (GD248 cells) (4, 5) yield a strong, thermostable, perinuclear U-antigen immunofluorescence, not only with GD248 cells or their isolated nuclei, but also with T19, a reticulum cell sarcoma derived from GD248 tumors,† and with SV40-transformed Balb/c 3T3 mouse fibroblasts. Rabbit antisera against T19 cells produced equivalent results. In addition, both sera produce distinct surface immunofluorescence not only with viable GD248 and T19 cells, but also with SV40-transformed mouse fibroblasts and SV40-transformed human W18 VA2 fibroblasts.

Abbreviation: SV40, simian virus 40.

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† P.-S. Lin, C. E. Butterfield, and D. F. H. Wallach, unpublished data.

MATERIALS AND METHODS

Cell Cultures. The following SV40-transformed cell lines were used: a human fibroblast cell line W18 VA2 (kindly provided by P. H. Black); a mouse cell line, Balb/c SV3T3 (Flow Laboratories, Rockville, MD); and a Syrian hamster cell line T19† isolated from a GD248-induced lymphoid tumor (6). Normal Balb/c 3T3 cells, kindly provided by P. H. Black, were used as controls. SV W18 VA2 and SV3T3 were propagated in Eagle's minimum essential medium plus glutamine, the 3T3 cells in Eagle's basal medium, and the T19 cells in RPMI 1640. These media, purchased from Associated Biomedic Systems, Inc. (Buffalo, NY), were supplemented with 10% heated, inactivated fetal calf serum (Grand Island Biological Co., Grand Island, NY). Cells were cultured on cover slips contained in petri dishes; they were used in various experiments within 72 hr after subculture.

Purification of GD248 Nuclei. GD248 cells were isolated as in refs. 4 and 5. Their nuclei were purified as in ref. 7, except for the following modifications: (*a*) the disruption medium was 65 mM NaCl/75 mM KCl/5 mM $MgCl_2$/10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.0; (*b*) disruption was by nitrogen decompression [450 lb/inch^2 (3.20 MPa), 20 min]; and (*c*) the ionic composition of the gradient steps was 70 mM KCl/2.5 mM $MgCl_2$/20 mM Hepes, pH 7.0.

Antisera. Antiserum against T was raised by inoculating 6-week-old Syrian hamsters subcutaneously with SV40 virus-induced GD248 tumors or T19 cells (4–5).† The tumor-bearing animals were bled 6–8 weeks after inoculation.

Antiserum against GD248 membrane was developed in guinea pigs as described (4). The animals were boosted three times and bled 10 days after the last booster.

Antiserum against T19 cells was prepared as follows: Cultured T19 cells were scraped off mechanically, washed twice with Dulbecco's phosphate-buffered saline (Grand Island Biological Co., Grand Island, NY), and inoculated intravenously into 4-month-old New Zealand White rabbits. The immunizing inocula contained 1.5 to 6 × 10^7 cells per injection. After 2 weeks, the animals were boosted at weekly intervals. Sera was obtained 1 week after boosting. The antiserum against T19 cells used in this study was a pool of four bleedings each from two rabbits.

Indirect Immunofluorescence. Fluorescein-labeled immunoglobulins (IgG) were obtained from Cappel Lab., Inc., (Downingtown, PA) and absorbed once with minced hamster liver and kidney before use. Immunofluorescence was observed in a Leitz Orthoplan microscope. Exciting illumination from a 200 W mercury light source was passed through a BG-24 heat filter, a BG-12 filter, and TK 500 dichromic mirror. A K 510 filter was used to eliminate stray exciting light in the emission path.

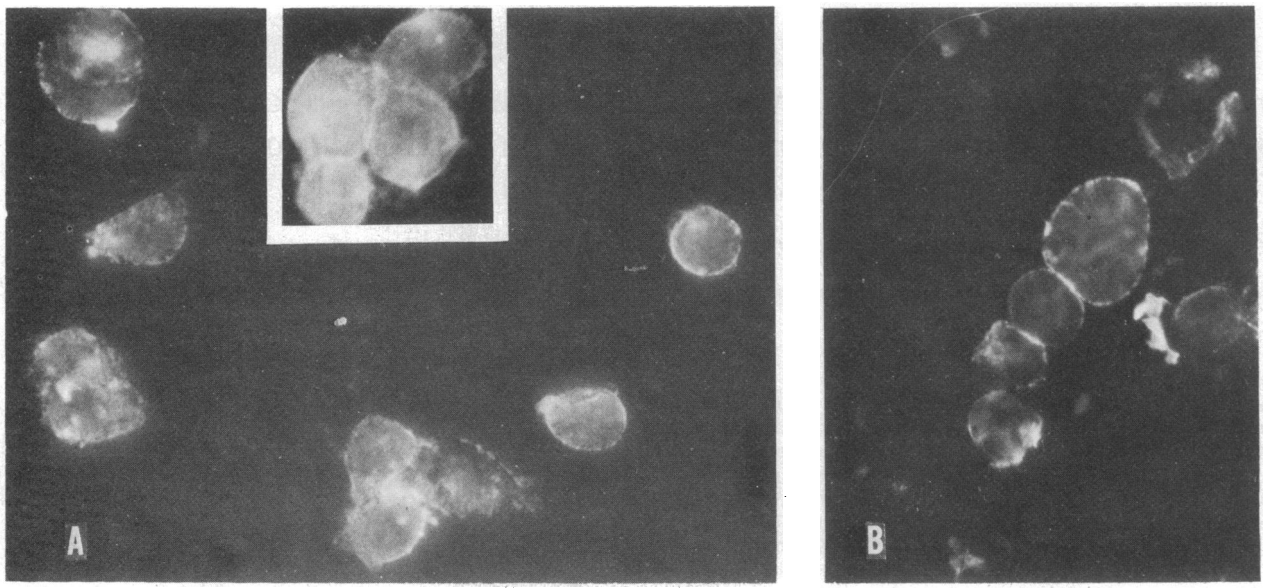

FIG. 1. (*A*) Viable SV3T3 cells in monolayer stained by indirect immunofluorescence at 4°, using antiserum against GD248 membrane, yield a prominent cell surface reaction. (*Inset*) Nuclear envelope and cytoplasmic staining produced when reaction is carried out at 20°. Intracellular staining at 20° can be time-monitored visually and is due to antibody internalization. (×750.) (*B*) Viable SV3T3 cells in suspension were stained by indirect immunofluorescence at 4° using antiserum against T19 cells, producing a prominent cell surface reaction. (×750.)

For detection of T-antigen, cells or GD248 nuclei, which had been fixed in cold acetone, on 1 × 1 cm cover slips were washed three to five times with phosphate-buffered saline, then incubated with antiserum against T for 30 min at 37°. The washes with phosphate-buffered saline were repeated and the cover slips were then incubated for 30 min at 37° with fluorescein-conjugated IgG fraction against antibody against hamster IgG, produced in rabbits. Before fluorescence microscopy, the cover slips were again rinsed three to five times with phosphate-buffered saline. Cells exposed to normal hamster sera were used as controls.

To stain living cells, viable (trypan blue-negative) cells as monolayers on coverslips or scraped off into suspension were washed with cold phosphate-buffered saline, then incubated with antiserum against GD248 membrane or antiserum against T19 cells for 30 min at 37°, 25°, or 4°. The cells were then washed with phosphate-buffered saline and incubated with fluorescein-conjugated IgG fraction against antibody against guinea pig IgG, produced in rabbits, or fluorescein-conjugated IgG fraction against antibody against rabbit IgG, produced in goats, for 30 min at 25° or 4°. The cells were then again washed with phosphate-buffered saline before fluorescence microscopy. Cells initially exposed to normal guinea pig or rabbit sera, respectively, were used as control.

For detection of U-antigen, cover slips bearing cells or GD248 nuclei that had been fixed in cold acetone were stained as described for viable cells except that the incubation temperature was 37°.

Heat Lability of T-Antigen and Heat Stability of U-Antigen. These antigens were tested using both the cover slip pro-

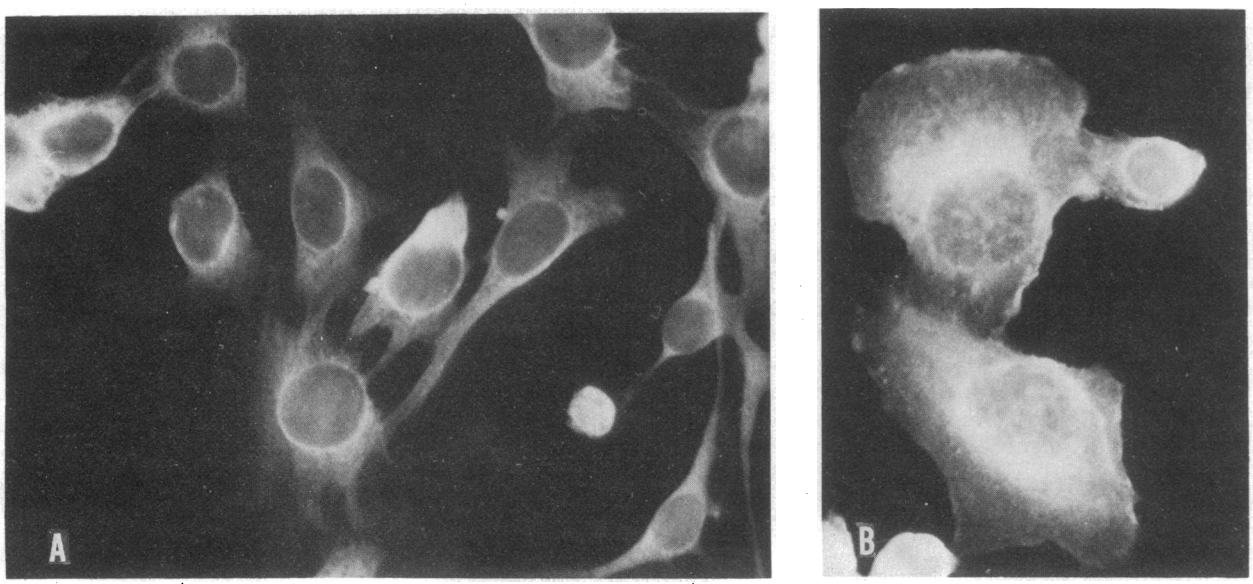

FIG. 2. Fixed SV3T3 cells (*A*) and fixed T19 cells (*B*) were treated as described in the legend for Fig. 1*A* and Fig. 1*B*, respectively, but at 37°. Note nuclear envelope and cytoplasmic fluorescence. (×750.)

Table 1. Indirect surface immunofluorescence in various living SV40-converted and normal cells*

Cell line	Antiserum	Surface fluorescence
T19	Rabbit anti-T19	Intense
T19	Normal rabbit	Negative
T19	Guinea pig anti-GD248 membrane	Intense
T19	Normal guinea pig	Negative
SV3T3	Rabbit anti-T19	Intense
SV3T3	Normal rabbit	Negative
SV3T3	Guinea pig anti-GD248 membrane	Intense
SV3T3	Normal guinea pig	Negative
W18 VA2	Rabbit anti-T19	Intense
W18 VA2	Guinea pig anti-GD248 membrane	Intense
3T3	Rabbit anti-T19	Negative
3T3	Guinea pig anti-GD248 membrane	Negative

* Obtained with guinea pig antisera against GD248 membrane and rabbit antisera against T19 cells.

cedure described in ref. 1 and the bulk procedure ("cell packs") described in ref. 2, but limiting heating to 50°. Both approaches fully abolished the T reaction.

RESULTS

Surface fluorescence of living SV40-transformed cells

Both guinea pig antisera against GD248 membrane and rabbit antisera against T19 cells, when used to stain living SV40-transformed cells by indirect immune fluorescence, produced vivid coloration of nearly all cells. This was limited to the cell surfaces when the reaction was carried out at 4° (Fig. 1; Table 1), but when living cells were processed at 37°, nuclear envelope staining was as prominent as surface fluorescence. Intermediate results were obtained at 20°.

No surface or cytoplasmic staining occurred with normal cells or with nonimmune sera. Surface staining was also not observed with the antisera against T. That the sera produced against membranes from SV40-transformed hamster cells or whole SV40-transformed cells gave the described reactions with both SV40-transformed mouse and human cells indicates that these reactions are due to SV40-specific antigen(s).

Nuclear envelope (perinuclear) and cytoplasmic fluorescence of fixed SV40-transformed cells

Both guinea pig antisera against GD248 membrane and antisera against T19 cells, when used to stain T19 and Balb/c SV3T3 cells, which had been fixed in cold acetone, by indirect immune fluorescence, produced an intense reaction (Fig. 2; Table 2) delineating all nuclei of the transformed cells. The fluorescence reaction was also observed with purified GD248 nuclei. The nuclear envelope staining observed in fixed cells was similar, but more intense than that observed using the same reagents on intact cells at 37°. Merging of the fluorescent zones about the individual nuclei was observed in multinucleate T19 cells. The staining was not observed with untransformed cells, normal guinea pig or rabbit sera, or our sera from tumor-bearing hamsters. The staining was not abolished by heating the cells or nuclei to 50°C for 30 min (Fig. 3).

Table 2. Indirect immunofluorescence of nuclear envelope and cytoplasm in various SV40-transformed and normal cells that had been fixed in cold acetone*

Cell line	Antiserum	Fluorescence: Cytoplasmic	Fluorescence: Nuclear envelope
T19	Rabbit anti-T19	+++	++++
SV3T3	Rabbit anti-T19	+++	++++
3T3	Rabbit anti-T19	−	−
Heated SV3T3	Rabbit anti-T19	++	+++
T19	Guinea pig anti-GD248 membrane	+++	++++
SV3T3	Guinea pig anti-GD248 membrane	+++	++++
3T3	Guinea pig anti-GD248 membrane	−	−
Heated SV3T3	Guinea pig anti-GD248 membrane	++	+++
T19	Hamster, SV40 tumor-bearing	±	−
SV3T3	Hamster, SV40 tumor-bearing	±	−
3T3	Hamster, SV40 tumor-bearing	−	−
Heated SV3T3	Hamster, SV40 tumor-bearing	±	−

* Produced with guinea pig antisera against GD248 membrane and rabbit antisera against T19 cells.

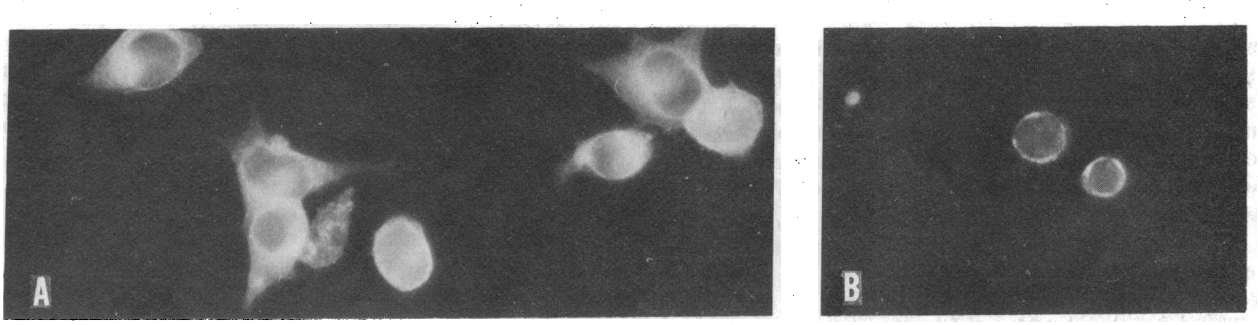

FIG. 3. (*A*) Same as in Fig. 2*A*, but the cells were heat-treated before they were incubated with antiserum against GD248 membrane. Staining characteristics as in Fig. 2*A*. (×750.) (*B*) Isolated GD248 nuclei were treated as described for *A*. Note the nuclear envelope fluorescence. (Unheated nuclei have the identical appearance.) (×750.)

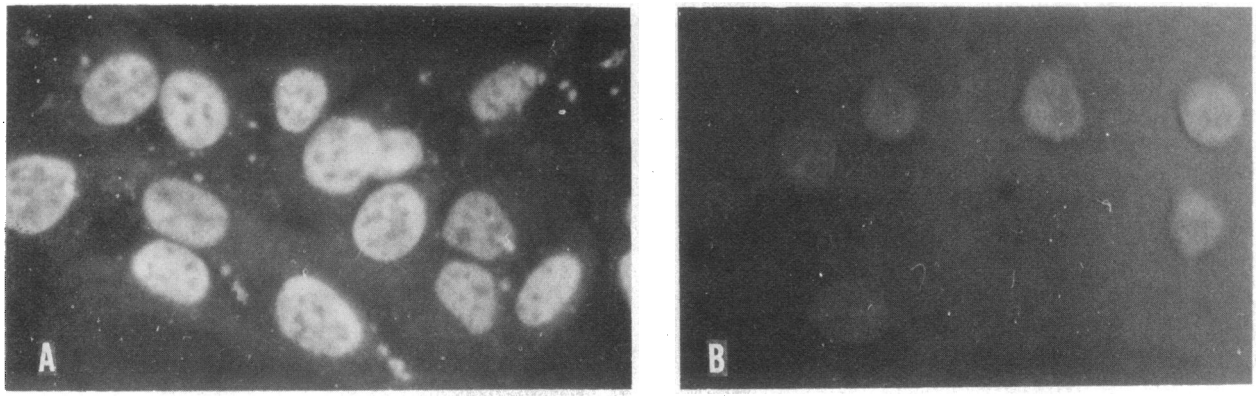

FIG. 4. (*A*) Fixed SV3T3 cells were stained by indirect immunofluorescence using antiserum against T. Note prominent nuclear fluorescence. (×750.) (*B*) Same as in *A*, but cells were heat-treated before they were incubated with antiserum against T. (×750.)

For isolated GD248 nuclei, the staining was discretely limited to the nuclear periphery (Fig. 3*B*). With whole cells, however, extranuclear cytoplasmic staining was also detected. This was always less intense than at the nuclear perimeter and too diffuse to allow localization to specific organelles. However, an invariable structuring of this staining suggested its association with particulate subcellular elements. In addition, extranuclear staining of T19 giant cells tended to accumulate in areas where we found a concentration of Golgi lamellae by electron microscopy.†

The nuclear envelope (perinuclear) staining described occurs only in SV40-transformed cells, can be detected only with sera directed against components of SV40-transformed cells, and is stable to heating. This identifies the reaction as due to the SV40 U-antigen, as defined in refs. 1 and 2.

Nuclear T-antigen

Indirect fluorescence tests with sera from hamsters bearing SV40-induced tumors gave a typical nuclear T-antigen fluorescence with all SV40-converted cells tested (Fig. 4*A*), as well as with isolated GD248 nuclei. Sera from normal hamsters gave no nuclear immunofluorescence. The T-antigen reaction was fully abolished by heating cells (Fig. 4*B*) or GD248 nuclei at 50° for 30 min. The characteristic intranuclear T-antigen staining pattern was *not* obtained with guinea pig antisera against GD248 membrane or rabbit antisera against T19 cells. Rather, both sera yielded a diffuse, intranuclear fluorescence that could not be securely distinguished from the intense nuclear envelope (perinuclear) fluorescence produced by these sera (Fig. 2), even after the cells were first heated at 50° (Fig. 3). However, neither of these sera, used conjointly or sequentially with hamster antiserum against T interfered with the typical T-antigen reaction of the latter.

DISCUSSION

Heretofore a perinuclear location of SV40 U-antigenicity has been satisfactorily demonstrated only for Ad2$^+$ND1-infected cells, where the antigen is part of a fragment of about 30,000 daltons (8–10), although synthesis of U-positive polypeptides has now also been documented for SV40-transformed cells (3). Our work, using antisera against membranes of SV40-transformed cells, shows the following:

(*a*) The sera are potent reagents for the localization of U-antigen by immune fluorescence.

(*b*) The sera lack interfering T-reactivity.

(*c*) The perinuclear location of U-antigenicity is an attribute also of SV40-transformed cells, where the antigen is on a 94,000-dalton polypeptide (3).

(*d*) The staining of isolated nuclei demonstrates a nuclear envelope association, rather than simple perinuclear accumulation.

Lewis and Rowe (2) attempted to produce antibody against U-antigen in African green monkeys using seven intramuscular injections of Ad2$^+$ND1-infected monkey cells at weekly intervals. However, this procedure yielded only sera giving low-titer immunofluorescence responses, without the perinuclear staining obtained with most sera from SV40 tumor-bearing hamsters. This result is not altogether surprising for reasons including the following: Ad2$^+$ND1 contains only about 16% of the SV40 genome (4) and, although the peptide segment bearing U-antigenicity is computed to have a molecular weight of 28,000–30,000 (9, 10), work by Robb (3) shows that the U-antigen in SV40-transformed cells has a molecular weight near 94,000. The 94,000-dalton fragment can be expected to be immunogenically superior than the 28,000 to 30,000-dalton fragments of the hybrid virus.

The results described herein should be viewed in the light of published experiments (4, 5), using guinea pig anti GD248-membrane serum in bidimensional immune electrophoresis. These immunochemical studies showed that the plasma membranes and mitochondria of the SV40-transformed lymphocytes contain two classes of antigenic protein not detectable in the membranes of normal lymphocytes. By isoelectric focusing (11) of Triton-solublized membranes these proteins were defined as two groups: The *first*, glycoprotein in nature, with an isoelectric point (pI) of pH 4.5, is chemically detectable only in the membranes of transformed cells. The *second*, with a pI of 4.7, overlaps, by chemical analysis, a component present also in normal membranes. These immunochemical analyses were carried out on neutral-detergent extracts which, according to recent work can be expected to preserve SV40-specific U-antigen (3) as well as transplantation (12) and surface-antigen (12, 13). We therefore suggest that the two categories of antigen identified in (4, 5) correspond to the SV40-specific surface- and U-antigenicities described herein by immunofluorescence. Immuno- and biochemical support for this suggestion is presented elsewhere (submitted for publication).

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