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NUCLEIC ACID HOMOLOGY STUDIES OF ADENOVIRUS TYPE 7-SV40 INTERACTIONS

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Adenovirus infection of monkey kidney cells in tissue culture is abortive; the early T antigens are formed efficiently and typical adenovirus cytopathic changes occur, but only limited amounts of viral structural antigens, morphologic particles, and infectious virus are produced.[1-3] In cells infected with SV40 virus, this block is eliminated; the viral antigens and infectivity are formed with efficiency comparable to that in human cells.[1-3] This potentiation of adenovirus growth by SV40 genetic material is also observed with the adenovirus type 7 (Ad. 7) strain E46$^+$ which carries the SV40 information for production of SV40 T antigen.[4-10]

This report describes the application of the nucleic acid homology technique to determine whether SV40 enhancement acted at a step before or after Ad. 7 DNA synthesis, and whether SV40 DNA could be demonstrated in E46$^+$.

Materials and Methods.—Tissue cultures: Primary monolayer cultures of African green monkey kidney (AGMK) and human embryonic kidney (HEK) cells were obtained from the Division of Biologic Standards, NIH, and Microbiological Associates, Inc. They were maintained in Eagle's basal medium #2 (BME) containing 2% agammaglobulinic, heated (56°C for 30 min) calf serum. The medium contained glutamine, penicillin, and streptomycin. Cultures were incubated at 37°C in air.

Viruses: The origin of the E46$^+$ and E46$^-$ strains of Ad. 7 have been described.[9] E46$^-$ is the Ad. 7 line (obtained from E46$^+$) which does not induce SV40 T antigen, and which cannot be propagated serially in AGMK cells. SV40 virus, strain 777,[11] and the Pinckney strain[12] of Ad. 7 were used. Virus inocula for E46$^+$ and SV40 were prepared in AGMK cells, E46$^-$ in HEK cells, and Pinckney strain in KB cells. The Pinckney and E46$^+$ strains were known to be contaminated with the 22-mμ diameter "adenovirus-associated virus," [13-15] but the E46$^-$ strain was free of the contaminant.

Virus infection of AGMK cells: Two 32-oz flask cultures of AGMK (approximately 10^7 cells) were used for each assay. The maintenance medium was decanted and 10 ml of virus suspension was added. Input multiplicity was about 2 plaque-forming units (PFU) per cell for SV40, and approximately 0.7 PFU per cell for E46$^+$ and E46$^-$. Cultures were held at 37°C for 4 hr; the fluid was then decanted and the cell sheet washed twice with 10 ml of BME. The cultures were fed with 40 ml of maintenance medium. Only the earliest changes characteristic of SV40 cytopathogenic effect were seen 72 hr after infection. At the time of harvesting, the cells were scraped into the fluid medium and centrifuged at 500 *g* for 15 min. The cell pack was resuspended in 2 ml of BME; a portion was taken for virus titration, and the cells were stored frozen (−20°C) prior to DNA extraction.

Adenovirus titrations: The cell packs were frozen and thawed twice for titration of adenovirus yield. Titrations were done in tube cultures of HEK cells in medium containing 1% SV40 rabbit antiserum. Cultures were observed for 21 days.

Cellular DNA preparation: For extraction of DNA from uninfected and infected AGMK cells, the frozen cell pack was suspended in 2 ml of 0.05 *M* tris (hydroxymethyl) aminomethane pH 8.5, with 0.1 *M* ethylenediamine tetraacetate, and sodium dodecyl sulfate (SDS; final concentration, 0.2%) was added after thawing. Pronase (Calbiochem, 45 PUK/mg; final concentration, 50 μg/ml) was then added, and the mixture held at 37°C for 30 min. DNA was then purified by a modification of the method described by Marmur.[16-18] An absorbancy of 0.025 optical density units (at 260 mμ) per μg of DNA was assumed. The amount of DNA isolated was almost identical from singly and doubly infected cell pack suspensions at any given time after virus infection.

Virus DNA preparation: Adenoviruses were grown in KB cell suspension cultures. Frozen-thawed cell packs were treated with Genetron (Matheson Co., East Rutherford, N.J.) and concentrated by two cycles of centrifugation in rubidium chloride density gradients.[19] The purified virus preparation was exposed to papain (0.2 mg/ml, Worthington Biochemical Corp.) and 0.3 *M* mercaptoethanol at 37°C for 5 hr. SDS (final concentration, 1.25%) was added, and the incubation continued at 50°C for 30 min. The concentration of NaCl was raised to 1.25 *M* and the mixture left at 4°C for 10 hr. DNA was precipitated by addition of 2 vol of 99% ethanol and then centrifuged (100,000 *g*) through cesium chloride (density 1.45).[17]

SV40 virus was grown in AGMK cultures and purified by a method described by Black *et al.*,[21] and the DNA extracted in a manner similar to that described for the adenoviruses.

DNA detection and assay by hybridization with complementary RNA: The quantity of specific virus DNA present in the DNA extracted from infected AGMK cells or purified viruses was assayed by reacting it with specific complementary ribonucleic acid (RNA) and measuring the amount of DNA-RNA hybrid formation. The extent of hybrid formation is proportional to both the concentration of DNA and the concentration of homologous complementary RNA employed. A detailed description of this homology technique is reported elsewhere.[17] Briefly, radioactive complementary RNA was synthesized *in vitro* with the enzyme RNA polymerase and the four tritiated ribonucleoside triphosphates (Schwarz BioResearch, Orangeburg, N.Y.) using DNA from purified virus or from uninfected AGMK cells as primer. Equal aliquots of the radioactive RNA were mixed with nonradioactive DNA in a total volume of 0.1 ml of 0.3 *M* NaCl, 0.03 *M* Na citrate pH 6.7, and 0.05% SDS. The mixture was heated to 100°C for 15 min and incubated at 67°C for 8 or 16 hr. Nucleic acid hybrid formation was detected by a modification of the nitrocellulose membrane filter method described by Nygaard and Hall.[17, 22] Radioactivity (assayed in a liquid scintillation spectrometer) retained on the filter after dilution and filtration of the DNA and RNA mixture measured the extent of DNA-RNA hybrid formation. Nonspecific retention of complementary RNA was determined by incubation and processing of the radioactive RNA without DNA. These latter values were less than 0.3% of the input RNA and were subtracted from the values obtained with DNA. All assays of adenovirus DNA were done in triplicate and the rest in duplicate.

Cellular DNA preparations were tested against virus-complementary RNA and AGMK-complementary RNA, and the results expressed as the ratio ($\times$100) of the counts/min retained by the filters in the two assays. This calculation served as a correction for errors in estimating DNA concentration by ultraviolet light absorption, and differences in the ability of the DNA's (virus and host) present to form DNA-RNA hybrids. Presumably, agents such as deoxyribonuclease would affect equally the ability of virus and AGMK DNA's to form these duplexes.

In one experiment a reference line relating known adenovirus DNA concentration to the "adenovirus/AGMK" ratio was constructed to enable estimation of the quantity of adenovirus DNA present.

Results.—Purified virus preparations: The specificity of the homology technique and the nature of the genetic material in DNA from purified adenovirus and SV40 virus preparations were investigated by reacting Ad. 7 and SV40-complementary RNA with DNA from these viruses (Table 1). DNA from Ad. 7 strains Pinckney, E46$^+$, and E46$^-$ reacted strongly with Ad. 7 (Pinckney)-complementary RNA, but there was essentially no cross-reaction with SV40-complementary RNA. SV40-complementary RNA reacted with SV40 and E46$^+$ DNA, but gave essentially no reaction with E46$^-$ or Ad. 7 (Pinckney) DNA's. The data did not permit an esti-

TABLE 1

BINDING OF RADIOACTIVE COMPLEMENTARY RNA, SYNTHESIZED WITH SV40 AND AD. 7 (PINCKNEY) DNA'S AS PRIMERS, TO DNA FROM PURIFIED VIRUS PREPARATIONS

Purified virus DNA*	Cpm Retained on Membrane Filter,† Virus-complementary RNA Employed SV40	Ad. 7
Ad. 7 (Pinckney)	30	28,000
E46$^-$	10	20,000
SV40	39,000	30
E46$^+$	640	4,000

* Five μg of DNA tested, except for E46$^+$ where quantity tested is unknown.
† Cpm of radioactive complementary RNA incubated with each DNA were: SV40, 100,000; Ad. 7, 100,000. The mixtures were incubated 8 hr.

mate of the relative amounts of SV40 and adenovirus nucleic acid in DNA from strain E46$^+$, since reference curves relating DNA concentration to extent of DNA-RNA hybrid formation were not constructed in this experiment.

Viral DNA's in infected AGMK cells: In view of the specificity of the homology technique with purified virus preparations, it was possible to apply it to the determination of the amount of specific virus DNA's produced by E46$^+$ and by E46$^-$ and SV40 infection alone or in combination. Table 2 shows the results of an initial experiment. Although SV40 preinfection enhanced the 72-hr yield of infectious E46$^-$ by greater than 1000-fold, the production of E46$^-$ DNA within the first 24 hr was not substantially affected, as indicated by the Ad. 7/AGMK ratios. DNA from AGMK cultures inoculated 24 hr previously with E46$^+$ reacted to a greater extent with adenovirus-complementary RNA than DNA from either E46$^-$ or SV40- and E46$^-$-infected AGMK cells. There was essentially no binding of DNA from AGMK cells harvested 4 hr after adenovirus inoculation to adenovirus-complementary RNA. Green[19] has shown that in infected KB cells, adenovirus DNA synthesis does not begin until 7 hr after infection. As expected, DNA from AGMK cultures singly inoculated with SV40 virus reacted insignificantly with adenovirus-complementary RNA.

TABLE 2

BINDING OF RADIOACTIVE COMPLEMENTARY RNA, SYNTHESIZED WITH SV40 AND AD. 7 (PINCKNEY) AND AGMK DNA'S AS PRIMERS, TO DNA FROM INFECTED AGMK CULTURES

AGMK DNA,* virus(es) inoculated into AGMK cells	Time of harvest (hr) After Ad. 7	After SV40	Cpm Retained on Membrane Filter, Complementary RNA Employed† SV40	Ad. 7	AGMK	SV40 AGMK ratio (×100)	Ad. 7 AGMK ratio (×100)	Adenovirus titer ($TCID_{50}$/0.1 ml)
E46$^-$	4		130	20	3600	3.6	0.6	
	24		150	150	3000	5.0	5.0	
	72							$10^{2.5}$
E46$^+$ and SV40‡	4	(28)	660	20	3400	19.4	0.6	
	24	(48)	3800	230	3300	115.1	7.0	
	72	(96)						$10^{5.8}$
E46$^+$	4		130	20	2100	6.2	1.0	
	24		230	390	2800	8.2	13.9	
SV40§		(28)	370	20	2000	18.5	1.0	
		(48)	3100	30	2900	106.8	1.0	
None			150	20	3200	4.7	0.6	

* Four μg of cellular DNA were incubated with RNA.
† The quantity of complementary RNA added and the length of incubation was the same as shown in Table 1. The cpm of AGMK complementary RNA added was 75,000.
‡ SV40 virus was inoculated 24 hr before the adenovirus. The SV40 virus for singly inoculated cultures was inoculated at the same time the SV40 virus was added to the doubly inoculated cultures.
§ Only one flask of AGMK tissue (approximately 5×10^6 cells) was inoculated and processed per time point.

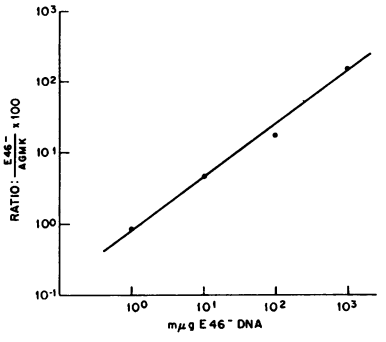

FIG. 1.—Relationship of the E46⁻/AGMK ratio (× 100) to known amounts of E46⁻ DNA. Dilutions of a measured amount of E46⁻ virus DNA were mixed with 4 μg of DNA from uninfected AGMK cultures and reacted with E46⁻-complementary RNA (100,000 cpm) and AGMK-complementary RNA (160,000 cpm). Assays and calculations necessary to determine the E46⁻/AGMK ratio were carried out as described in *Materials and Methods*. The data were obtained on the same date and under the conditions described in the legend to Table 3.

SV40-complementary RNA was also reacted with DNA from these infected AGMK tissue cultures (Table 2). Significant reactions occurred between this RNA and DNA extracted from the AGMK cells inoculated 28 and 48 hr previously with SV40 virus (the amount not being affected by subsequent E46⁻ infection) and from cells inoculated 24 hr previously with E46⁺. No significant reaction occurred with DNA from cells singly infected with E46⁻.

In the second experiment, AGMK cultures were inoculated simultaneously with SV40 and E46⁻ singly and as a mixture. The quantity of adenovirus DNA present in the cultures at 24, 48, and 72 hr was determined by the nucleic acid homology technique. The E46⁻/AGMK ratios were determined, and these ratios converted to quantity of E46⁻ DNA present in the 4 μg of DNA from the infected AGMK cultures. A reference line (Fig. 1) relating the E46⁻/AGMK ratio to known E46⁻ DNA concentration was constructed in the same experiment and was used to make these conversions. The results (Table 3 and Fig. 2) indicated that, although there was 100- to 3000-fold enhancement of adenovirus yield by SV40 infection, production of E46⁻ DNA was increased only two- to threefold in the doubly infected cultures.

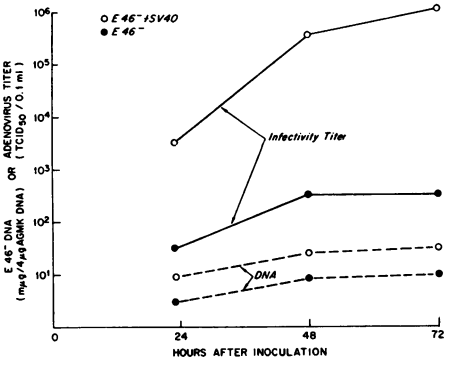

FIG. 2.—Time course of development of infectious adenovirus and E46⁻ DNA in virus-infected AGMK cultures. Graphic presentation of data in the last two columns of Table 3.

Discussion.—With respect to virologic parameters of adenovirus growth, normal AGMK cells resemble 5-fluoro-deoxyuridine (FUDR)-treated human cells (in which viral DNA synthesis is blocked[19, 20]), while SV40-infected AGMK cells resemble normal human cells. That is, in the former systems, T antigen formation (at 6–8 hr) and cytopathogenicity occur, but viral structural antigens, particles, and infectivity (all commencing after the 12th hr) are produced to only a limited degree;[1–3] in adenovirus infection of human cells and SV40-infected AGMK cells, the later events occur with much greater efficiency. Thus, it was surprising to find that SV40 infection did not exert its enhancing effect by accelerating adenovirus DNA synthesis by AGMK cells, but exerted its primary effect at a different stage

TABLE 3

BINDING OF RADIOACTIVE COMPLEMENTARY RNA, SYNTHESIZED WITH E46$^-$ AND AGMK DNA'S AS PRIMERS, TO DNA FROM INFECTED AGMK CULTURES

AGMK DNA,* virus(es) inoculated into AGMK cells	Time of harvest (hr)	Cpm Retained on Membrane Filter,† Complementary RNA Employed		E46$^-$ AGMK ratio (×100)	E46$^-$ DNA/ 4 μg AGMK DNA‡ (mμg)	Adenovirus titer ($TCID_{50}$/ 0.1 ml)
		E46$^-$	AGMK			
E46$^-$	24	370	19,000	1.9	4	$10^{1.5}$
	48	880	21,000	4.2	9	$10^{2.5}$
	72	620	14,000	4.4	10	$10^{2.5}$
E46$^-$ and SV40§	24	660	16,000	4.1	9	$10^{3.5}$
	48	1600	17,000	9.4	26	$10^{5.5}$
	72	1500	14,000	10.7	32	$10^{6.0}$
SV40	24	70	18,000	0.4	0	
	72	80	16,000	0.5	0	
None		50	18,000	0.3	0	

* Four μg of cellular DNA tested.
† Cpm of radioactive complementary RNA incubated with each DNA were: E46$^-$, 100,000; AGMK, 160,000. The mixtures were incubated 16 hr.
‡ The E46$^-$ DNA concentration was calculated using the E46$^-$/AGMK ratio and the reference line plotted in Fig. 1.
§ E46$^-$ and SV40 inoculated simultaneously.

in virus growth. The precise step at which the SV40 infection exerts its enhancing effect may be one or more of the following: (*a*) transcription of information contained in adenovirus DNA into messenger RNA; (*b*) translation of messenger RNA base sequences into amino acid sequences in peptides; and (*c*) changing of peptide conformation in ways necessary for virus encapsidation and detection as an antigen. Selective replication of defective DNA or of a portion of the adenovirus DNA, or inactivation of adenovirus DNA before its transcription into RNA appear less likely as the defect, but they cannot be eliminated on the basis of present data.

The parallel increase in adenovirus DNA production in singly and doubly infected AGMK cultures (see Fig. 2) indicates that any mechanisms limiting adenovirus DNA synthesis, particularly in the latter stages of infection, were equally effective in these differentially inoculated cultures. That is, a "compensatory" increase in DNA production in the presence of limited conversion of preformed adenovirus DNA into infectious particles was not seen.

The detection of DNA reactive with SV40-complementary RNA in purified E46$^+$ virus and in AGMK cells infected with E46$^+$ provides definitive evidence for the presence of SV40 DNA in this Ad. 7 strain, and further supports the conclusion that SV40 T antigen is formed from information in the SV40 DNA. Likewise, the failure to find SV40 DNA in E46$^-$ supports the previous view that this subline is typical adenovirus and is not carrying SV40 genetic material in a form which cannot be expressed by T antigen synthesis.

The present data do not permit any statement about the relative quantities of adenovirus and SV40 DNA in E46$^+$ or the relationship between the two types of DNA.

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*IMMUNOFLUORESCENT STUDIES OF GROUP-SPECIFIC ANTIGEN OF THE AVIAN SARCOMA-LEUKOSIS VIRUSES**

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The sera of hamsters bearing tumors induced by the Schmidt-Ruppin (S-R) strain of Rous sarcoma virus (RSV) contain antibody which fixes complement when allowed to react with antigen prepared from cells infected with any one of several representative viruses of the avian sarcoma-leukosis group. The reacting antigen appears to be common to all members of the avian sarcoma-leukosis group and specific for this group.[1, 2]

In the present report, the sera of hamsters bearing tumors induced by the S-R virus were used in an immunofluorescent staining technique to examine cells infected *in vitro* with various viruses of the avian sarcoma-leukosis group and with other viruses. The results indicate that the antigen stained is probably the same