Proc. Natl. Acad. Sci. USA
Vol. 75, No. 3, pp. 1279–1283, March 1978
Biochemistry

Biological activity of purified simian virus 40 T antigen proteins

(adenovirus–simian virus 40 hybrids/microinjection/DNA replication/helper function)

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Communicated by J. D. Watson, January 9, 1978

ABSTRACT **Proteins related to simian virus 40 (SV40) T antigen were isolated from cells infected with adenovirus 2/SV40 hybrids Ad2⁺D2 and Ad2⁺ND1 dp2 as well as from a line of human cells (SV80) transformed by SV40. The 96,000- and 107,000-dalton proteins of SV80 and Ad2⁺D2, after injection into the cytoplasm of cultured cells, rapidly accumulate in the nuclei, where they remain antigenically reactive for at least 20 hr and trigger DNA synthesis in quiescent cells. By contrast, the 23,000-dalton protein coded by Ad2⁺ND1 dp2 does not stimulate cellular DNA synthesis. However, all three purified proteins are able to provide helper function for the growth of adenovirus 2 in monkey cells. Thus, purified SV40 T antigen and proteins that share sequences with it retain the ability to carry out at least two functions associated with the product of the A gene of SV40.**

The product of the A gene of simian virus 40 (SV40), a polypeptide with an apparent molecular weight of 96,000, plays a pivotal role in the biology of the virus (1–4). It has been implicated in the initiation of cellular (5) and viral (6) DNA replication, in the regulation of viral transcription (4, 7–9), and in the initiation and maintenance of the transformed (3, 10–13) state; it carries antigenic determinants that elicit, from the host animal, immunological responses directed against SV40-induced tumors (14–16). Finally, it is thought to provide a function that helps the growth of human adenoviruses in monkey cells (17–19). At least some of these effects seem to result from binding of the protein to specific sequences of viral DNA (20–22). However, the details of the mechanisms that are involved remain obscure, chiefly because reconstruction *in vitro* of eukaryotic DNA replication and transcription systems has not been successful.

To circumvent this difficulty, we have used microinjection to assay the biological activity of purified T antigen injected into individual cells in culture. Microinjection experiments were carried out with proteins isolated from three different sources and coded at least in part by the A gene of SV40:

1. SV80 cells, a line of human fibroblast cells stably transformed by SV40 (23), synthesize a 96,000-dalton protein that is phosphorylated and crossreacts specifically with sera from hamsters bearing tumors induced by SV40.

2. Ad2⁺D2 is a defective adenovirus/SV40 hybrid lacking adenovirus 2 (Ad2) sequences that map between coordinates 76 and 96 and contains an insertion of DNA encompassing the entire genome of SV40 except for those sequences between map positions 54 and 63 (24) (Fig. 1). Cells infected with Ad2⁺D2 produce large quantities of a 107,000-dalton phosphorylated protein that is specifically immunoprecipitated by anti-T serum, shares extensive structural homology with authentic SV40 T antigen, and consists of approximately 10,000 daltons of an Ad2 protein at its NH_2 terminus and approximately 90,000–100,000 daltons of the SV40 A gene protein at its COOH terminus. In view of recent findings concerning the little t and big T early gene products of SV40 (ref. 25; M. Sleigh, personal communication), the 107,000-dalton protein from Ad2⁺D2 may well be missing some SV40-coded amino acids. This 107,000-dalton protein, which will be referred to as the D2 hybrid protein, has been purified to near homogeneity and shown to bind in a sequential manner to tandem recognition sites at a sequence of 120 bases near or at the origin of SV40 DNA replication (22).

3. Ad2⁺ND1 dp2 is a nondefective hybrid virus that carries two insertions of SV40 sequences: one from positions 11 to 28 and another from 11 to 21 (Fig. 1). Late in infection, cells containing Ad2⁺ND1 dp2 produce large amounts of a 23,000-dalton protein that is the product from the righthand insertion of SV40. This small protein is coded partly by the fiber gene of Ad2 and partly by the A gene of SV40 (G. Fey, A. Bothwell, and J. B. Lewis, unpublished data). Although this 23,000-dalton fusion protein contains peptides in common with the COOH terminus of T antigen, it does not react with anti-T serum and has no detectable affinity for DNA. However, the 23,000-dalton T antigen-related protein is believed to contain helper function because Ad⁺ND1 dp2 grows efficiently in monkey cells whereas revertants of Ad2⁺ND1 dp2 that have lost the gene that codes for SV40 simultaneously lose the capacity to replicate in monkey cells (Fey et al., unpublished data).

MATERIALS AND METHODS

Cells. Monolayers of TC7 (a subline of CV1 monkey cells), SV80 (a line of human fibroblasts transformed by SV40), and HeLa cells were cultured in plastic dishes in Dulbecco's modification of Eagle's medium containing 5% (vol/vol) fetal bovine serum and streptomycin and penicillin at 100 μg/ml. For microinjection, cells were grown on glass slides (1 × 5 cm) imprinted with 1-mm^2 grids.

Viruses. Plaque-purified hybrid viruses Ad2⁺D2 and Ad2⁺ND1 dp2 were propagated in monolayers of CV1 monkey cells as described (24).

Purification of T Antigen and Related Proteins. The 96,000-dalton and 107,000-dalton T antigen proteins were purified from SV80 cells and HeLa cells infected with Ad2⁺D2, respectively, as described (22). The 23,000-dalton protein from Ad2⁺ND1 dp2 was isolated by a similar procedure except that the cytoplasmic extract from infected cells was first fractionated by polyethylene glycol/dextran phase partition according to the procedure of Babinet (26). The Ad2⁺ND1 dp2 protein was found in the low-salt (0.1 M NaCl) polyethylene glycol fraction and was recovered by ammonium sulfate precipitation (60%

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Abbreviation: SV40, simian virus 40.

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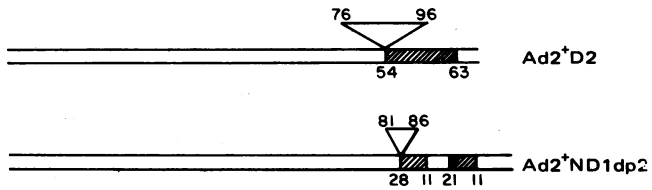

FIG. 1. The genome structure of Ad2+D2 and Ad2+ND1 dp2. The triangle represents the deleted portions of adenovirus 2 DNA and the hatched area, the inserted SV40 sequences. Numbers above the triangle indicate the coordinates of the Ad2 deletion; those below the hatched area show the coordinates of the SV40 insertion. The genome structure of Ad2+D2 was reproduced from Hassell *et al.* (24) and the structure of Ad2+ND1 dp2 was based on the data from E. Lukanidin (personal communication).

saturation). The 23,000-dalton protein was further purified by chromatography through Ultragel A44 and DEAE-Sephadex A-25 (22). At every stage of purification, the T antigen proteins were assayed by complement fixation, antibody precipitation followed by sodium dodecyl sulfate/polyacrylamide gel analysis, or, as in the case of the Ad2+ND1 dp2 polypeptide, by direct sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The purified polypeptides were concentrated to 1–2 mg/ml by DEAE-cellulose chromatography as described (22) and, in some cases, the proteins were further concentrated by dialysis against Ficoll.

Microinjection. Delivery of purified antigen into cells was by means of a glass capillary drawn out to a tip 0.5–1.0 μm in diameter. The capillaries were treated with a dilute solution (5%) of HF for 1 sec and subsequently washed with water and ethanol (100%) as described (5) except that the capillaries were not treated with tetrahydrofuran and dimethyldichlorosilane. The injections were visually monitored by phase-contrast microscopy, and micromanipulation was carried out with the aid of a Leitz micromanipulator.

Assay for Stimulation of DNA Synthesis. The T antigen proteins purified from various sources were injected into confluent monolayers of cells that had been incubated in medium containing <1% serum, such that >90% of the cells became quiescent. After injection, cells were incubated in medium containing [^{3}H]thymidine, 0.1 μCi/ml. Sixteen to 18 hr after injection, the cells were fixed in acetone/methanol, 1:1 (vol/vol) at −20°. Those cells that contained injected material were visualized by indirect immunofluorescence using sera from hamsters bearing tumors induced by SV40 and commercial rabbit anti-hamster gamma globulin coupled to fluorescein. Injected cells that had incorporated [^{3}H]thymidine were assayed by dipping the glass slide containing fixed cells into emulsion and developing the film after 2–4 days of exposure.

Helper Function Assay. Confluent monolayers of TC7 monkey cells that had been infected for 24 hr with adenovirus 2 (50–100 plaque-forming units/ml) were injected with T antigen proteins from various sources as described above. Twenty hours after injection, injected cells in a 1-mm^2 field were harvested by micro-aspiration through a capillary with a 2 to 4 μm aperture (the volume of cell suspension recovered by this procedure is 0.1 ± 0.05 ml). The recovered cells were diluted 1:10 with phosphate-buffered saline, freeze-thawed three times, and sonicated twice for 1 min in a Raytheon model DF101 sonicator. Aliquots (0.1 ml) of these lysates were subsequently titered directly on HeLa cells as described (27, 28). Duplicate values were obtained for each titration point, and three dilutions of the virus lysates were used. In an alternate assay for helper activity of purified T antigen proteins, TC7 cells infected with adenovirus 2 and injected with protein were fixed in acetone/methanol as described above and stained with rabbit anti-fiber gamma globulin and fluorescein-conjugated goat anti-rabbit gamma globulin.

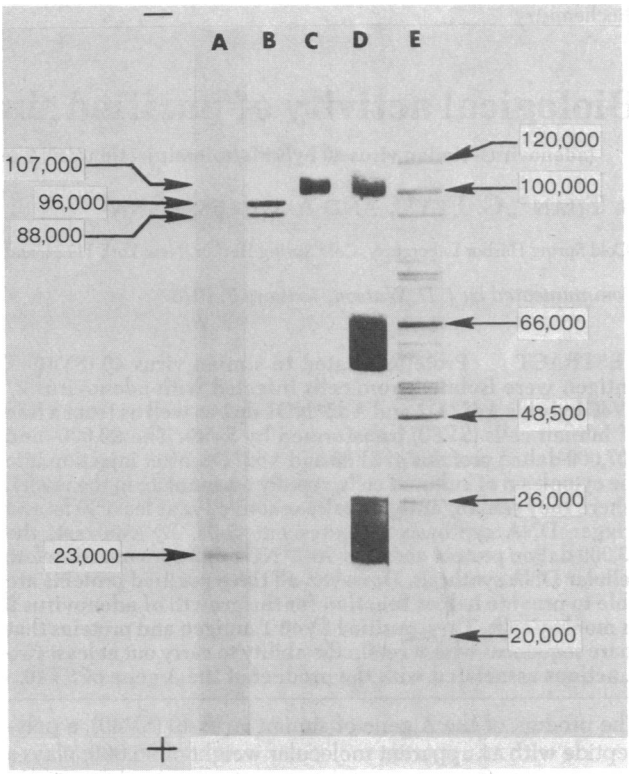

FIG. 2. Purified T antigen proteins. Electrophoresis of purified T antigen proteins (2–8 μg) on a 25-cm slab gel containing sodium dodecyl sulfate in Tris-glycine buffer and a 7–15% gradient of acrylamide (31). Lanes: A, purified 23,000-dalton protein from cells infected with Ad2+ND1 dp2; B, purified 96,000- and 88,000-dalton T antigen from SV80 cells (the 88,000-dalton protein is a proteolytic degradation product of the 96,000-dalton T antigen); C, purified D2 hybrid protein; D, antibody precipitate of purified D2 hybrid protein (the heavy and light chains of the gamma globulin are seen migrating as two very heavily stained bands at 50,000 and 25,000 daltons); E, proteins from a total extract of HeLa cells infected with Ad2+D2. In all cases, the protein bands in the gel have been visualized by staining with Coomassie brilliant blue.

RESULTS AND DISCUSSION

Although proteins related to SV40 T antigen have been isolated since 1967 (20, 29, 30) it has previously not been possible to determine whether such purified proteins retain any biological activity other than immunological reactivity and their ability to bind DNA. In an attempt to devise a suitable biological assay, a new approach was taken which required no prior knowledge of how T antigen might operate and involved quantitating the effects of injecting purified T antigen and proteins related to it into individual cells in culture.

Stability and Intracellular Location of the D2 Hybrid Protein after Injection into Cells. In a preliminary characterization of the D2 hybrid protein, we determined how long the antigen persists and where it accumulates after injection into cells. The 107,000-dalton protein isolated from cells infected with Ad2+D2 was purified to near homogeneity (>95% pure) by gel filtration and chromatography in DEAE-Sephadex and phosphocellulose (Fig. 2) (22). Before injection the purified D2 hybrid protein was concentrated by chromatography on

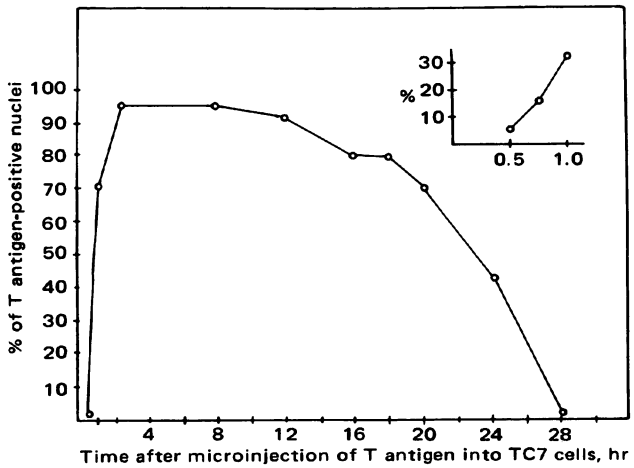

FIG. 3. Intracellular localization and antigenic stability of injected D2 hybrid proteins. Approximately 0.2–1.0 × 10^6 molecules of purified D2 hybrid protein were injected into the cytoplasm of TC7 monkey cells. At various times after injection, the cells were fixed in acetone/methanol, 1:1 (vol/vol) at −20° and allowed to react (at 37°) with 20 μl of hamster anti-T gamma globulin (diluted 1:100) for 45 min. Nuclei containing antigenically active D2 hybrid protein were subsequently visualized by staining the cells with 20 μl of fluorescein-conjugated rabbit anti-hamster gamma globulin (diluted 1:20). (*Inset*) Kinetics of T antigen accumulation in the nucleus at short times after injection.

DEAE-cellulose to 1–2 mg/ml and shown to be antigenically active. Thus, the highly concentrated preparation of D2 hybrid protein was quantitatively immunoprecipitable by sera raised in hamsters bearing tumors induced by SV40 (Fig. 2) and 1 unit of guinea pig complement was fully inhibited by 2.5 nl of the antigen in a standard microcomplement fixation assay.

Because SV40 T antigen is known to accumulate in the nucleus, the D2 hybrid protein injected into the cytoplasm of cells was expected to migrate into and accumulate in the nucleus. Approximately 10^6 molecules of D2 hybrid proteins in 10–50 fl (femtoliters, 10^{-12} liter) were delivered into the cytoplasm of the TC7 line of monkey cells by the technique of microinjection using a microcapillary with a 0.5-μm tip (5). At various times after injection, glass slides containing injected cells were removed from the culture dish and the number of cells antigenically reactive with anti-T serum was determined by the method of indirect immunofluorescence. In general, 100 cells in a field of 600–800 cells were injected with the purified protein. In the first hour after injection a large proportion of the injected cells exhibited diffuse cytoplasmic staining and 10–20% of the cells displayed nuclear fluorescence (Fig. 3). Three hours after injection, 90% of the injected cells displayed a characteristic nuclear fluorescence when challenged with anti-T serum. Most of the cells that had been injected retained antigenically active protein for 20 hr. Thereafter, the reactivity of the nuclear D2 hybrid protein decreased rapidly and only 20% of the cells retained antigenically active D2 hybrid protein at 26 hr after injection. Similar results were obtained with T antigen isolated from SV80 cells (data not shown). Thus, it appears that purified T antigen, when injected into the cytoplasm of cells, migrates across the nuclear membrane and remains antigenically stable in the nucleus for up to 20 hr under physiological conditions.

Purified T Antigen and Related Proteins Stimulate Cellular DNA Synthesis. The ability of T antigen to stimulate DNA synthesis was determined by injecting the purified proteins into quiescent cells and measuring the uptake of [^{3}H]-

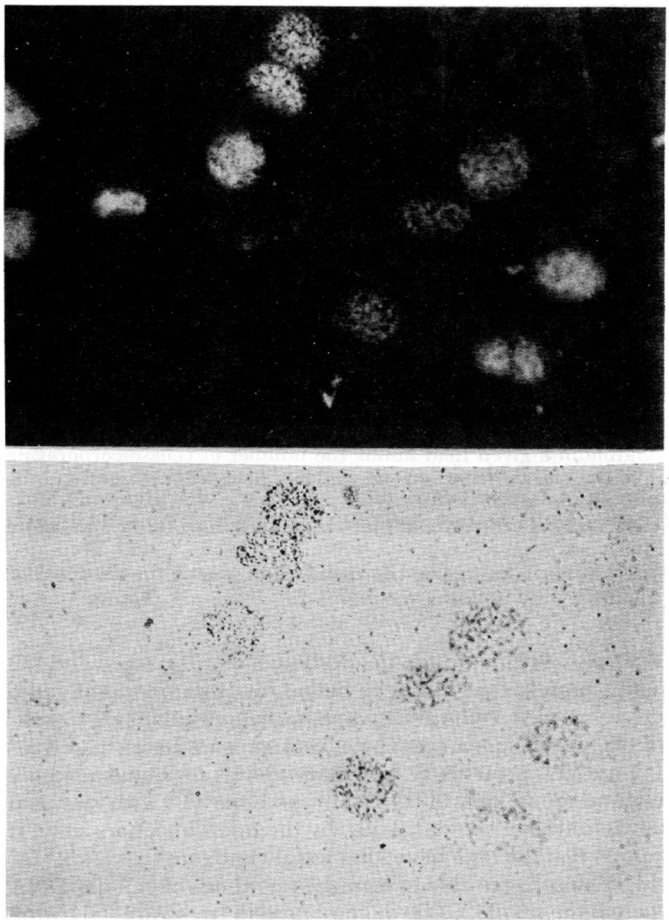

FIG. 4. Incorporation of [^{3}H]thymidine in cells displaying positive T-antigen immunofluorescence. Cells (primary mouse kidney) in a confluent monolayer kept in medium supplemented with <1% calf serum for at least 24 hr were injected with D2 hybrid protein. After injection, the cells were incubated for 18–20 hr at 37° in medium containing [^{3}H]thymidine at 0.1 μCi/ml. In addition to staining by indirect immunofluorescence (*Upper*) with anti-T serum, the fixed cells were overlayed with a thin film of photographic emulsion (5) and the incorporation of [^{3}H]thymidine was visualized by autoradiography (*Lower*).

thymidine. To reduce the number of cells synthesizing DNA, confluent monolayers of TC7 monkey cells were incubated for at least 24 hr in medium containing <1% serum. Under these conditions, >90% of the cells became quiescent. In a typical experiment, purified antigen was injected into either the nucleus or cytoplasm of 100–200 cells and the injected cells were subsequently incubated for 18 hr in medium containing [^{3}H]-thymidine. Cells that contained T antigen proteins were later identified by indirect immunofluorescence (staining with anti-T serum) and cells that incorporated thymidine were identified by autoradiography (Fig. 4). Of 100 cells injected with purified D2 hybrid protein, 57 exhibited a strong nuclear fluorescence and, of these, 45 also incorporated [^{3}H]thymidine (Table 1). Similarly, 50% of the cells infected with T antigen from SV80 also displayed positive immunofluorescence when challenged with anti-T serum and >80% of these cells were stimulated to synthesize DNA. Cells injected with purified SV40 DNA form I (100 μg/ml) also displayed T-antigen immunofluorescence and were stimulated to synthesized DNA. By contrast, cells injected either with the 23,000-dalton fusion protein from Ad2$^+$ND1 dp2 or "mock T-antigen" isolated from cells infected with Ad2 displayed no nuclear fluorescence and did not in-

Table 1. Stimulation of cellular DNA synthesis by injection of purified T antigen

Source of T antigen	Cells injected	Cells T antigen-pos. (fluorescence)	Cells with [^{3}H]thymidine*	T antigen-pos. cells with [^{3}H]thymidine
Ad2$^+$D2	100	57	45	45
SV80	100	46	38	38
Ad2$^+$ND1 dp2	200	0	0	—
Ad2$^+$D2, boiled†	200	0	0	—
SV80, boiled†	100	0	0	—
Ad2‡	200	0	0	—

* Values represent the total number of cells incorporating [^{3}H]thymidine in a given field minus the number of cells incorporating [^{3}H]thymidine from an equivalent field of uninjected cells (<2.5%).

† As a negative control, heat-inactivated by boiling for 10 min before injection.

‡ Ad2 cell extract that had been purified in exactly the same way as the D2 hybrid protein from Ad2$^+$D2.

corporate [^{3}H]thymidine. In addition, SV80 T antigen and D2 hybrid protein that had been boiled for 10 min lost antigenic activity as well as the ability to stimulate cellular DNA replication. These experiments have been repeated with injections of T-antigen proteins into primary cultures of mouse kidney cells and mouse cells of the established line 3T3 with identical results (data not shown). Thus, by the microinjection assay, it appears that the high molecular weight antigens from Ad2$^+$D2 and SV80 cells are able to stimulate synthesis of cellular DNA whereas the small (23,000 daltons) protein from Ad2$^+$ND1 dp2 is not.

Although a wealth of evidence has implicated the involvement of the SV40 T antigen in regulating cellular replication, the data presented here provide the strongest evidence, to date, that the A gene product of SV40 is directly involved in controlling cellular DNA synthesis. These results taken together with the recent finding that the D2 hybrid protein recognizes and binds tightly to a specific sequence of nucleotides near or at the origin of SV40 DNA replication suggest that a similar protein–DNA interaction might occur between T antigen and cellular DNA sequences that contain origins of replication.

Purified T Antigen Proteins Contain Helper Function Activity. The ability of T antigen to help adenovirus 2 grow in monkey cells was determined directly by injecting purified protein into TC7 monkey cells that had been infected with a moderate multiplicity of adenovirus 2 (50 plaque-forming units per cell) such that >95% of the cells contained viral DNA. In general, 100 cells were injected with purified antigen 24 hr after infection by adenovirus 2. Approximately 18–20 hr after injection, cells in a region containing the injected cells were removed from the glass slide by micromanipulation with an aspirator with a 2 to 4 μm tip. Cells recovered by this procedure were lysed by freeze-thawing followed by sonication, and the yield of adenovirus 2 lysate was determined by plaque formation on monolayers of HeLa cells. Eighty cells injected with D2 hybrid protein produced a total of 2700 infectious particles—a yield of approximately 35 plaque-forming units per injected cell (Table 2). Similarly, 90 cells injected with either the 23,000-dalton fusion protein from Ad2$^+$ND1 dp2 or T antigen from SV80 cells yielded a total of 900 plaque-forming units. Moreover, 23,000-dalton protein that had been treated with RNase also retained the ability to provide helper function

Table 2. Virus yield from TC7 cells infected with adenovirus 2 and injected with T antigen

Source of T antigen	Cells injected	Total plaques*	Plaques/ injected cell
Ad2$^+$D2	80	2750	35
SV80	90	875	9
Ad2$^+$ND1 dp2	90	900	10
H71	150	240	1.5
Ad2$^+$ND1 dp2, boiled	90	150	1.6
Ad2$^+$D2, boiled	100	80	0.8
SV80, boiled	90	50	0.5
Background	—	100	—
SV40 DNA I	64	2700	40

* The virus yield represents the total number of plaque-forming units recovered from one field of injected cells containing approximately 600–800 cells, of which 100–200 had been injected with antigen.

and enhance the growth of Ad2 in monkey cells (data not shown). By contrast, a protein related to T antigen isolated from a mutant adenovirus/SV40 hybrid (H71) lacking helper function (19) was incapable of enhancing the growth of adenovirus 2 in monkey cells. Similarly, cells not injected or injected with heat-inactivated antigen produced only background levels of adenovirus 2 virus.

As an independent test of helper function, injected cells were also assayed by indirect immunofluorescence with antibody directed against fiber, an adenovirus 2 viral capsid protein whose synthesis in monkey cells is inhibited in the absence of helper activity (19). Greater than 70% of the monkey cells infected with adenovirus 2 and injected with D2 hybrid protein, SV80 T antigen, or the 23,000-dalton protein of Ad2$^+$ND1 dp2 displayed bright nuclear fluorescence when challenged with anti-fiber serum (Table 3). Less than 1 cell in 600 exhibited any fluorescence when not injected with T antigen protein or injected with protein isolated from cells infected with H71. Taken together, these results provide strong support for the idea that all three purified T antigen-related proteins are able to provide helper function as determined by microinjection.

Although genetic evidence had strongly suggested that no more than 50–60 amino acids from the COOH terminus of the SV40 A gene protein are required for helper activity (ref. 19; E. Lukanidin, personal communication), the microinjection data reported here constitute direct evidence that the purified antigen can, by itself, promote the growth of adenovirus 2 in monkey cells.

Table 3. Fiber immunofluorescence after injection of T antigen into TC7 cells infected with adenovirus 2

Source of T antigen	% of injected cells producing fiber*
Ad2$^+$D2	85
SV80	80
Ad2$^+$ND1 dp2†	70
H71†	<1
Background	<1

* A total of 100 cells were injected per 1-mm^2 field containing 600–800 cells. The percentage of cells containing fiber immunofluorescence represents the average value from two independent sets of injections in separate fields.

† Anti-fiber serum does not crossreact strongly with purified antigen from Ad2$^+$ND1 dp2 and H71 even though these peptides may contain some amino acids from fiber.

We thank P. Reichel and E. Guhl for their excellent technical assistance. This project was facilitated by travel funds to R.T. and G.F. from the International Union Against Cancer. Part of this work was made possible by a grant from the Inter-Boro Leukemia Organization. This work was funded by a grant from the National Cancer Institute and by the Deutsche Forschungsgemeinschaft. R.T. is a Junior Fellow of the Harvard Society of Fellows.

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