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*SV40-ADENOVIRUS "HYBRID" POPULATIONS: TRANSFER OF SV40 DETERMINANTS FROM ONE TYPE OF ADENOVIRUS TO ANOTHER**

BY FRED RAPP, JANET S. BUTEL, AND JOSEPH L. MELNICK

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

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Recent reports have documented the existence of an adenovirus type 7 strain (stocks E46 and SP2) which can induce the synthesis of papovavirus SV40 tumor, or T, antigen in green monkey kidney (GMK) cells, or in tumors induced in hamsters by this adenovirus population.[1-3] (This antigen was discovered by Huebner who named it the tumor or T antigen. It has since been referred to as INCA, ICFA, or NEO. To avoid the tendency of each new worker introducing his own "exotica," we have continued to use the original model T terminology. It has the advantage of priority and simplicity, and furthermore, regardless of whether present in tumor cells or in virus-infected cells, the T antigen thus far can be detected only by using antibodies present in tumor-bearing animals.) The antigens induced by this adenovirus are similar or identical to those present in GMK cells during the early phases of the SV40 cytolytic cycle[4-7] and to those synthesized in all cells transformed by SV40.[8-10] SV40 viral antigens or infectious SV40 are never detected, and both the *in vitro* and *in vivo* SV40-related activities of the SP2 stock can be abolished by neutralization with adenovirus type 7 antiserum.[1-3] It was therefore postulated that during a previous joint infection, the adenovirus 7 had incorporated a portion of the SV40 genome into its own.

Although SV40 enhances the growth of adenoviruses in GMK cells,[11-14] the SP2 strain of adenovirus 7 grows equally well in GMK cells in either the presence or absence of SV40.[13] However, the SV40 tumor antigen determinant is lost after

plaquing in human cells[15] and the resulting progeny grow poorly, if at all, in GMK cells. This suggested the possibility of the presence of two distinct particles in the SP2 population.

Recent studies on the nature of the SV40-adenovirus type 7 interaction in this "hybrid" virus population have revealed that the population contains two distinct types of particles: (1) a conventional adenovirus, and (2) a deficient SV40 genome encased in an adenovirus capsid.[16, 17] The present report shows that adenovirus type 2, and other adenoviruses, can substitute for type 7 with the subsequent formation of type 2 or other type adenoviruses that now carry the SV40 tumor antigen determinant; the hybridized type 2 populations then breed true on passage in GMK cells. Similar independent observations are reported in the accompanying paper by Rowe.[18]

Materials and Methods.—The origin and history of the SV40-adenovirus type 7 hybrid (strain LL, stock E46, and its next passage stock SP2) has been described in detail.[1-3] It was used in this study after two additional passages of the SP2 stock in GMK cells.

Two pure adenovirus type 7 strains were employed. The first was an isolate derived from the SP2 stock and plaque-purified three times in human embryonic kidney cells by Boeyé *et al.*;[15] this virus no longer carries the T antigen determinant of SV40. The second was a strain of adenovirus 7 (hu) isolated from a fatal case of pneumonia[19] and passed only in human embryonic kidney (HEK) cells. It is also free of SV40 determinants.

Adenovirus type 2 was obtained from Dr. Benyesh-Melnick. It was also a fresh human isolate and was used after three passages in KB cells.

The Baylor reference strain of SV40 used in previous studies was employed.[5, 13, 20] It was used in its sixth passage level in GMK cells.

All virus stocks were prepared in 16-oz bottles and harvested by 2 alternate cycles of quick-freezing and thawing. The cell debris was removed by low-speed centrifugation and the supernatant fluid dispensed in 1-ml amounts in glass ampules. These were sealed, quick-frozen, and stored at −90°C.

Antiserum: Neutralizing antisera were prepared in rabbits against the adenovirus type 7 (hu) and the adenovirus type 2 described above. The rabbits received 1 injection weekly for 3 weeks, a 4th inoculation 2 weeks later, and antiserum was obtained 2 weeks following the final inoculation.

Cells: Primary cultures of African green monkey kidney cells were grown at 37°C in 60-mm plastic Petri dishes in a 5% CO_2 incubator. The nutrient medium, M-H,[21] contained lactalbumin hydrolysate supplemented with 2% calf serum.

Immunofluorescent techniques: GMK cells were grown as secondary cultures on 15-mm coverglasses in plastic Petri dishes in an atmosphere of 5% CO_2. The coverslips were harvested 24 hr after infection and the cells stained for the detection of SV40 tumor antigen following the procedure reported previously.[9] Sera from hamsters bearing SV40-induced tumors and fluorescein-labeled antihamster rabbit globulin were used. Detailed fractionation and labeling methods for the preparation of the fluorescent antibody have been described.[20] The preparations were viewed with a Zeiss fluorescence microscope utilizing an Osram HBO-200 mercury arc vapor lamp for illumination. All the tests included known positive and negative controls.

Plaque assay: Quantitative assays were carried out on GMK monolayers in 60-mm plastic Petri dishes. The inoculum (0.1 ml) was allowed to adsorb for 1 hr at 37°C; 0.2 ml tris buffer (pH 7.4) was used as carrier fluid to allow even distribution of the virus. The cells were then overlaid with 5 ml of the following mixture: Eagle's basal medium, 10% fetal calf serum, 1% agar, and 0.23% bicarbonate. The cells were incubated at 37°C in a 5% CO_2 incubator for 7 days, at which time a second overlay containing a 1:20,000 dilution of neutral red was added. Plaques were visible and could be counted the following day; maximum titers were usually reached by the 11th day.

The enhancement experiments were performed in the same manner as the assay, except that 0.1 ml of each virus dilution was inoculated and allowed to adsorb simultaneously.

Results.—Enhancement of SP2 plaque titers by adenovirus types 2 and 7: SP2 was

TABLE 1

ENHANCEMENT OF SP2 PLAQUE TITERS IN GREEN MONKEY KIDNEY CELLS BY THE ADDITION OF ADENOVIRUSES TYPE 7 OR 2

Enhancing virus	Log_{10} PFU/ml Expt. 1	Expt. 2	Expt. 3
None	5.40	4.70	4.93
Adenovirus type 7*	7.08	6.04	6.60
Adenovirus type 2	6.81	Not done	6.38
SV40 virus	4.90	4.30	Not done

* Results obtained for adenovirus type 7 isolated from the SP2 population. Similar results were obtained with a fresh human isolate, adeno 7 (hu).

titrated on monolayers of GMK cells, some of which were simultaneously infected with high concentrations of either adenovirus type 2, adenovirus type 7, or with SV40. In the absence of SP2 neither the adenovirus nor SV40 caused the formation of plaques in the GMK cells during the observation period. As shown by representative data in Table 1, joint infection with another adenovirus enhanced the SP2 plaque titer by approximately 20–50-fold. In three separate experiments, the plaque titer in GMK cells of SP2 was increased by adenovirus type 7 or type 2; this finding was reproducible, and a number of strains of adenovirus type 7, as well as adenovirus types 3 and 12, also yielded similar results. However, the addition of SV40 did not increase the titer. The component with which the adenovirus interacted to initiate plaque formation will be referred to in this and future publications as "particle aiding (and aided by) replication of adenovirus" (PARA). (The dictionary meaning of the prefix "para" is beside, alongside of, amiss, faulty, disordered condition, and abnormal. This describes the PARA and its relation to adenovirus replication in simian cell systems.)

Properties of progeny from enhanced plaques: Plaques appearing as a result of the adenovirus enhancement were picked from plates receiving SP2 diluted 100-fold beyond the end point in the absence of enhancement. The progeny from these plaques were passed once in green monkey kidney cells. The progeny were then tested for their ability to induce the synthesis of SV40 T antigen and were treated with various antisera to determine the type of protein coat carried by PARA.

TABLE 2

ABILITY OF PROGENY FROM ADENOVIRUS ENHANCED SP2 PLAQUES TO INDUCE SV40 TUMOR ANTIGEN IN GREEN MONKEY KIDNEY CELLS

Enhancing virus	Plaque Progeny No. tested	No. positive*
Adenovirus type 7	18	18
Adenovirus type 2	12	12
Totals	30	30

* Positive = ability to induce synthesis of SV40 tumor antigen in green monkey kidney cells.

(a) *Induction of T antigen:* GMK cells were infected with plaque progeny and stained 24 hr later with immunofluorescent reagents to detect SV40 T antigen. The data in Table 2 show that all of the 30 plaque progeny tested were capable of inducing the synthesis of SV40 T antigen. These experiments included viruses derived both from plaques that had been enhanced by adenovirus type 7 and by adenovirus type 2.

(b) *Antigenic analysis of adenovirus type 2-enhanced plaque progeny:* The type of protein coat carried by PARA progeny was identified by neutralization of two different properties of the virus: (1) ability to induce synthesis of T antigen, and (2) ability to cause plaque formation on GMK cell monolayers. Equal volumes of the virus suspension and either tris buffer or the appropriate rabbit antiserum were incubated together and then inoculated onto GMK cells. The tumor antigen

TABLE 3

EFFECT OF ADENOVIRUS TYPE 7 AND TYPE 2 ANTISERA IN INDUCTION OF TUMOR ANTIGEN SYNTHESIS BY ADENOVIRUS TYPE 2-ENHANCED PLAQUE PROGENY

Plaque no.	Tumor Antigen Induction Following Treatment with: Tris	Adeno 7 antiserum	Adeno 2 antiserum
A23G5P1	+	+	0
A23G5P2	+	+	+
A23G5P3	+	+	0
A23G5P4	+	+	0
A23G5P6	+	+	0
A30H5P2	+	+	0
A30I5P1	+	+	0
A30I5P2	+	+	0
A30I5P3	+	+	0
A30I5P4	+	+	0
A30J4P1	+	+	0
A30K4P3	+	+	0

+ = Evidence of tumor antigen synthesis 24 hr following inoculation of green monkey kidney cultures.
0 = No evidence of tumor antigen synthesis.

induction results are listed in Table 3. None of the 12 plaque isolates tested was affected by adenovirus type 7 antiserum. Only the progeny from one plaque were able to induce the synthesis of SV40 T antigen after treatment with adenovirus type 2 antiserum; this was probably due to incomplete neutralization of the virus present, although mixed infection is another possible explanation. These results indicate that the particles responsible for tumor antigen induction had become encased in an adenovirus type 2 protein coat during the course of replication in the presence of that virus.

Similar neutralization experiments with adenoviruses 7 and 12 enhanced plaque progeny have identified such progeny as being antigenically type 7 or 12 adenoviruses, respectively.

Neutralization of plaque formation in GMK cells by selected plaques is illustrated in Table 4. It is evident from the data that the PARA responsible for plaque formation by adenovirus type 2 had been converted to a type 2 adenovirus; neither adenovirus type 12 nor adenovirus type 7 antiserum had any inhibitory effect although adenovirus 7 antiserum neutralized the parent hybrid. Plaque progeny from a plaque obtained by enhancement with adenovirus type 12 now were neutralized only by adenovirus type 12 antiserum.

Since these progeny were tested after additional passages in GMK cells, they breed true as adenovirus type 2 or type 12 populations and do not revert to adenovirus type 7.

Demonstration of coat antigens carried by PARA in SP2 virus population: Experiments described above revealed that the component designated as PARA responsible

TABLE 4

EFFECT OF ANTISERA ON PLAQUE FORMATION IN GREEN MONKEY KIDNEY CELLS BY ADENOVIRUS TYPE 2 OR TYPE 12-ENHANCED PLAQUE PROGENY

Neutralized with:	Average Number of Plaques per Plate: Parent SP2	Adeno 2-PARA	Adeno 12-PARA
Tris	>100	>100	>100
Adenovirus type 7 antiserum	0	>100	>100
Adenovirus type 2 antiserum	>100	3	>100
Adenovirus type 12 antiserum	>100	>100	0

for T antigen induction and plaque formation in GMK cells by the SP2 stock did not invariably breed as an adenovirus type 7, but could be transferred to a type 2 adenovirus. The possibility existed that PARA was distinct antigenically and the neutralization experiments were merely affecting the helper adenovirus. It was necessary to show that PARA itself was neutralized. This was accomplished by first reacting SP2 with adenovirus type 7 antiserum, followed by plating the neutralized mixture on GMK cells carrying a lawn of adenovirus type 2. If PARA were not neutralized, extensive plaquing should occur in the presence of the provided helper virus. As shown in Table 5, however, plaque formation did *not* occur, indicating that PARA was actually encased in an adenovirus type 7 protein coat.

TABLE 5

NEUTRALIZATION OF PARTICLE IN SP2 (PARA) RESPONSIBLE FOR PLAQUE FORMATION BY ADENOVIRUSES ON GREEN MONKEY KIDNEY CELLS

Inoculum	Average No. of Plaques per Plate: No helper virus	Average No. of Plaques per Plate: Adenovirus type 2 lawn
SP2 + tris buffer	3	>100
SP2 + adenovirus type 7 antiserum	0	1

The SP2 stock neutralized by adeno-7 antiserum was also tested for T antigen induction in the presence of adenovirus type 2 and again yielded negative results. This was further evidence that the particle carrying the SV40 determinant was neutralized by type-specific adenovirus antiserum.

Discussion.—The ability of adenoviruses types 7, 2, 12, and others, to enhance the plaque titer of the SV40-adenovirus "hybrid" virus population reveals the presence of a defective particle (PARA) which is unable to induce plaque formation without the help of an adenovirus. In the study by Boeyé, Melnick, and Rapp[17] on the SP2 population, the particle described as P_2 is such a PARA particle. This defective particle carries the SV40 T antigen determinant, since the plaque progeny from all enhanced plaques possess the property of T antigen induction in susceptible cells.

Previous investigations in this laboratory,[15] and by Rowe and Baum,[16] have shown that progeny from all GMK plaques carry the SV40 T antigen determinant while plaque progeny from HEK cells no longer possess this marker. It therefore appears that SP2 and the progeny descended from it carry the genetic markers delineated in Figure 1. As illustrated, the T+ marker is lost in HEK cells and the resulting virus has the properties of a normal adenovirus. Passage of the hybrid in GMK cells, however, results in a retention of the T antigen determinant, along with all the other characteristics of the parent virus. The only alteration which has been detected seems to be the antigenicity of the protein coat, and this is dependent upon the type of helper adenovirus present.

It is being reported elsewhere[16, 17] that two particles in the SV40-adenovirus 7 population are necessary for plaque formation in GMK cells. Since the present study indicates that the coat of PARA is provided by the co-infecting helper adenovirus and since PARA is required for plaque formation by adenoviruses in GMK cells, a mutual dependence exists between adenovirus and PARA for replication in simian cells. The known properties of PARA, of adenoviruses, and of SV40 virus are summarized in Table 6.

The mutual dependence of the 2 particles in GMK cells is one major point which distinguishes the PARA-adenovirus system from that of the defective Rous sarcoma virus (RSV). Although Rous-associated virus (RAV) does similarly provide the

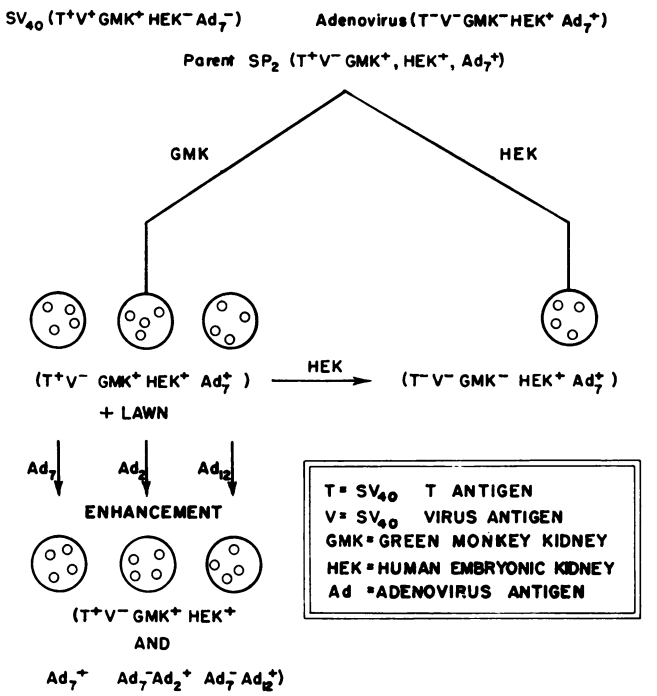

FIG. 1.—Genetic markers of SP2 and plaque progeny.

protein coat for RSV,[22, 23] both RSV and RAV can exercise independent functions in the absence of the other.[24] These include cell transformation for RSV and replication for RAV. Another difference is that RAV and RSV are RNA viruses of the same avian leucosis complex, while papovavirus SV40 and adenoviruses are unrelated DNA viruses.

The observations reported here are not compatible with the previous suggestions that the SP2 stock consists uniformly of type 7 adenovirus with a portion of SV40 DNA attached to its genome. However, we cannot rule out the possibility that PARA contains defective adenovirus as well as defective SV40 DNA. It appears that the hybrid population contains a defective mutant of SV40 which cannot direct the synthesis of a protein coat, either of SV40 or adenovirus, and that the hybrid is dependent on a superinfecting adenovirus for its provision. Adenoviruses 2 and 12 serve this purpose as well as the original adenovirus 7, and other results show that adenovirus 3 can also substitute for type 7. Indeed, many such adenoviruses (and

TABLE 6

PROPERTIES OF ADENOVIRUS, PARA,* AND SV40

Property	Designation of genetic markers	Adeno-virus	PARA	PARA + adenovirus	SV40
Induction of SV40 T antigen	T	0	Probably 0	+	+
Induction of SV40 V antigen	V	0	0	0	+
Induction of adeno 7 antigen	Ad7	+	0	+	0
Replication in GMK cells	GMK	0	0	+	+
Replication in HEK cells	HEK	+	Probably 0	+	±
Presence of adenovirus capsid		+	+	+	0

* Para = beside, alongside of, amiss; PARA = P (particle) A (aiding) R (replication of) A (adenovirus).

perhaps other viruses also) may be able to serve as helpers. The progeny of enhanced plaques breed true, yielding progeny which (1) produce the cytopathic effect of an adenovirus, (2) grow well on monkey kidney cells (unlike the parent enhancing adenoviruses), (3) carry the SV40 T antigen determinant, and (4) are neutralized by antiserum against the enhancing adenovirus.

The fact that the newly formed PARA-adenovirus 2 populations breed true indicates that the SV40 genome is not irreversibly linked to a complete adenovirus 7 genome. We cannot rule out the possibility that a portion of the adenovirus 7 DNA is being carried along, but we have not been able to detect any manifestation of such a segment.

We also cannot exclude the possibility that the DNA of adenovirus and of PARA might occasionally be enclosed in the same capsid, space permitting. This would be an example of genotypic mixing; to detect it, one would have to study the minor components of the mixed population.

The proposed hypothesis as an explanation for the actual physical state existing in the hybrid population is compatible with the available data. It seems to be a phenomenon unique in animal virology. The possibility exists that such interactions are more common than we realize among different viruses, but that they are undetected because of a lack of specific recognizable markers.

The results described here reveal that genetic determinants of one virus (SV40) can be carried in the capsid of an unrelated virus (adenovirus); such a population breeds true. The capsid can be changed, however, by the addition of an heterologous adenovirus during the replication cycle. For the phenomenon whereby a foreign virus determinant is carried in a stable form within the capsid of an unrelated virus, we propose the term *transcapsidation*.

Summary.—Plaque formation in GMK cells by an SV40-adenovirus 7 "hybrid" population can be enhanced by the addition of additional adenovirus type 7 or by other adenovirus types. The progeny from these enhanced plaques are coated by protein antigenically identical to that of the co-infecting adenovirus. These new adenovirus type "hybrid" populations (types 2, 3, 12, etc.) then breed true on further passage in GMK cells. It appears that the "hybrid" populations consist of at least two types of particles. One of these particles is the normal adenovirus and the second particle is a defective virus (termed PARA) which contains a portion of the SV40 genome and which is necessary for adenovirus replication in GMK cells. In the process of replication, PARA acquires the capsid of the helper adenovirus.

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*GENE CONSERVATION IN BACILLUS SPECIES, II. THE LOCATION OF GENES CONCERNED WITH THE SYNTHESIS OF RIBOSOMAL COMPONENTS AND SOLUBLE RNA**

BY DAVID DUBNAU, ISSAR SMITH, AND JULIUS MARMUR

DEPARTMENTS OF BIOCHEMISTRY AND PATHOLOGY, ALBERT EINSTEIN COLLEGE OF MEDICINE, YESHIVA UNIVERSITY, NEW YORK

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The previous paper in this series[1] demonstrated that the genes determining resistance of *Bacillus subtilis* to streptomycin (S), erythromycin (E), micrococcin (M), and bryamycin (B), as well as those coding for soluble (4*S*) and ribosomal (16*S* and 23*S*) RNA's, form part of a core of genetic material which has diverged relatively slowly during evolution among the members of the genus *Bacillus*. It was postulated on the basis of these and other data[2–6] that the S^r and E^r mutations occur in genes which code for ribosomal components.

In this paper an attempt is made to map the S-, E-, and M-resistance markers and the genes which code for soluble and ribosomal RNA's in *B. subtilis*, using the D_2O transfer method developed by Yoshikawa and Sueoka.[7] If the S and E genes are involved in the synthesis of ribosomes, their chromosomal location may be the same as, or adjacent to, that of the rRNA cistrons. An attempt to map the rRNA cistrons might also provide some information as to whether coding segments for each of these species of RNA are clustered or dispersed on the *B. subtilis* chromosome.

Evidence is presented below, suggesting that the S^r and E^r markers, and at least