A NONDEFECTIVE (COMPETENT) ADENOVIRUS-SV40 HYBRID ISOLATED FROM THE AD.2-SV40 HYBRID POPULATION

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Communicated by Robert J. Huebner, April 18, 1969

Abstract.—A new nondefective hybrid virus has been plaque-isolated from the Ad.2-SV40 hybrid population. This virus replicates efficiently with one-hit kinetics in both human embryonic kidney and African green monkey kidney cells, induces an SV40 specific antigen which is detectable by immunofluorescence and complement-fixation using sera from SV40 tumor-bearing hamsters, and produces SV40-specific RNA detectable by DNA-RNA hybridization. The SV40-specific antigen induced by this virus is heat-stable, sensitive to inhibitors of DNA synthesis, serologically different from SV40 T and viral antigens, and is an unrecognized SV40 antigen.

Since the initial reports describing the hybridization of human adenoviruses (Ad.) and SV40, two types of hybrid populations have been described: those free of detectable SV40 virions represented by the Ad.3 and Ad.7 populations; and those which release detectable SV40 virions represented by the Ad.1, 2, 4, 5, and 12 populations.[1-3] Characterization of the infectivity and the SV40 T antigen-inducing capacity of the progeny of plaques isolated from human embryonic kidney (HEK) and African green monkey kidney (AGMK) cells have established that these hybrid populations consist of a mixture of nonhybrid Ad. virions and hybrid particles containing SV40 genome in Ad. capsids.[4-7]

Studies on adenovirus plaque formation by the two types of Ad.-SV40 hybrid populations have shown that the nonhybrid component induces adenovirus plaques by one-hit kinetics in human embryonic kidney cells, indicating that one virion initiates plaque formation. In green monkey kidney cells, however, the induction of adenovirus plaques by these populations proceeds by two-hit kinetics, indicating that both a nonhybrid adenovirion and a defective hybrid particle (i.e., a particle requiring nonhybrid adenovirus to replicate in either human embryonic or green monkey kidney cells) are required to initiate adenovirus plaque formation.[2, 4-7]

Biological and biophysical studies on the Ad.7-SV40 hybrid population, E46+,‡ have shown that the adenovirus and SV40 DNA in these hybrid particles is covalently linked; thus, these particles are true molecular hybrids.[8-10] All efforts to obtain a pure clone of E46+ hybrid particles free of nonhybrid adenovirions have failed.[4]

Biological studies on the Ad.2-SV40 (Ad.2++)‡ population have demonstrated that, in addition to nonhybrid Ad.2 virions, there are adenovirus encapsidated particles which are capable of producing SV40 plaques on AGMK monolayers by one-hit kinetics.[2] These particles contain the infectious SV40 genome.

The purpose of this paper is to describe the isolation from the Ad.2++ popula-

tion of a new type of Ad.-SV40 hybrid virus. Designated Ad.2^{+}ND$_1$, this virus is nondefective, produces SV40-specific RNA, a previously unrecognized SV40-specific antigen, and does not yield infectious SV40 virus.

Materials and Methods.—Virus: Pool 2 of the Ad.2^{++} population has been previously described.[2] Pools B56 and B55, representing the progeny from plaques 1208 (described in detail elsewhere)[11] and 1562 (later designated the Ad.2^{+}ND$_1$P1 pool), constitute the third and fourth AGMK passages, respectively, of plaques that were isolated from two-hit titrations in AGMK cells of Ad.2^{++} pool 2. The infectivity characteristics of these pools are presented in Table 2.

Ad.2 (strain Ad.6) and Ad.12 (Huie strain) were maintained by serial passage in either HEK or KB cells. The pools used for DNA extraction were purified by Dr. Paul Burnett of Eli Lilly Co., Indianapolis, Indiana.[12] SV40 strain 777,[13] maintained by serial passage in BSC-1 cells, was used for preparation of SV40 DNA. Prior to extraction of the viral DNA, the virus was purified by the method of Black *et al.*[14]

Plaque assays: The techniques for HEK and AGMK plaque assays with and without adenovirus lawns, and the procedures used to isolate and pass plaque isolates, have been described in detail elsewhere.[2, 11]

In order to disperse aggregates prior to plaque titrations, an aliquot of each virus pool was treated with 1% sodium deoxycholate and 0.1% trypsin for 30 min at 37°C,[14] extracted for 15 min at 4°C with an equal volume of cold chloroform, clarified at 1500 rpm × 10 min, diluted 1–10 in medium containing 10% fetal bovine serum, and stored until assayed at −70°C.

Antigen preparation: The techniques used for preparing coverslip antigens with and without 5-fluorodeoxyuridine (FUDR) or cytosine arabinoside have been described.[15]

Immune sera and immunofluorescent tests: Pooled and individual sera used for immunofluorescent (FA) tests were obtained from hamsters bearing either large primary tumors induced by SV40 strain 776 (obtained from Dr. M. D. Hoggan), or tumors induced by the THK-3 line of transformed hamster embryo cells (provided by Drs. P. H. Black and A. E. Freeman).[13]

The techniques used to prepare the hyperimmune adenovirus rabbit antiserum (RAS) and SV40 RAS specific for SV40 viral (V) antigen have been described elsewhere.[11, 15] The SV40 RAS used was from a single rabbit and had an FA titer of 1-160 anti-SV40 V antibody and no reaction by FA at 1-10 with SV40 T antigen.

The guinea pig serum specific for SV40 V antigen (obtained from Dr. M. D. Hoggan) failed to react by complement fixation (CF) at a 1/20 dilution with SV40 T antigen but had a titer of 1/2560 when tested against SV40 cell pack antigen.

The FA procedure used in these experiments was the method of Pope and Rowe for staining T antigen.[16]

Nucleic acid extraction and RNA-DNA hybridization techniques: Confluent monolayers of primary HEK cells or the Vero line (a mycoplasma-free line at 130th passage level) of AGMK cells[17] in 32-oz bottles were inoculated with Ad.2^{+}ND$_1$ virus at multiplicities of 10–25 pfu/cell. At 12 hr after infection (expts. 2–3) or 18 hr after infection (expt. 1), the medium was decanted and 15 ml of Eagle's minimal essential medium containing uridine-5-^{3}H (Nuclear-Chicago, 29,600 mCi/mM) 15 μCi/ml were added. The cultures were incubated at 37°C for 10 hr (expts. 2–3), or 4 hr (expt. 1). At 22 hr after infection, cells were removed by scraping and pelleted at 1500 rpm × 10 min. The procedures for extraction of RNA from these cells, the technique of RNA-DNA hybridization, and the method of elution and rehybridization have been previously described.[18] KB and Vero cell DNA was extracted by the method of Marmur,[19] and DNA from purified virus was prepared by papain digestion followed by SDS-phenol extraction.[20]

Results.—Isolation of the Ad.2^{+}ND$_1$ virion in HEK: Ten selected adenovirus plaques were isolated from two-hit titrations in AGMK monolayers infected with the parent Ad.2^{++} pool 2. All ten pools prepared from the progeny of

these plaques induced SV40 antigen detectable by immunofluorescence. Infectivity studies on one of these pools suggested the existence of nondefective virions. Pool B55, designated the Ad.2$^+$ND$_1$P1 pool, plaqued with nearly equal efficiency in both HEK and AGMK monolayers with dose-response curves following one-hit kinetics. This was in marked contrast to the pools prepared from the progeny of the other nine plaques; each formed adenovirus plaques at high dilutions by one-hit kinetics in HEK monolayers, while forming such plaques at lower dilutions, and by two-hit kinetics in AGMK monolayers. Moreover, five of five subplaques of Ad.2$^+$ND$_1$P1 isolated from the HEK monolayers induced SV40 antigens detectable by immunofluorescence in 2–70 per cent of the nuclei, whereas none of 28 HEK plaque progeny of the parent Ad.2^{++} pool 2 induced FA detectable SV40 antigen in HEK.

To determine whether the Ad.2$^+$ND$_1$P1 pool did contain Ad.2-SV40 hybrid particles capable of independent replication in both HEK and AGMK cells, a series of three plaque isolation procedures on HEK monolayers was performed. Before each subsequent plaque isolation step, the isolated plaques were passed in HEK cells and their progeny were tested by immunofluorescence for induction of SV40 antigens. By these procedures the plaque isolation series provided the conditions necessary for the simultaneous selection of nondefective hybrid virions and the detection of nonhybrid Ad.2 which may have been present in the Ad.2$^+$-ND$_1$P1 pool. A summary of these plaque isolation studies is presented in Table 1.

To initiate the plaque isolation series, the HEK plaque of Ad.2$^+$ND$_1$P1 which induced FA-detectable SV40 antigen in the highest percentage of cells was selected. A 1-ml aliquot of the progeny from this plaque was treated to disperse aggregates and plaqued on HEK monolayers. Ten well-isolated plaques were picked, passed in HEK tubes, and the progeny from each examined by immunofluorescence for SV40 antigen induction. The progeny of the plaque producing the highest percentage of cells positive for SV40 antigen was again selected and an aliquot treated to disperse aggregates and plaques in human embryo kidney cells. These procedures were repeated for a total of three cycles. The progeny of 42 of 45 plaques examined induced SV40 antigen detectable by immunofluorescence in a high percentage of cells. In addition, the virions present in each pool prepared during the plaque isolation procedures replicated efficiently in both human embryo and green monkey kidney cells (see below). Thus, the plaque isolation series demonstrates that nondefective virions which induce SV40 antigens can be plaque-isolated from the Ad.2$^+$ND$_1$P1 pool.

Three of the ten plaques induced by Ad.2$^+$ND$_1$P3 (i.e., progeny grown from the first cycle) in HEK monolayers failed to induce antigen detectable by immunofluorescence when pooled SV40 sera from tumor-bearing hamsters were used. Progeny of these three plaques failed to plaque in AGMK monolayers (titers of $< 10^4$ pfu/ml), demonstrating that the Ad.2$^+$ND$_1$P3 pool contained nonhybrid virions after one-plaque isolation. The origin of these virions remains unexplained.

Infectivity characteristics of the Ad.2$^+$ND$_1$ virus: Infectivity studies were done on the progeny from all pools prepared during the plaque-isolation series. Representative titers are presented in Table 2 and are compared with similar

TABLE 1. *Summary of the tissue culture procedures used to isolate Ad.2^{+}ND$_1$ virus.*

Origin and passage level of Ad.2^{+}ND$_1$ pools	Plaque isolation steps in establishing Ad.2^{+}ND$_1$ pools*	Induction of SV40 antigen by HEK plaque progeny† (no. positive/ no. tested)	Morphology of SV40 antigen staining
Ad.2^{++} Pool 2	Plaque (AGMK)‡		
Plaque 1562	Undiluted (AGMK)		
Ad.2^{+}ND$_1$ P1	Undiluted (AGMK)		
	Plaque (HEK)	5/5	Intranuclear staining (SV40 T)
Ad.2^{+}ND$_1$ P3	Undiluted (HEK)		
	Plaque (HEK)	7/10	Nuclear membrane staining
Ad.2^{+}ND$_1$ P5	Undiluted (HEK)		
	Plaque (HEK)	10/10	" " "
Ad.2^{+}ND$_1$ P8§	Undiluted (HEK)		
	Plaque (HEK)	10/10	" " "
Ad.2^{+}ND$_1$ P9	Undiluted (HEK)		
Ad.2^{+}ND$_1$ P10	Undiluted (HEK)		
	Plaque (HEK)	10/10	" " "

* Arrows indicate the sequence of the plaque isolation and HEK passage steps.
† All cover slips were stained with SV40 tumor-bearing hamster sera FA pool 3.
‡ Type of cell used in the indicated passage.
§ P8 was established by growing the plaque from P5 in HEK tubes followed by a 2nd passage in 32-oz HEK bottles.

studies on the parent Ad.2^{++} pool 2 and the progeny (Ad.2^{++} pool B56) of another plaque (1208) which had infectious characteristics similar to the parent population. Adenovirus plaque formation in HEK cells by all pools proceeded by one-hit kinetics In AGMK cells, however, Ad.2^{++} pools 2 and B56 formed adenovirus plaques by two-hit kinetics, while pools representing Ad.2^{+}ND$_1$P1 or its subplaques formed adenovirus plaques efficiently by one-hit kinetics. Furthermore, it was shown that adenovirus plaque induction by both Ad.2^{++} pools 2 and B56 and the progeny from Ad.2^{+}ND$_1$P1 were neutralized by Ad.2 RAS and not by SV40 RAS. Plaque induction in AGMK cells by Ad.2^{++} pool B56 was enhanced by a lawn of nonhybrid Ad.2, and the slope of the dose-response curve changed from 2 to 1, while the same lawn had no apparent effect on adenovirus plaque formation in AGMK cells by pool Ad.2^{+}ND$_1$P1 or P8. Only Ad.2^{++} pools 2 and B56 produced SV40 plaques in AGMK cells and contained detectable quan-

TABLE 2. *Infectivity assays comparing Ad.2^{+}ND$_1$ pools with Ad.2^{++} pools 2 and B56.*

Virus	Titer: HEK Ad. plaque	AGMK Ad. plaques	AGMK SV40 plaques[a]	AGMK with Ad. lawn Ad. plaques	AGMK non-hybrid SV40[b]
Ad.2^{+}ND$_1$P1[c] (pool B55)	6.1 (0.8)[d]	6.1 (0.9)	<4.0	6.6 (0.9)	0
" P3	7.9	7.2 (1.0)	<4.0		
" P8	8.2 (1.0)	7.4 (1.1)	<4.0	7.4 (0.9)	
Ad.2^{++} pool 2	8.7 (1.1)	(2.1)[e]	7.7 (0.9)		4.0
" pool B56	8.2 (1.0)	(2.5)[e]	7.8 (0.9)	6.7 (0.7)	2.7[f]

[a] SV40 plaques were induced by particles with Ad.2 capsids.
[b] SV40 plaques appearing in the presence of Ad.2 RAS.
[c] See Table 1 for origin of Ad.2^{+}ND$_1$ pools.
[d] Titer expressed as $\log_{10}$ pfu/ml with slope of the dose-response curve indicated in parentheses. Slope of the dose-response curve was determined by visual curve fitting.
[e] Calculation of the titer is dependent on dilution when dose-response curve is not 1.0.
[f] Ad.2^{++} pool B56 was prepared with media containing SV40 RAS.

tities of SV40 virions. The induction of SV40 plaques by these pools followed one-hit kinetics, and the formation of these SV40 plaques was neutralized by Ad.2 RAS. To demonstrate that pools of the Ad.2^{+}ND$_1$ virus were free of SV40 virions, 50-ml aliquots of the Ad.2^{+}ND$_1$P1 and P9 pools were freed of Ad.2 encapsidated virions by heat inactivation and were passed in 32-oz bottle cultures of AGMK cells for 30 days with a subsequent blind passage in one experiment. No SV40 virions were detected in either of these pools. Thus, the Ad.2^{+}ND$_1$ virus is not defective in either human embryo or African green monkey kidney cells, replicating with one-hit efficiency in both cell types in the absence of non-hybrid Ad. virions. To prove that this virus was an Ad.-SV40 hybrid, it was necessary to show that it contained SV40 genome. This was done by demonstrating that it induced both SV40-specific antigen and SV40-specific RNA.

Production of SV40-specific antigen by the Ad.2^{+}ND$_1$ virus: During the immunofluorescent evaluation of the progeny from plaque isolates from the Ad.2^{+}ND$_1$P3 pool, a dramatic difference was noted in the morphology of the immunofluorescent staining reaction detected with SV40 serum from tumor-bearing hamsters (Table 1). The Ad.2^{+}ND$_1$P1 pool had induced an FUDR-resistant, intranuclear antigen which was morphologically indistinguishable from that previously described for SV40 T antigen. The five subplaques from this pool induced a morphologically similar antigen. However, seven of ten subplaques from the progeny (the Ad.2^{+}ND$_1$P3 pool) of one of the Ad.2^{+}ND$_1$P1 plaques induced an antigen with an entirely different morphology when stained with the same pool of SV40 hamster serum. The immunofluorescent staining reaction of the antigen induced by these plaques was seen only at the nuclear membrane and perinuclear area of the cell. This unusual morphologic pattern was observed in immunofluorescent studies of all the subsequent subplaques from the Ad.2^{+}ND$_1$ population regardless of whether HEK, AGMK, or rat embryo cells were used to prepare the antigens. Cells infected with Ad.2^{+}ND$_1$ virus failed to react with a variety of pooled and individual sera from hamsters bearing transplanted tumors induced by Ad.7, 12, 18, polyoma, and Rous sarcoma virus. Additional immunofluorescent studies demonstrated that this antigen reacted with a great majority of sera from SV40 tumor-bearing ham-

sters. In contrast to SV40 T antigen, it was sensitive to inhibitors of viral DNA synthesis, FUDR, or cytosine arabinoside, and was not inactivated by heating coverslips to 56°C for 30 minutes. In contrast to the SV40 V antigen, which is induced in AGMK cells by SV40 virus or in HEK cells by either Ad.2^{++} pools 2 or B56,[2, 11, 13] the antigen induced by Ad. 2^{+}ND$_1$ virus did not react with SV40 hyperimmune rabbit or guinea pig sera specific for SV40 V antigen. The nuclear membrane staining was reduced 90–97% per cent by mixing the Ad.2^{+}ND$_1$ virus with Ad.2 RAS, while no reduction in per cent staining was detected by mixing the inoculum with either a preimmune serum from the same rabbit, SV40 RAS, or a complement-fixation reactive pool of sera from SV40 tumor-bearing hamsters. The SV40 specificity and the general properties of this antigen were confirmed by complement-fixation testing. Serologic reagents specific for other agents including adeno-associated viruses 1-4, SV5, and lymphocytic choriomeningitis failed to react with this antigen by either immunofluorescence or complement fixation. This SV40-specific antigen, which differs strikingly from both SV40 T and V antigen by immunofluorescence and complement fixation, is most likely a previously unrecognized SV40 antigen and has been designated the SV40 "U" antigen. A more detailed characterization of this antigen will be presented in a subsequent report.

Detection of SV40-specific RNA in cells infected with Ad.2^{+}ND$_1$ virus: Biochemical confirmation that Ad.2^{+}ND$_1$ virus carries SV40 genetic information was obtained by RNA-DNA hybridization experiments with ^{3}H-RNA from cells acutely infected with Ad.2^{+}ND$_1$ virus (Table 3). ^{3}H-RNA extracted from Ad.2^{+}ND$_1$-infected cells reacted specifically with both Ad.2 and SV40 DNA. Similar results were obtained in three separate experiments using HEK and Vero cells and three different passages of Ad.2^{+}ND$_1$ virus.

In contrast, ^{3}H-RNA extracted from cells acutely infected with Ad.2 virus reacted extensively only with Ad.2 DNA. Similarly, ^{3}H-RNA from SV40-infected cells reacted only with SV40 DNA. ^{3}H-RNA from uninfected Vero or KB cells did not react significantly with any of the viral DNA's; this indicates that the SV40 DNA used in these experiments was free of any contaminating cellular DNA.

The specificity of the reaction between ^{3}H-RNA from Ad.2^{+}ND$_1$-infected cells and SV40 DNA was also tested by eluting the hybridized RNA and rehybridizing it with new SV40 and Ad.12 (control) filters. The eluted ^{3}H-RNA rehybridized specifically only with SV40 DNA.

Discussion.—A new, nondefective Ad.2-SV40 hybrid virus has been isolated from the Ad.2^{++} hybrid population. This virus replicates with one-hit efficiency in both HEK and AGMK cells and induces SV40-specific antigens detectable by immunofluorescence or complement fixation when pooled or individual sera from SV40 tumor-bearing hamsters are used. The plaque studies were especially meaningful, since the isolation procedures were adjusted to allow the emergence of any nonhybrid Ad.2 virions. No such nonhybrid Ad.2 virions were detected beyond the first HEK plaque isolation. The SV40 specificity of the antigen induced by the Ad.2^{+}ND$_1$ virus is indicated by the following: First, the antigen reacts by immunofluorescence and complement fixation only with serum from

TABLE 3. *Virus-specific RNA in acutely infected human and monkey cells.*

Expt.	RNA Source: Infecting virus†	RNA Source: Cell type	RNA input‡ (cpm × 10^5)	Net Cpm Retained on Virus DNA*: SV40	Ad.2	Ad.12
1	Ad.2+ND_1P10	HEK	1.0	86	2,012	40
	Ad.2	"	1.0	1	1,034	8
	None	"	1.0	2	0	0
2	Ad.2+ND_1P12	Vero	17.0	1,353	13,185	100
	Ad.2	"	5.8	0	2,680	21
3	Ad.2+ND_1P13	Vero	13.6	208	3,179	54
	SV40	"	5.2	2,269	3	4
	None	"	17.5	2		

* Total virus-specific RNA cpm bound to 1.0 μg filters was determined in duplicate after a 20-hr hybridization. Determination was done in quadruplicate in expt. 1. Background (cpm bound to 1.0 μg *E. coli* DNA ($<2 \times 10^{-4}$%) has been subtracted.

† Multiplicity of infection was 10 pfu/cell in expt. 1, and 25 pfu/cell in expts. 2 and 3. Approximately 25% of cells exhibited Ad. CPE at time of harvest.

‡ Specific activity (cpm/μg RNA) was 2.0×10^4 in expt. 1, $1.0–3.0 \times 10^4$ in expts. 2 and 3.

hamsters bearing SV40-induced tumors; second, a morphologically similar antigen has been detected by immunofluorescence in acutely infected cells from three species, including humans, monkeys, and rats; third, the particles inducing this antigen are neutralized only with Ad.2 RAS; finally, cells infected with these virions contain SV40-specific RNA.

The isolation of the Ad.2+ND_1 virus, which induces an unrecognized SV40 antigen and does not yield infectious SV40 virions, provides several striking insights into the nature of Ad.-SV40 hybrid viruses. Most significantly, it points out the genetic heterogeneity between different hybrid populations and within a single hybrid population. This heterogeneity can be exploited for genetic studies on both adenovirus and SV40 virus, as has been illustrated by the detection of the unrecognized SV40 antigen. Such heterogeneity could be accounted for either by the occurrence of breaks or deletions at varying points and subsequent recombinations between portions of the adenovirus and SV40 genomes. An alternative model is that the entire SV40 genome is included within each hybrid particle with different portions of the genome being expressed.

The finding that a non-T antigen-inducing Ad.-SV40 hybrid particle will replicate efficiently in AGMK cells also suggests that the portion of the SV40 genome responsible for enhancement of adenoviruses in AGMK cells is different from that which induces T antigen. It cannot be ruled out that the Ad.2+ND_1 virus is a monkey cell-adapted Ad.2 mutant that contains a portion of the SV40 genome but lacks the segment responsible for enhancing the replication of human adenoviruses in monkey cells.

The Ad.2+ND_1 virus is a laboratory-created hybrid virion which is unique because of its nondefective nature; this virus should prove to be much more useful in studying the molecular interaction of the adenovirus and SV40 DNA's than the hybrid particles described heretofore.

The pathogenicity of nondefective viruses which are hybrids between human pathogens and other viral agents such as SV40 must be considered. Such viruses could be maintained in human and subhuman populations and as pathogens would represent unknown hazards. Preliminary studies in hamsters have indi-

cated that the Ad.2+ND_1 virus is not oncogenic since after 360 days it has produced no tumors in 28 hamsters inoculated as newborns with 10^7 pfu. Likewise, no evidence of transformation has been detected in hamster embryo cultures after 90 days.

The authors would like to acknowledge the support and critical appraisal of Drs. W. P. Rowe, M. D. Hoggan, and M. N. Oxman, and the technical assistance of Mrs. Xandra Funk, Mrs. Jean Noe, Miss Aurella Grochal, and Mr. John Jones.

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‡ Ad.2 refers to the virion phenotype. The "plus" indicates that some particles in the hybrid population contain the entire SV40 genome or a portion of it but do not produce infectious SV40 progeny, while "double plus" indicates that some particles not only contain SV40 genome but produce SV40 progeny as well; "ND" indicates that the particle is nondefective (i.e., it replicates without helper, a single particle being competent to initiate a productive infection). Suffix "P" with a number refers to passage level starting with the Ad.2+ND_1P1 pool.

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