T Antigen from Nuclear and Cytoplasmic Extracts from an Adenovirus Type 12 Transformed Cell Line[1] (34252)

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In our laboratory a line of hamster kidney cells transformed *in vitro* by adenovirus type 12, designated as 0-66 (1), had shown a consistent increase in complement-fixing (CF) T antigen titer at different passage levels. The staining patterns of the T antigen varied in the indirect fluorescent antibody technic (IFA) when sera from different hamsters bearing tumors induced by these cells were used. Some sera stained flecks of cytoplasmic antigen while others stained also antigen which was diffuse in the nucleus and cytoplasm. Such results suggested the presence of two or more different antigenic moieties within the same cell. To investigate this possibility, T antigen was extracted from nuclear and cytoplasmic fractions of the 0-66 cell line, and studied by starch block electrophoresis and hydroxylapatite column chromatography.

Materials and Methods. Preparation of T antigen from 0-66 cell line. From whole cell lysates. Crude T antigen was extracted from monolayer cultures by sonication of a 10% cell pack suspended in Kolmer's saline buffered with 0.01 *M* Tris–HCl, pH 8.1 (TBKS).

From nuclear fractions. Nuclei were isolated by homogenization in 2.3 *M* sucrose solution according to the method of Chauveau *et al.* (2). The nuclei were then suspended in 1 *M* sucrose solution and treated with a mixture of three detergents, according to Taub *et al.* (3), in order to remove more cytoplasmic contamination. The nuclear fractions were collected by centrifugation.

The whole cell lysates and nuclear preparations after sonication (10 min 20 Kcps, Bronwill Biosonik) and centrifugation (1 hr, 78,000*g*) were concentrated by ultrafiltration (Diaflo, Amicon Corp.) at 4°.

From cytoplasm. Cytoplasm was obtained from the supernatant fluid after removal of the crude nuclei, by precipitation with 50% ammonium sulfate. The precipitate was dialyzed against TBKS for 24 hr and concentrated by ultrafiltration.

Preparation of extracts of control cells. Similar extracts of whole cell lysates from a continuous line of normal baby hamster kidney cells (cell line 0-853) were also prepared.

Preparation of antiserum to T antigen. Three–5-day-old hamsters were inoculated subcutaneously with 10^6 cells of the 0-66 cell line. When tumor size was maximal the sera were collected from the animals and assayed for CF activity and for staining in the IFA technic. Pools with CF titers of 1:128–1:256 were used in this study.

Fluorescent antibody technic. The indirect fluorescent antibody procedure was used as previously described (4). The antihamster gamma globulin used for labeling was prepared in a goat.

Assay procedures. Complement fixation assays were done by block titration in the microtiter test (5) using 0.025 ml of antigen and antibody. A unit of antigen is the reciprocal of the highest dilution giving a 3 or 4+ reaction by block titration with the highest dilution of standardized antiserum. Protein was estimated by the Lowry method (6).

Hydroxylapatite fractionation. Columns

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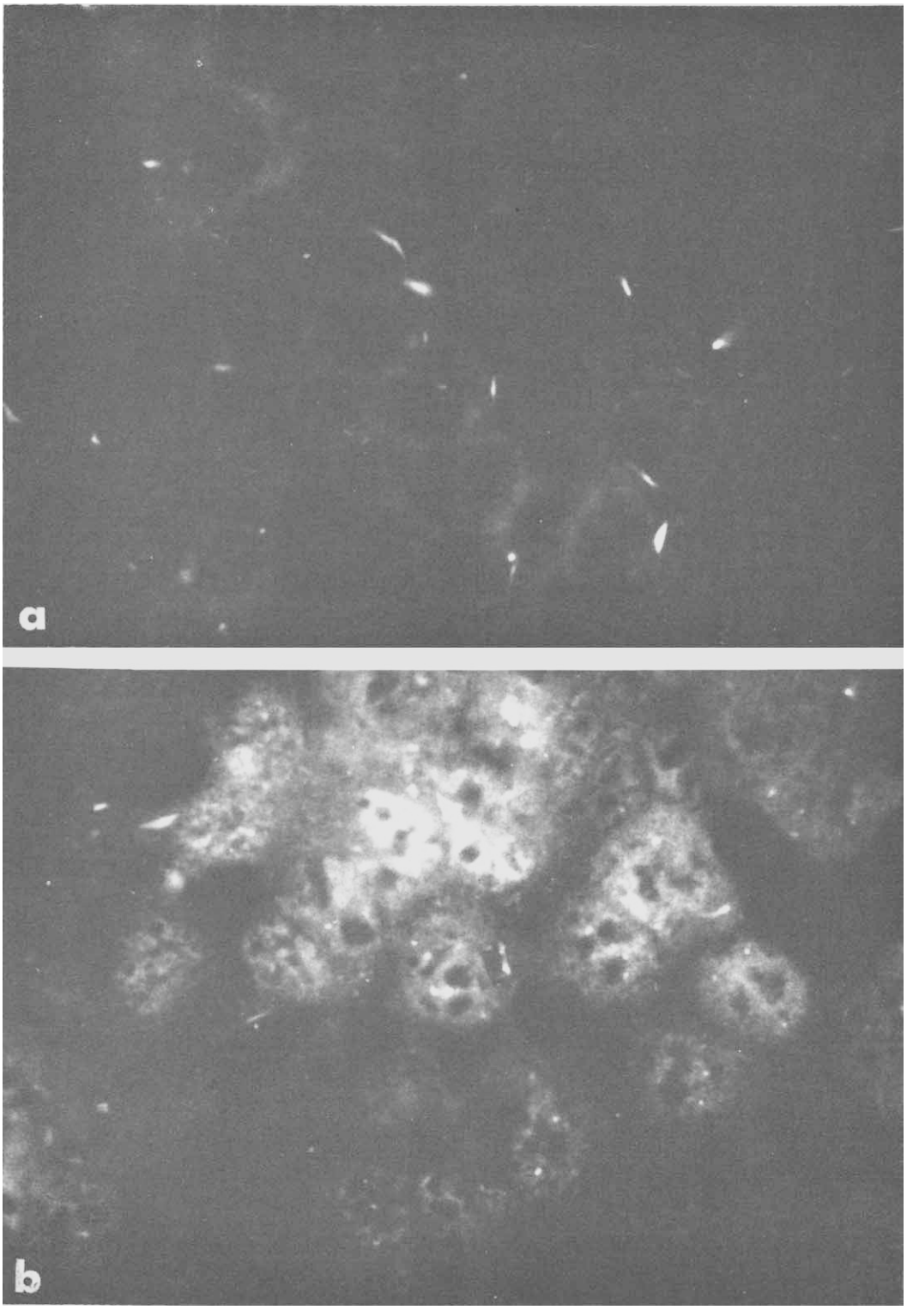
a
b

FIG. 1. Demonstration of adenovirus type 12 T antigen in 0-66 cell line by the indirect fluorescent antibody procedure. (a) Individual TBHS staining only the cytoplasmic flecks; (b) Individual TBHS staining cytoplasmic flecks and diffuse intranuclear antigen (×1000).

(1.0 × 15 cm) were packed with commercially prepared hydroxylapatite (Bio-Rad Laboratories, Richmond, California), suspended in 0.005 *M* potassium phosphate buffer, pH 7.4, containing 10% glycerol and 0.005 *M* mercaptoethanol. The applied sample was eluted stepwise with increasing molarities of buffer (0.005, 0.1, 0.2, and 0.3 *M*).

Starch block electrophoresis. Electrophoresis was conducted at 450 V, 12 Ma, with 0.1 *M* Tris–HCl buffer, pH 8.6, for 23 hr at 4°. At the end of the run, 1-cm wide sections were cut and eluted with TBKS.

Results. Characteristics of 0-66 cell line. Infectious virus could not be isolated from these cells either by utilizing extracts of the cells as inoculum on human embryonic kidney cells (HEK), or by cocultivation with HEK cells. During early passages of the cells the usual CF titer of the T antigen was 1:16 (640 units/ml). With further passage levels the titer increased until at passage level 240 the titer was 1:256 (10,240 units/ml).

The cells have retained their oncogenic potential in hamsters with 80–90% of the inoculated animals producing tumors by 6 months.

By IFA test some sera stained only cytoplasmic T antigen, which appeared as flecks or long slender rods (Fig. 1a). Other sera stained in addition to the cytoplasmic T antigen, an intranuclear antigen. The intranuclear T antigen appeared as a bright diffuse, amorphous mass usually throughout the nucleus with only the nucleolus remaining unstained (Fig. 1b). A dull diffuse staining of the entire cytoplasm often occurred, which although specific was not as intense as either the cytoplasmic fleck shaped antigen nor the diffuse intranuclear antigen. The cells would not stain with potent labeled antivirion serum.

The control cell line, 0-853 showed no CF activity and did not stain by the IFA procedure.

Assay of T antigen preparations. After concentration by ultrafiltration, protein content of whole cell lysates and cytoplasmic preparations ranged from 80 to 120 mg/ml and of nuclear preparations 9 to 11 mg/ml. CF units/ml were comparable in the various preparations ranging from 10,240 to 51,200 units/ml with an average of 24,000. Therefore, specific CF activity was always higher in nuclear extracts as compared to whole cell lysates and cytoplasmic extracts (Tables I, II).

TABLE I. CF Activity of T Antigen(s) by Hydroxylapatite Chromatography.

Sample (*M*)	CF (units/ml)		CF (units/mg of protein)	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
Original	10,240	40,960	850	420
0.005	160	640	250	120
0.1	320	640	450	370
0.2	1280	2560	2000	2000
0.3	160	320	300	300
Protein (mg) placed on column			22	151
% yield CF (all fractions)			68	66
% protein removed (all fractions)			32	33
% yield CF (0.2 *M* fraction)			45	55
% protein removed (0.2 *M* fraction)			80	92

Hydroxylapatite column chromatography. Using stepwise elution of hydroxylapatite columns, peaks of protein and CF activity were obtained with each increasing molarity (0.005–0.3). No more activity was obtained above 0.3 *M* as the molarity of the elutant was increased to the limit of saturation of salts in the buffer at 4°. The majority of the CF activity was recovered in the 0.2 *M* fraction, the yield ranging from 30 to 50% in different experiments.

Table I compares the results obtained in typical experiments utilizing concentrated nuclear extract and concentrated cytoplasmic extract. In the fraction eluting with 0.2 *M* salt, specific activity, as determined by the

TABLE II. CF Activity of T Antigen(s) by Starch Block Electrophoresis.

	Whole cell lysate; CF units per		Cytoplasm; CF units per		Nuclei; CF units per	
Fraction no.	ml	mg	ml	mg	ml	mg
Sample[a]	20,480	200	51,200	400	20,480	2200
42[b]	160	300	—	—	—	—
43	160	300	—	—	—	—
44	320	400	—	—	—	—
45	640	400	—	—	—	—
46	1280	400	—	—	160	2000
47	2560	1000	160	1800	320	2000
48	2560	1100	160	900	640	5100
49	1280	1100	640	3400	1280	22,000
50	1280	2800	1280	3700	640	18,300
51	640	1800	320	1700	320	8900
52	320	1600	<160	—	320	9100
53	<160	—	—	—	160	5300
Protein (mg) electrophoresed	658 (7 ml)		149 (1.1 ml)		52 (5.5 ml)	

[a] Starting material.
[b] Ten-ml fractions.

number of CF units/mg of protein was greater than two times that obtained in the original sample of nuclear extract, while it was almost five times that of the original sample obtained from the concentrated cytoplasmic preparation.

Starch block electrophoresis. After electrophoresis all of the recovered CF activity of the various T antigen preparations was associated with a protein peak located about 10 cm (varying from 8 to 12) cathodal to the point of origin (Fig. 2). Some or all of this movement could be due to electro-osmosis. The fractions comprising the entire peak contained 10–20% of the protein electrophoresed and they contained 35–75% of the CF activity. The fraction with the highest specific CF activity contained 0.5–3.0% of the protein electrophoresed and 10–25% of the CF activity. The fraction with the highest specific activity and maximal units of CF/ml usually did not coincide with the fraction absorbing maximally at 280 mμ, rather it was displaced 1–2 cm farther toward the cathode (Fig. 2). Table II shows the results of representative electrophoretic runs.

Electrophoresis of the extracts from the normal baby hamster kidney cell line (0-853) resulted in the separation of a protein peak in the same position as the protein peak from the adenovirus transformed cell line (0-66). However it was devoid of CF activity.

Discussion. Staining results by the IFA test reported here are in agreement with those of Pope and Rowe (7), who described three different patterns: cytoplasmic flecks, nuclear and cytoplasmic flecks, or homogenous staining of nucleus and cytoplasm. These staining patterns suggest different nuclear and cytoplasmic T antigens.

By hydroxylapatite chromatography the majority of the recovered CF activity from nuclear, cytoplasmic, or whole lysate was associated with one peak similar to the findings of Giliad (8). By starch block electrophoresis where separation is by charge, all of the T antigen appeared in a few fractions (Fig. 2), achieving in one step, separation from the majority of host material. The overall yield of CF activity by hydroxylapatite and starch block was approximately the same but increase in specific activity in any one fraction was greater with the latter.

Because after electrophoresis of T antigen for 22 hr the CF activity was present primar-

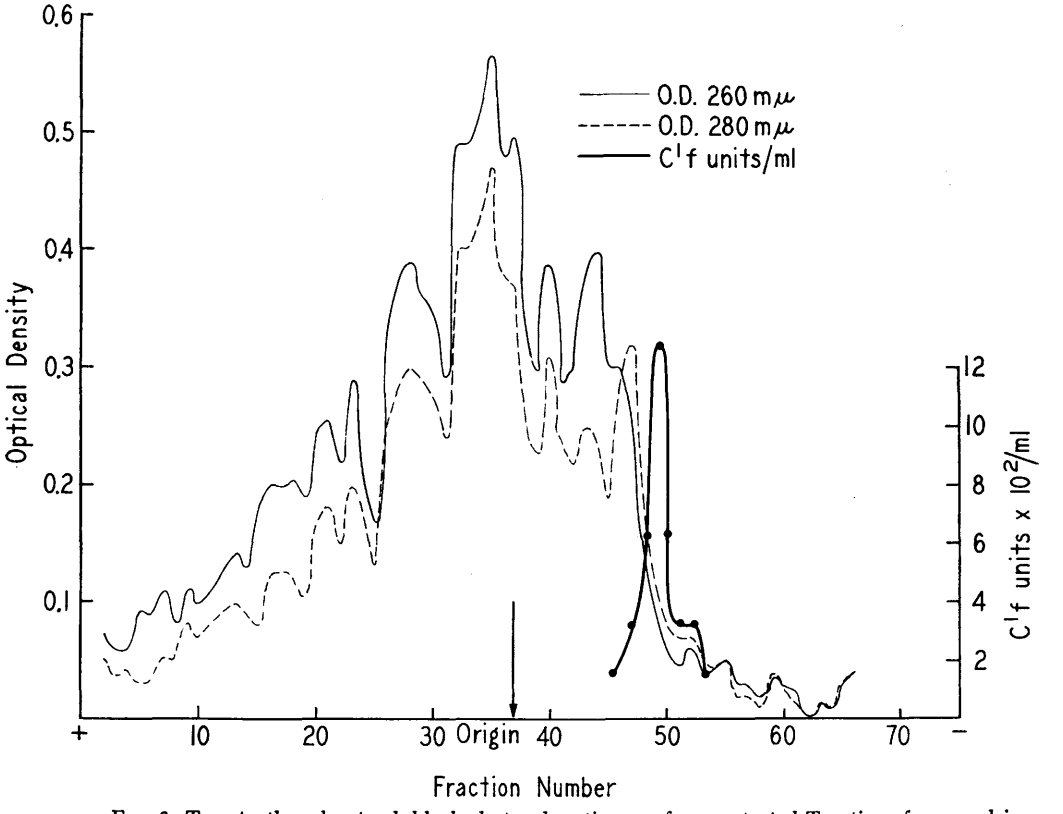

FIG. 2. Twenty-three-hr starch block electrophoretic run of concentrated T antigen from nuclei.

ily at the cathodally advancing portion of the protein peak (Fig. 2), several runs were made for 46–48 hr in order to achieve further separation. This however did not result in either two peaks of CF activity or in further separation of T antigen from host material. Whether T antigen migrates to this extent because of its own properties or because of its close association with host-cell constituents is not clear. Results obtained with the lysates of the control cell line 0-853 show that the cathodally moving protein peak is not unique to the lysates of the transformed cell line 0-66.

The T antigen preparations from the nuclear fraction showed a higher specific activity as compared to the T antigen preparations from the whole cell lysate or the cytoplasm suggesting that the isolation of the nuclei by detergent treatment served as a step for purification of T antigen.

The T antigen found in 0-66 cells seemed to be found quite firmly within the nuclei. Various attempts to extract the T antigen from intact nuclei using different chemical solvents such as 0.5% Tween 20, 0.04% sodium lauryl sulfate, 0.5% deoxycholate, 0.02 *M* Versene or 0.5 *M* NaCl failed. Solubilization of the T antigen occurred only by mechanical means such as sonication.

Previous studies on isolation of adenovirus type 12 T antigen from whole cell lysates or nuclei (8–11) utilized primarily columns of hydroxylapatite and Sephadex or centrifugation. This study reports the separation on hydroxylapatite columns and by starch block electrophoresis of antigen from cytoplasmic and nuclear fractions as well as whole cell lysates obtained from an adenovirus type 12 transformed cell line. By the methods used, no differences in the cytoplasmic and nuclear T antigen were observed.

Summary. A baby hamster kidney cell line transformed *in vitro* by adenovirus type 12 produced high titer T antigen. Using different tumor-bearing hamster sera in the indirect

fluorescent antibody technic the resultant staining was either confined to the cytoplasm or was present also in the nucleus. Both the intranuclear T antigen and intracytoplasmic T antigen when isolated behaved similarly on hydroxylapatite columns and on starch block electrophoresis. On hydroxylapatite columns the majority of the recovered CF activity was eluted with 0.2 *M* salt. On starch block electrophoresis all of the recovered CF activity was present in one peak effecting a 10-fold increase in specific activity by a one-step procedure. Preparations with highest specific activity were obtained from fractions of nuclear preparations separated by starch block electrophoresis.

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