*THE INCORPORATION OF SV40 GENETIC MATERIAL INTO ADENOVIRUS 7 AS MEASURED BY INTRANUCLEAR SYNTHESIS OF SV40 TUMOR ANTIGEN**

By F. Rapp, J. L. Melnick, J. S. Butel, and T. Kitahara

DEPARTMENT OF VIROLOGY AND EPIDEMIOLOGY,
BAYLOR UNIVERSITY COLLEGE OF MEDICINE, HOUSTON, TEXAS

Communicated by Robert J. Huebner, October 14, 1964

The surprising finding of Huebner *et al.*[1] that tumors developing in hamsters after inoculation with adenovirus 7 contain the complement-fixing cellular antigen[2] of SV40 transformed cells suggested that preparations of adenovirus 7 should be studied for ability to induce similar antigens in cells growing in culture. Recent observations using the immunofluorescence technique had confirmed that cells transformed by SV40 contain a new cellular antigen; this antigen appears to be synthesized in the nucleus and is present in all cells of cultures derived from SV40-transformed systems.[3, 4] These sensitive techniques have made it possible to detect the tumor antigens even when they are synthesized in relatively few cells in the culture. The same techniques have been used to demonstrate that the SV40 tumor antigens occur not only in virus-free transformed cells but are also synthesized in cells infected with SV40 prior to the appearance of the virus antigens.[5, 6]

This report will document the finding that preparations of adenovirus 7 grown in monkey cells and free of infectious SV40 can induce the synthesis of a new intranuclear antigen unrelated to adenovirus in a wide variety of cells. This antigen is similar to or identical with the tumor antigens induced by SV40 virus. These results suggest the incorporation and transmission of a portion of the SV40 genome by the unrelated adenovirus.

Materials and Methods.—Viruses: The L. L. strain of adenovirus 7 used in these studies, kindly made available by Wyeth Laboratories, was originally isolated and passed serially in primary rhesus kidney cells. Stocks of the virus were subsequently found to be contaminated with SV40 and were therefore treated with SV40 antiserum and passed twice in primary green monkey kidney (GMK) cells in the presence of the antiserum. The virus was then passed 4 times in the absence of antiserum in GMK cells; large volumes of each virus yield were tested and found to be free of infectious SV40. This virus stock was designated E46. A stock of virus consisting of several liters was prepared by passing E46 in GMK, and the harvest was designated SP2. Samples of lots E46 and SP2 were kindly supplied by Dr. B. A. Rubin of Wyeth Laboratories. Repeated attempts to isolate SV40 from E46 and SP2 have been unsuccessful. Complement fixation tests with SV40 antiserum have also failed to detect the presence of SV40 antigen. A fresh isolate of adenovirus 7 from a fatal human case[7] passed only in human embryonic kidney (HEK) cells was also employed.

Cells: The various cells utilized were grown in Eagle's basal medium fortified by varying concentrations of calf serum ranging from 2 to 10%; GMK cells were grown in Melnick's medium.

Immunofluorescence tests: The cells were trypsinized and grown on 15-mm cover glasses in 60-mm plastic Petri dishes in 5% CO_2 at 37°C. When monolayers had formed, 0.1 ml of virus inoculum was added per cover glass, adsorption allowed to occur for 1 hr at 37°C, and 5 ml of maintenance fluid added per Petri dish. The cultures were reincubated at 37°C in 5% CO_2 and harvested at the times stated in *Results*. At time of harvest, the cultures were rinsed with warm tris saline (pH 7.4), air-dried, fixed for 3 min in acetone at room temperature, air-dried, and reacted with the immunofluorescent reagents.

Tests for the presence of SV40-induced antigens were carried out using sera from hamsters bearing SV40-induced tumors followed by antihamster globulin labeled with fluorescein isothio-

cyanate. Detection of SV40 virus antigen was attempted using a labeled anti-SV40 monkey serum that had been prepared for a previous study.[8] All tests included uninoculated control cells that were allowed to react with positive hamster sera, and inoculated cells allowed to react with negative hamster sera.

Complement fixation tests: The tests were carried out as previously described.[6] Cells in 16-oz bottles were dislodged with a rubber policeman, suspended in 1-ml volumes, and disrupted by 2 cycles of rapid freezing and thawing or by treatment in a sonic oscillator. The suspensions were clarified by centrifugation at 2500 rpm for 10 min and the supernates were used as antigen. Uninfected cells were treated in the same manner to serve as controls. A semimicro complement fixation test was employed; reactions were carried out in plastic dishes. All tests included antigen, serum, complement, and cell controls. In addition, a standardized positive and negative serum was included with each test.

Results.—The immunofluorescent method employed revealed that both adenovirus 7 preparations E46 and SP2 were capable of inducing the SV40 tumor antigen in cells derived from a variety of species (Table 1). The cells included primary kidney cultures derived from the African green monkey (GMK), a stable line of cells derived from the same species (BSC-1), primary hamster embryo fibroblasts (HEF), rabbit kidney (RK) cells, and cells obtained from human embryonic kidneys (HEK). SV40 virus antigen was not present at the time the SV40 tumor antigen was detected. The ability of the adenovirus preparations to induce the SV40 tumor antigen appeared to be related to the replication of the adenovirus; thus, approximately 25 per cent of cells in GMK cultures contained the antigen, and cytopathic effects (CPE) due to adenovirus were pronounced. In most of the other cultures, only a rare cell was "induced" in the absence of adenovirus CPE. Human embryonic lung (HEL) cultures were negative, but it is possible that sampling of the cells was not extensive enough to detect the rare cell that may have synthesized the new cellular antigen. In addition to GMK cells, HEK cells also showed abundant adenovirus CPE and SV40 tumor antigen. As adenovirus CPE became widespread, the number of cells present and synthesizing the new antigen decreased.

Complement fixation tests confirmed these results (Table 2). Six and 10 days after the inoculation of GMK cells with SP2, SV40 tumor antigen was detected when the cell extracts were reacted with serum from SV40-tumor-bearing hamsters. Adenovirus antigens were also being synthesized at this time (Table 2). Cytopathic changes due to adenovirus became pronounced after the sixth day postinoculation, and 50 per cent of the cells were affected 10 days following inoculation of the cultures. At no time did the cell extracts react with anti-SV40 GMK serum. Tests carried out with the SV40-transformed but virus-free 2X-10 hamster cells yielded reactions only with the SV40 tumor serum (Table 2); this serum does not react with either SV40 or adenovirus 7 antigens (Table 2).

TABLE 1

INDUCTION OF SV40 TUMOR ANTIGEN BY PREPARATIONS OF ADENOVIRUS 7

Cells	Inoculum	Days postinoc.	Adeno CPE	Immunofluorescence: Anti-SV40 tumor	Immunofluorescence: Anti-SV40 virus
GMK	E46	3	Present	Common	None
HEF	E46	3	Absent	Rare	None
GMK	SP2	1, 2, 3	Present	Common	None
BSC-1	SP2	1	Absent	Rare	None
HEF	SP2	3	Absent	Rare	None
RK	SP2	7	Absent	Rare	None
HEK	SP2	2	Present	Common	None
HEL	SP2	3	Absent	None	None
GMK	Human ad 7	2, 3	Present	None	None

TABLE 2

DETECTION OF SV40 TUMOR ANTIGEN BY COMPLEMENT FIXATION FOLLOWING INOCULATION OF GREEN MONKEY CELLS BY ADENOVIRUS 7

Material	Days postinoc.	Adeno CPE	Complement Fixation Titers* of Antigens† vs. Anti-SV40 GMK serum, 1:40	SV40 tumor hamster serum 1:40	Antiadeno, human conval. 1:10
GMK + SP2	1	Absent	0	0	0
GMK + SP2	2	Absent	0	0	0
GMK + SP2	6	Absent	0	8	8
GMK + SP2	10	Present	0	8	32
2X-10 Cells			0	8	0
SV40 virus			32	0	0
Human adeno 7			0	0	64

* Numbers represent highest dilution of antigen yielding less than 50% hemolysis; 0 = no reaction with undiluted antigen.

† Antigens prepared from 16-oz bottle cultures and concentrated in 1-ml volumes; results similar when cells were disrupted by freezing and thawing or sonication.

Antiadenovirus 7 rabbit serum neutralized the ability of SP2 to induce the SV40 tumor antigen in GMK cells. Induction of the tumor antigen was not inhibited, however, when SP2 was exposed to (1) anti-SV40 monkey serum, (2) serum obtained from hamsters bearing tumors induced by SV40, or (3) serum from uninoculated hamsters. The anti-SV40 monkey serum used neutralized 500 plaque-forming units of SV40 when diluted 1:31,250. The serum from the hamsters bearing SV40-induced tumors is the one employed to detect SV40 tumor antigen in this study.

A preparation of adenovirus 7 that had been propagated exclusively in human tissue was unable to induce the SV40 tumor cell antigen in primary GMK cells (Tables 1 and 2). Serum obtained from hamsters not bearing SV40 tumors failed to react with any of the test systems employed.

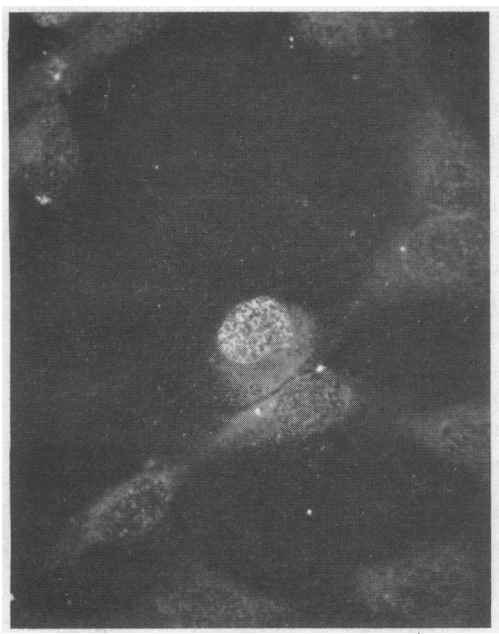

FIG. 1.—Immunofluorescent photomicrograph of GMK cells inoculated 24 hr previously with SP2 stock of adenovirus 7. Reacted with serum from hamster bearing SV40 tumor. Note single cell in center containing intranuclear antigen. ×425.

The SV40 tumor antigen was entirely localized within the nuclei. Generally, antigen detected one day postinoculation was present in single cells (Fig. 1). However, the antigen was readily detected in the daughter nuclei of dividing cells (Fig. 2), and with the passage of time following inoculation of the cultures, antigen was seen in the nuclei of increasingly larger clusters of cells (Figs. 2 and 3). The antigen appeared to be concentrated in particulate, coccal-shaped bodies varying in size (Figs. 1–3) and was seen along the nuclear membrane of some cells (Figs. 2 and 3).

Discussion.—The initial finding that hamsters inoculated with a papovavirus are resistant to challenge with cells transformed by the homologous virus[9–15] led to the detection of a new

complement-fixing antigen in cells transformed by SV40.[2] Immunofluorescent procedures have been used to detect this antigen both in the nuclei of cells transformed,[3, 4] as well as in cells infected,[5, 6] by SV40. It seems increasingly likely that the complement-fixing antigen and the antigens detected by immunofluorescence are similar or identical.[16] Detection of this antigen in cells no longer able to synthesize infectious SV40 virus[17] suggests that under certain conditions, part of the SV40 genome can integrate with cellular DNA. That the genetic information leading to synthesis of this new antigen derives from the virus is supported (1) by the observation that cells from different species transformed by the virus synthesize the same antigen, and (2) by detection of the new antigen in a variety of cells during early stages of infection with SV40.[5, 6] Normally, however, this antigen then decreases and the SV40 viral antigen appears.[6]

Detection of the same or similar antigens in cultures exposed to preparations of adenovirus 7 that once were contaminated with SV40[18] but now are free of infectious SV40 suggests that at least a portion of the SV40 genome is in some way carried by the adenovirus. It would appear to differ from genotypic heterozygosis since five passages of the virus, representing many more cycles of multiplication, did not result in segregation of the genomes. Only a portion of the SV40 genome seems to be involved since neither whole infectious SV40 is formed, nor is the virus protein antigen produced, as is the case of SV40 infection in the presence of fluorouracil.[8] Whether hybridization of the DNA of SV40 and that of adenovirus has occurred is not known at present, but the ob-

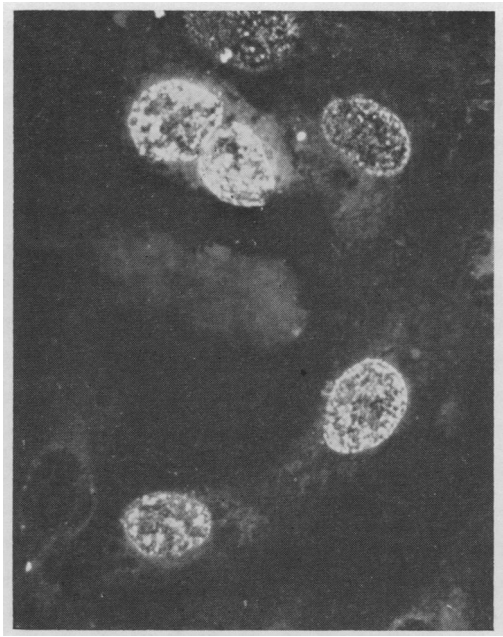

FIG. 2.—Immunofluorescent photomicrograph of GMK cells inoculated 72 hr previously with E46 stock of adenovirus 7. Reacted with serum from hamster bearing SV40 tumor. ×425.

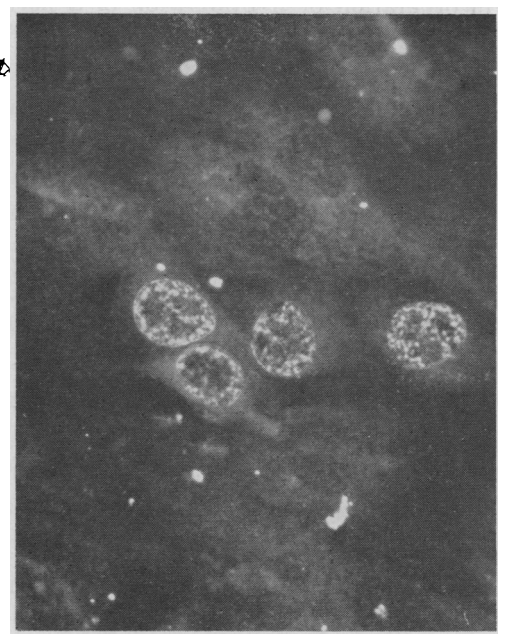

FIG. 3.—Immunofluorescent photomicrograph of HEF cells inoculated 72 hr previously with SP2 stock of adenovirus 7. Reacted with serum from hamster bearing SV40 tumor. ×425.

servations reported here and by Huebner *et al.*[1] suggest such a possibility.

The intranuclear localization of the new antigen described in this report and its particulate structure are not distinguishable from the antigen seen in cells transformed by SV40 or from that seen in the early stages of SV40 replication in monkey cells. However, SV40 viral antigen appears in larger particulates[8] and its distribution is somewhat different from that of the SV40-induced cellular antigen. The findings of the SV40 tumor antigen in the daughter nuclei of cells in division and in the nuclei of cells distributed in a focal pattern in the culture, as well as the observation that the number of antigen-containing cells in a focus increase with time, suggest strongly that information for the synthesis of the new antigen is transmitted to daughter cells during the mitotic cycle. No information is at hand about the role, if any, of the new antigen in starting or maintaining neoplasia, but it does furnish a handy marker of one activity of the SV40 papovavirus.

Summary.—A strain of adenovirus 7 once contaminated with SV40 but now free of the infectious papovavirus was found to contain the genetic information necessary to induce synthesis of a new intranuclear antigen. The antigen, which can be detected by immunofluorescence and complement fixation is immunologically identical with the tumor antigen induced by infectious SV40 in cells transformed by the papovavirus. The SV40 tumor antigen is synthesized in monkey, human, rabbit, and hamster cells following exposure of the cells to the virus; however, adenovirus penetration or replication seems required for synthesis of the new antigen to take place. Information for synthesis of the antigen is transmitted to daughter cells during mitosis. One explanation of the observations recorded might be incorporation or hybridization of a portion of a virus genome (SV40) with the genome of an unrelated virus (adenovirus).

* This investigation was aided by USPHS research grants CA-04600 from the National Cancer Institute, and AI 05382 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

[1] Huebner, R. J., R. M. Chanock, B. A. Rubin, and M. J. Casey, these PROCEEDINGS, **52**, 1333 (1964).

[2] Black, P. H., W. P. Rowe, H. C. Turner, and R. J. Huebner, these PROCEEDINGS, **50**, 1148 (1963).

[3] Pope, J. H., and W. P. Rowe, *J. Exptl. Med.*, **120**, 121 (1964).

[4] Rapp, F., J. S. Butel, and J. L. Melnick, *Proc. Soc. Exptl. Biol. Med.*, **116**, 1131 (1964).

[5] Sabin, A. B., and M. A. Koch, these PROCEEDINGS, **52**, 1131 (1964).

[6] Rapp, F., T. Kitahara, J. S. Butel, and J. L. Melnick, these PROCEEDINGS, **52**, 1138 (1964).

[7] Benyesh-Melnick, M., and H. S. Rosenberg, *J. Pediat.*, **64**, 83 (1964).

[8] Melnick, J. L., S. E. Stinebaugh, and F. Rapp, *J. Exptl. Med.*, **119**, 313 (1964).

[9] Sjögren, H. O., I. Hellström, and G. Klein, *Cancer Res.*, **21**, 329 (1961).

[10] Sachs, L., *Exptl. Cell Res.*, **24**, 185 (1961).

[11] Habel, K., *J. Exptl. Med.*, **115**, 181 (1962).

[12] Khera, K. S., A. Ashkenazi, F. Rapp, and J. L. Melnick, *J. Immunol.*, **91**, 604 (1963).

[13] Habel, K., and B. E. Eddy, *Proc. Soc. Exptl. Biol. Med.*, **113**, 1 (1963).

[14] Koch, M. A., and A. B. Sabin, *Proc. Soc. Exptl. Biol. Med.*, **113**, 4 (1963).

[15] Defendi, V., *Proc. Soc. Exptl. Biol. Med.*, **113**, 12 (1963).

[16] Kitahara, T., J. S. Butel, F. Rapp, and J. L. Melnick, *Nature*, in press.

[17] Melnick, J. L., K. S. Khera, and F. Rapp, *Virology*, **23**, 430 (1964).

[18] Yang, C., and J. L. Melnick, *Proc. Soc. Exptl. Biol. Med.*, **113**, 339 (1963).