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Two tumor antigens and their polypeptides in adenovirus type 12-infected and transformed cells

(indirect immunofluorescence/immunoprecipitation/transforming gene product)

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ABSTRACT A tumor (T) antigen, designated T antigen g, was visualized as fine fluorescent granules in nuclei of adenovirus type 12 (Ad12)-infected cells by immunofluorescence with sera from rats bearing HY cell tumors (H sera). HY cells are rat cells incompletely transformed by the *Acc* I-H endonuclease fragment (0–4.7 map units) of Ad12 DNA. The antigen is different from the usually described T antigen, designated T antigen f, which is visualized as fluorescent flecks or filaments in both nucleus and cytoplasm of Ad12-infected cells when tested with narrowly reacting T sera. Extracts of [^{35}S]methionine-labeled infected cells were immunoprecipitated with H sera, and the resultant precipitate was analyzed by the two-dimensional gel electrophoresis technique of O'Farrell. The autoradiogram showed the presence of a cluster of several polypeptides (M_r 35,000–40,000, pI 5.0–5.5) that was absent in extracts of mock-infected cells. A similar autoradiogram of infected cells analyzed with narrowly reacting T sera showed the presence of a small polypeptide (M_r 10,000, pI 6.4), that was absent in extracts of mock-infected cells. The results show that M_r 35,000–40,000 polypeptides are components of T antigen g and a M_r 10,000 polypeptide is a component of T antigen f. Ad12-transformed cells showed a similar result. T antigen g was present and T antigen f was absent in HY cells. Both T antigen g and T antigen f were present in CY cells, which are rat cells completely transformed by the *Eco*RI-C endonuclease fragment (0–16 map units) of Ad12 DNA. The possible functions of these proteins are discussed.

T (tumor) antigen in adenovirus (Ad) 12 tumors was found by complement fixation tests with sera from hamsters bearing Ad12 tumors (1). The antigen was subsequently visualized by immunofluorescence as unique flecks or filaments in both the cytoplasm and nucleus of Ad12-infected or transformed cells (2, 3). Since then, T antigen has been used as a marker for Ad12-infected or transformed cells. Recently, evidence has accumulated showing that the transforming genes of human adenovirus are in only a small region of the left end in the viral genome (4–9). T antigen is considered to be the product of the adenovirus transforming genes as analyzed in Ad2- (10–12), Ad5- (13, 14), or Ad12-infected cells (15, 16).

The transforming genes of the highly oncogenic Ad12 reside in the restriction endonuclease *Eco*RI-C fragment (0–16 map units) and *Hin*dIII-G fragment (0–6.8 map units) of Ad12 DNA. Rat cells transformed by the *Eco*RI-C fragment (CY cells), and by the *Hin*dIII-G fragment (GY cells), were established (7, 8). T antigen was detected in both CY and GY cells by immunofluorescence or complement fixation tests. Tumors were induced efficiently in rats after transplantation of CY and GY cells and antibody to T antigen was detected in sera from these tumor-bearing rats (8).

Rat cells were transformed by the smaller *Acc* I-H fragment (0–4.7 map units) of Ad12 DNA, and the cells, designated as HY cells, were established (17). [In the previous paper (17), "*Bpa* I" was used instead of "*Acc* I." *Bpa* I was corrected to *Acc* I after the first description of the enzyme.] However, HY cells showed properties intermediate between untransformed and transformed cells. The cells were T-antigen negative by immunofluorescence and complement fixation tests. Tumors were formed inefficiently in rats after transplantation of HY cells, and antibody to T antigen was not detected in sera from rats bearing HY cell tumors (17). With the background described above, we once again tried to detect antibody in sera from rats bearing HY cell tumors. We used indirect immunofluorescence, in which Ad12-infected cells were stained with rat sera, followed by dye-conjugated anti-rat IgG. After examination of many rat sera, we found that a small number of sera stained the antigen in Ad12-infected cells. However, the morphology of the antigen (tentatively named T antigen g) was different from that of T antigen (tentatively named T antigen f) reported until now (2, 3). It was fluorescent granules confined to the nucleus, in contrast to fluorescent flecks or filaments in the nucleus and cytoplasm. We concluded that the new T antigen must be different from the classic T antigen of Ad12.

On the basis of this finding, we tried to identify T antigen polypeptides with the use of sera from hamsters bearing tumors or sera from rats bearing CY or HY cell tumors. The results show that at least two polypeptides (M_r 35,000–40,000 and 10,000) are involved in transformation of cells by Ad12.

EXPERIMENTAL PROCEDURE

Cells. Human KB cells and secondary cultures of human embryo kidney (HEK) cells were used for propagation and assay of Ad12. The following untransformed and transformed cells were used. A rat cell line, 3Y1, was established from a Fischer rat embryo (18). WY3 cells were 3Y1 cells transformed by whole Ad12 DNA (7). CY1 cells were 3Y1 cells transformed by the *Eco*RI-C fragment (0–16 map units) of Ad12 DNA (7). GY1 cells were 3Y1 cells transformed by the *Hin*dIII-G fragment (0–6.8 map units) of Ad12 DNA (8). HY1 to HY7 cells were 3Y1 cells incompletely transformed by the *Acc* I-H fragment (0–4.7 map units) of Ad12 DNA (17). Cha1 cells were hamster embryo cells transformed by the *Eco*RI-C fragment of Ad12 DNA. These transformed cells were cultured in low-calcium (0.1 mM Ca^{2+}) Eagle's minimal essential medium supplemented with 10% fetal calf serum.

Antisera. Narrowly reacting sera of tumor-bearing hamsters (nT sera) were obtained from hamsters bearing Cha1 cell tumors. The sera contained antibody only to T antigen. Broadly

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Abbreviations: T antigen, tumor antigen; Ad, adenovirus.

reacting sera of tumor-bearing hamsters (bT sera) were obtained from hamsters bearing tumors after injection of Ad12 into newborn hamsters and contained antibody to both T antigen and other viral early proteins such as single-strand DNA-binding protein (19). Sera from Fischer rats bearing CY1 cell tumors (C sera) were narrowly reacting. Sera from Fischer rats bearing HY cell tumors were screened for antibody-positive sera by indirect immunofluorescence described below. Three antibody-positive sera were selected from 60 sera and used as H sera. Properties of these sera are summarized in Table 1.

Immunofluorescence. HEK cells on a coverslip were infected with Ad12 at a multiplicity of 50 plaque-forming units per cell in the presence of cytosine arabinonucleoside (20 μg/ml). At 16 hr after infection, the coverslip was rinsed with phosphate-buffered saline, dried, treated with acetone at room temperature for 30 min, and stored at −20°C. For staining, the cells were allowed to react with either hamster or rat sera at room temperature for 2 hr, washed, and stained by either anti-hamster IgG or anti-rat IgG rabbit immunoglobulin conjugated with the fluorescent dye fluorescein isothiocyanate. Stained cells were examined under a fluorescence microscope (Standard 18, Zeiss).

Preparation of Extracts of the Infected and Transformed Cells. KB or HEK cells cultured in 90-mm plastic dishes were infected with Ad12 at 50 plaque-forming units per cell. For controls, cells in some dishes were mock-infected with medium only. After adsorption at 37°C for 2 hr, the cells were incubated in maintenance medium (Eagle's minimal essential medium supplemented with 2% fetal calf serum) containing cytosine arabinonucleoside (20 μg/ml) at 37°C. Twelve hours after infection, the cells were labeled with [^{35}S]methionine (265 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) at 50 μCi/ml in methionine-free Eagle's minimal essential medium for 3–4 hr. The cells were then washed with phosphate-buffered saline, harvested, collected by centrifugation, frozen quickly, and stored at −70 °C. After thawing, the cells were suspended in IM buffer (10 mM sodium phosphate, pH 7.2/0.15 M NaCl/1% Triton X-100/0.5% deoxycholate), sonicated for 0.5–1 min, and centrifuged at 30,000 rpm at 4°C for 30 min in a Beckman SW 50L rotor. The supernatant was used as the infected cell extract for immunoprecipitation. Transformed cells were cultured in low-calcium Eagle's minimal essential medium with 10% fetal calf serum and labeled with [^{35}S]methionine at 50 μCi/ml for 24 hr. The transformed cell extract was prepared in the same way.

Immunoprecipitation. A sample (0.2–0.3 ml) of the labeled cell extract was mixed with 10–50 μl of antiserum (either hamster or rat) and incubated at 4°C for 4 hr. Unlabeled extract of mock-infected cells was added to reduce the background. Four volumes of 10% suspension of formaldehyde-fixed *Staphylococcus aureus* [protein A on the surface of the bacteria (20, 21), prepared in the Laboratory of Biological Products of this institute] was added and the mixture was incubated at 4°C for 2 hr. The bacterial cells were then collected by centrifugation at 3000 rpm for 15 min and washed three times with IM buffer and finally with cold water. The bacterial cells were suspended in lysis buffer A [9 M urea/2% (vol/vol) Nonidet P-40/5% (vol/vol) 2-mercaptoethanol/2% Ampholine pH 3.5–10] (22) and incubated at 37°C for 2 hr to dissociate antigens from bacterial cells. After removal of bacterial cells by centrifugation, the supernatant was used in two-dimensional electrophoresis.

Two-Dimensional Gel Electrophoresis. This analysis was carried out as described by O'Farrell (22). The immunoprecipitated sample was dissolved in 25 μl of lysis buffer A and applied to the prefocused Ampholine gel. The gel was subjected to electrophoresis at 350 V for 12 hr. The focused gel was placed in 5 ml of sodium dodecyl sulfate sample buffer O [10% (vol/vol) glycerol/5% (vol/vol) 2-mercaptoethanol/2.3% sodium dodecyl sulfate/62.5 mM Tris·HCl, pH 6.8] for 90 min at room temperature. The second-dimensional electrophoresis was carried out in a vertical slab gel (0.8 mm thick, 13% polyacrylamide) as described by Laemmli (23). The isoelectric focused gel was fixed with 1% agarose on the top of the stacking gel. After electrophoresis, the gel was stained with Coomassie brilliant blue and immersed in 20% (wt/wt) 2,5-diphenyloxazole as described by Bonner and Laskey (24). The gel was dried under reduced pressure and autoradiographed for about 7 days.

RESULTS

Immunofluorescence

Ad12-infected cells were stained with H and nT sera. When the cells were stained with H sera, fine fluorescent granules confined to the nucleus were observed (Fig. 1*A*). In contrast, fluorescent flecks and filaments scattered in cytoplasm and nucleus were observed in cells stained with nT sera (Fig. 1*B*). Staining of cells with C sera resulted in a staining pattern similar to that observed with nT sera. Because the morphology and distribution of fluorescence were different between cells stained with H and nT sera, fluorescent granules stained with H sera have been tentatively designated as T antigen g (granules) and fluorescent flecks or filaments stained with nT sera as T antigen f (flecks). Ad12-infected cells stained with bT sera showed T antigen f as well as fluorescent dots and granules in the nucleus, which may be Ad12 early proteins other than T antigen, such as DNA-binding protein (25).

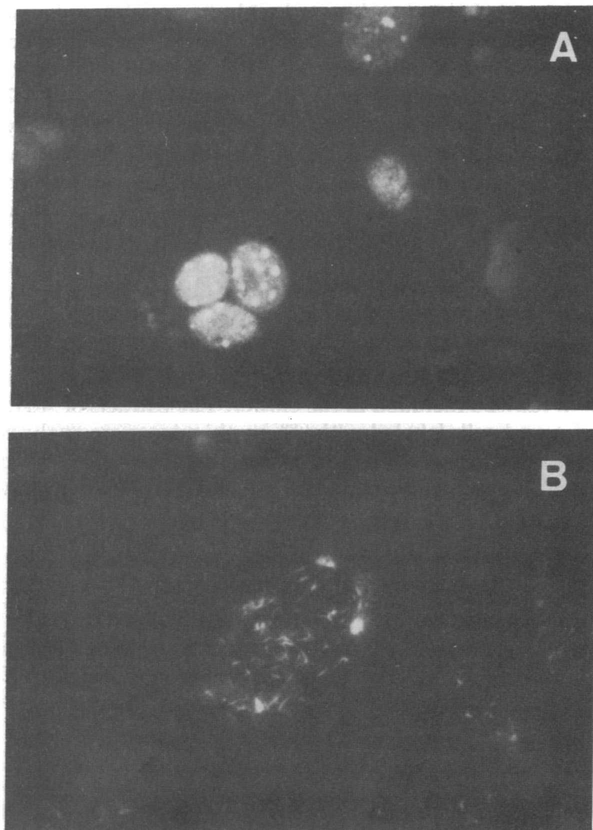

FIG. 1. Immunofluorescent T antigens g and f. Ad12-infected cells in the early phase stained with H (*A*) and nT (*B*) sera.

T antigen g was observed in the nucleus of CY and HY cells stained with H sera, but the fluorescence was far less intense than that seen in Ad12-infected cells. T antigen f was stained with nT sera in CY cells but not in HY cells. The results are summarized in Table 1 and show that two kinds of T antigens, g and f, are present in Ad12-infected and transformed cells.

T antigen f has been visualized by Pope and Rowe (2), but T antigen g has not been visualized until now. This may be due to the small amount of T antigen g in Ad12-transformed cells. Antibody to T antigen f is predominant in sera from animals bearing Ad12 tumors (nt, bT, C sera), making it difficult to detect T antigen g. T antigen f is absent in HY cells, and H sera contain antibody to T antigen g only. Antibody to T antigen g was detected in sera of rats bearing HY cell tumors for a period longer than 3 months. When hamsters or rats became tumor-bearing after virus injection or after transplantation of completely transformed cells, the growth of tumors was rapid and the animals became moribund, usually in 2 months. In sera from these animals (nT, bT, and C sera) antibody to T antigen f was detected but antibody to T antigen g was not, possibly due to the shorter period of the tumor-bearing state. The availability of HY cells and H sera enabled us to detect T antigen g.

Polypeptides analysis by immunoprecipitation

Extracts of Ad12-infected and [^{35}S]methionine-labeled cells were analyzed by immunoprecipitation and two-dimensional gel electrophoresis. A cluster of polypeptides (M_r 35,000–40,000, pI 5.0–5.5) was detected in the sample immunoprecipitated with H sera (Fig. 2*A*). The cluster (35,000–40,000) may be components of T antigen g, because this cluster was not observed with the use of sera devoid of antibody to T antigen g. A small polypeptide (M_r 10,000, pI 6.4) was detected in addition in the sample immunoprecipitated with H and nT sera (Fig. 2*B*). The polypeptide (10,000) may be a component of T antigen f, because it was not detected with the use of sera devoid of antibody to T antigen f. These specific polypeptides were not detected in mock-infected cells (Fig. 2*C*). With the use of bT sera, an additional small polypeptide (M_r 12,000, pI 5.7) (Fig. 3*A*, shown by a lower right arrow) and a cluster of polypeptides (M_r 45,000–60,000, pI 7.5–7.9) (Fig. 3*A*, shown by an upper arrow) were detected. These polypeptides were absent in the extract of mock-infected cells (Fig. 3*B*). The small polypeptide (12,000) may be one of the Ad12 early proteins, although it was absent in CY1 cell extract and may not be involved in transformation. The most probable candidate for the cluster (45,000–60,000) may be single-strand DNA-binding protein and its degraded products (19). However, it cannot be excluded that Ad12 early proteins other than DNA binding protein may be included in the cluster. The extracts of Ad12-transformed cells labeled with [^{35}S]methionine were analyzed by immunoprecipitation and two-dimensional gel electrophoresis. A small polypeptide (10,000) and a cluster of polypeptides (35,000–40,000) were detected in the extract of CY1 cells immunoprecipitated with nT and H sera (Fig. 4*A*). The same cluster of polypeptides (35,000–40,000) was immunoprecipitated by H sera in the HY cell extract (Fig. 4*B*). However, the small polypeptide (10,000) was not immunoprecipitated by nT sera in the HY cell extract. These observations agree with the immunofluorescent results, in which CY1 cells contain both T antigens f and g, whereas HY cells contain T antigen g only.

Polypeptides bigger than 10,000 or other than 35,000–40,000 were detected in Figs. 2, 3, and 4. Some of them may not be virus specific, because they were observed in mock-infected cells. However, we would like to reserve our conclusion until further work is done on them.

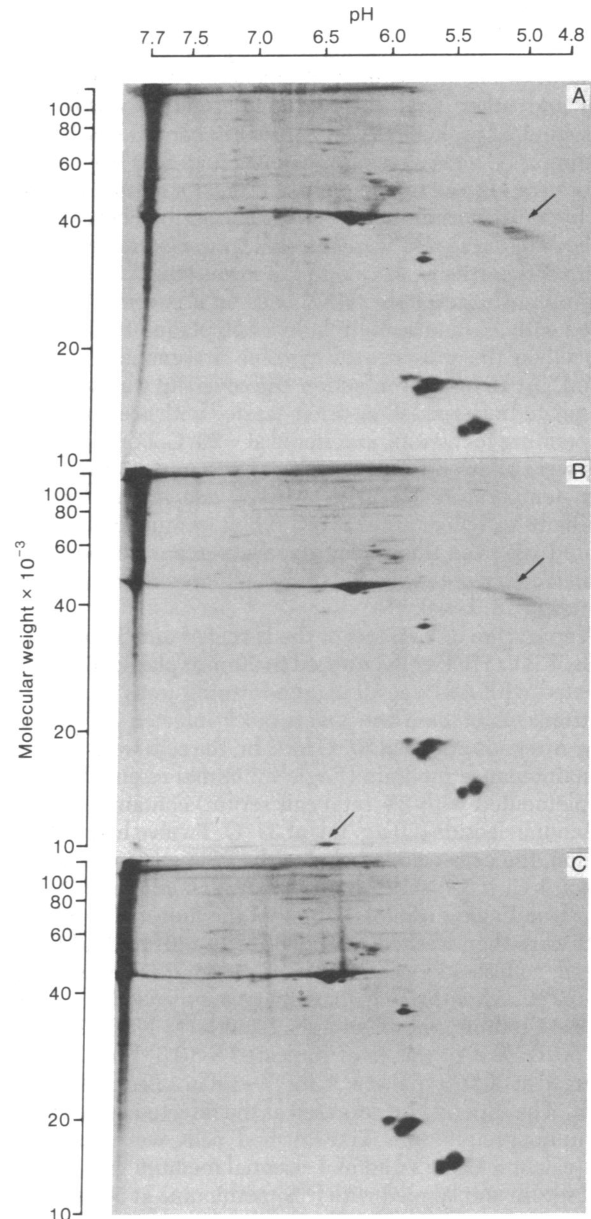

FIG. 2. Autoradiogram of two-dimensional gel electrophoresis of proteins immunoprecipitated from extracts of [^{35}S]methionine-labeled Ad12-infected cells. (*A*) Immunoprecipitated with H sera. (*B*) Immunoprecipitated with H and nT sera. (*C*) Mock-infected cell extract immunoprecipitated with H and nT sera. The infection and mock infection and subsequent operations were carried out simultaneously under the same conditions.

Table 1. Properties of antisera and detection of T antigens f and g

Antisera	Ad12-infected cells	Ad12-transformed cells CY1	GY1	HY1	HY2
nT	f	f	f	–	–
bT	f, EP	f	f	–	–
C	f	f	f	–	–
H	g	g*	g*	g*	g*

Results are given in terms of the antigens detected by the sera. EP, Ad12 early proteins. A – means that no antigen is detected.

* The fluorescence of T antigen g is far less intense in Ad12-transformed cells than in Ad12-infected cells.

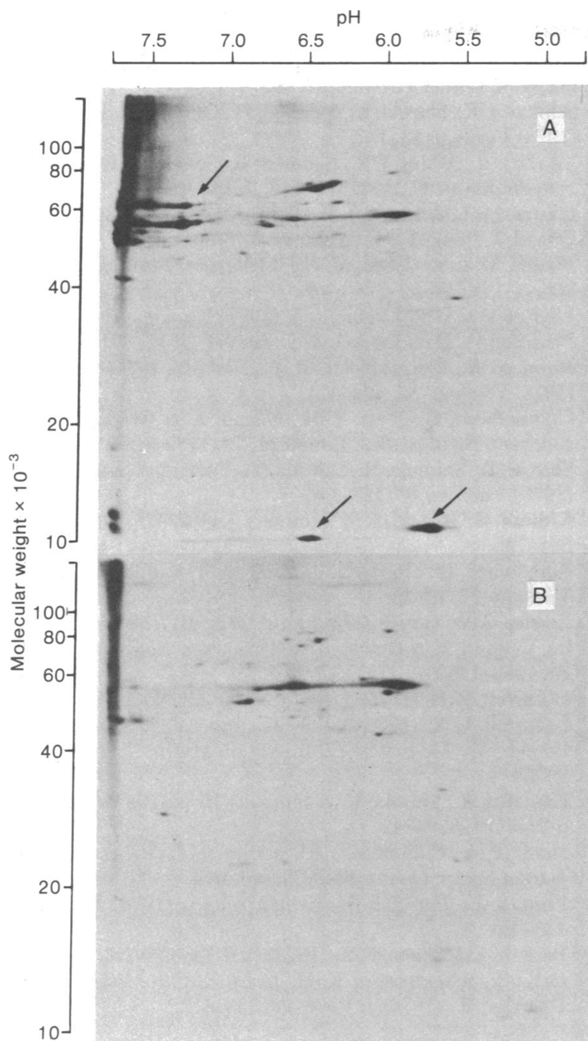

FIG. 3. Autoradiogram of two-dimensional gel electrophoresis of proteins immunoprecipitated with bT sera from extracts of [^{35}S]-methionine-labeled Ad12-infected cells (*A*) and mock-infected cells (*B*).

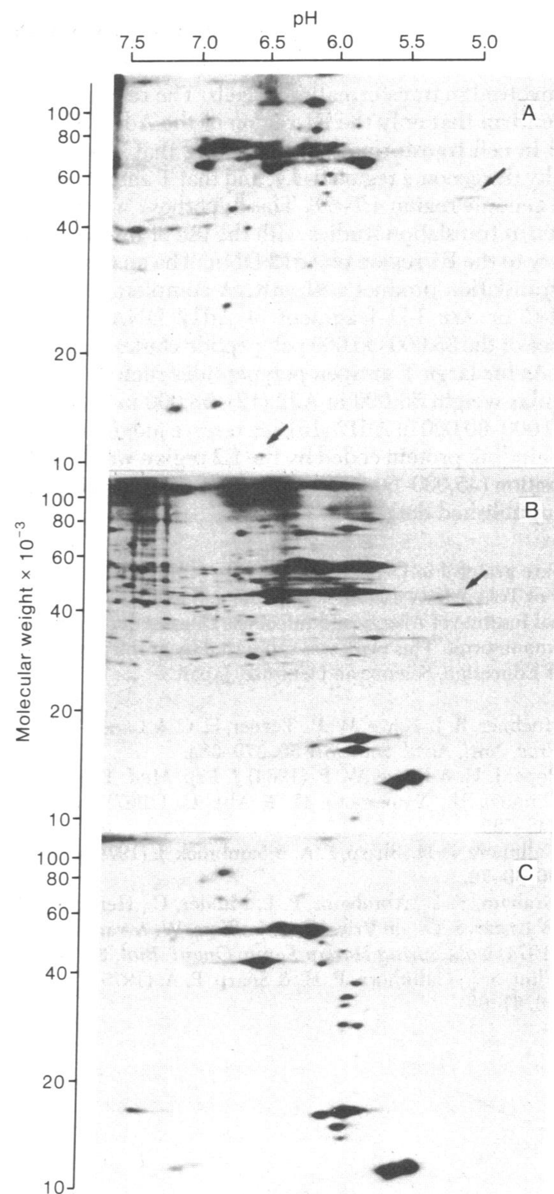

FIG. 4. Autoradiogram of two-dimensional gel electrophoresis of proteins immunoprecipitated with nT and H sera from extracts of [^{35}S]methionine-labeled transformed cells. (*A*) CY1 cell extract. (*B*) HY2 cell extract. (*C*) Untransformed 3Y1 cell extract.

DISCUSSION

The transforming genes of Ad12 reside on a small region of the left end of the viral genome, such as the *Eco*RI-C (0–16 map units) or the *Hin*dIII-G fragment (0–6.8 map units) of Ad12 DNA. CY cells, rat cells transformed by the *Eco*RI-C fragment and GY cells, rat cells transformed by the *Hin*dIII-G fragment, were established (7, 8). Both CY and GY cells are completely transformed, T antigen-positive, cells, which form tumors efficiently in rats after transplantation. Antibody to T antigens is detected in sera from rats bearing CY or GY cell tumors (8).

Infection of rat cells with a small *Acc* I-H fragment (0–4.7 map units) resulted in the formation of foci of transformed cells. These cells were established in culture and designated as HY cells. However, HY cells are incompletely transformed, T antigen-negative, cells, which form tumors inefficiently when transplanted in rats. Antibody to T antigens was not detected in sera from rats bearing HY cell tumors (17). Most of sera from rats bearing HY cell tumors were negative in staining the antigen in Ad12-infected cells, but some sera gave positive reactions. These were selected and used as H sera. The morphology of the antigen stained by H sera was different from that of T antigen heretofore reported (2, 3). Fluorescent granules confined to the nucleus were stained by H sera, whereas the reaction with nT or C sera showed fluorescent flecks or filaments scattered in the cytoplasm and nucleus of Ad12-infected cells (Fig. 1). This observation suggested that the two antigens were different. The antigens were tentatively designated as T antigens g (granules) and f (flecks). The suggestion was confirmed by immunoprecipitation and two-dimensional gel electrophoresis. H sera precipitated a cluster of polypeptides (M_r 35,000–40,000, pI 5.0–5.5), whereas nT or C sera precipitated a small polypeptide (M_r 10,000, pI 6.4). The cluster of polypeptides may be components of T antigen g and the small polypeptide a component of T antigen f. Two kinds of Ad12 T antigens were thus confirmed by immunoprecipitation.

CY or GY cells are completely transformed, whereas HY cells are incompletely transformed (17). Concomitantly, CY cells contain both T antigen f and T antigen g, whereas HY cells have T antigen g only. It is, therefore, suggested that T antigen g is involved in initiation and T antigen f in maintenance or promotion of transformation. The regions of early proteins are

located on four regions (E1, E2, E3, and E4) of Ad2 and Ad5 genomes (26–28). Similar early regions of the Ad12 genome have been reported (29). Of these regions, only E1 is thought to be involved in transformation of cells. The results presented here confirm that only the E1 region of the Ad12 DNA is involved in cell transformation, suggesting that T antigen g is coded by the genome region 0–4.7, and that T antigen f is coded by the genome region 4.7–6.8. This hypothesis was confirmed by *in vitro* translation studies with the use of mRNA complementary to the E1 region of Ad12 DNA. The analysis of the *in vitro* translation product with mRNA complementary to the *Eco*RI-C or *Acc* I-H fragment of Ad12 DNA showed the presence of the 35,000–40,000 polypeptide cluster (unpublished data). As for large T antigen polypeptides such as those with molecular weight 53,000 in Ad2 (12), 58,000 in Ad5 (13, 14), and 50,000–60,000 in Ad12 (15), we reserve judgment, because DNA-binding protein coded by the E2 region was detected in the position (45,000–60,000) shown by the upper arrow in Fig. 3A (unpublished data).

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