

## STUDIES OF ADENOVIRUS SV40 HYBRID VIRUSES

### I. ASSAY SYSTEM AND FURTHER EVIDENCE FOR HYBRIDIZATION\*

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Recent studies (1-3) have produced presumptive evidence of a genetic hybrid between adenovirus type 7 (Ad. 7) strain L.L. and the portion of the SV40 genome coding for formation of SV40 neoantigen (4) (the antigen also referred to as "tumor" or "T" antigen). This hybrid strain will be referred to here as E46<sup>+</sup>. The evidence for hybridization as against some type of mixed infection consisted of failure to isolate SV40 virus, failure of E46<sup>+</sup> to induce SV40 viral antigen, differences in kinetics and host range of SV40 antigen formation, and prevention of SV40 neoantigen formation by procedures which specifically eliminated adenovirus infectivity, namely, neutralization with Ad. 7 antiserum, ultrafiltration, or heating at 56°C for 10 minutes.

This report describes a procedure for quantitation of both adenovirus and SV40 antigen-inducing particles in E46<sup>+</sup>, by means of fluorescent antibody (FA) staining, and its use for quantitative evaluation of attempts to dissociate SV40 neoantigen induction from adenovirus particles in E46<sup>+</sup> preparations.

#### *Materials and Methods*

*Viruses.*—The E46<sup>+</sup> strain of Ad. 7 was used after 5 to 8 undiluted virus passages in African green monkey kidney (AGMK) tissue cultures beginning with preparation E46 (1). Virus pools were prepared by scraping the cells into the fluid at the time of complete cytopathic effects (CPE), freezing and thawing twice, and distributing small aliquots into vials which were stored at -60°C. E46<sup>-</sup> virus (2), the virus recovered from pool E46 by 2 HEK-limiting dilution passages which does not induce SV40 neoantigen, was grown in human embryonic kidney (HEK) tissue cultures and harvested as above.

For comparing E46<sup>+</sup> virus with mixed SV40 and Ad. 7, the latter two viruses were grown as a mixed infection. Flask cultures of AGMK were infected first with 10<sup>8</sup> tissue culture infectious dose (TCID<sub>50</sub>) of SV40 strain 776 (5) and 24 hours later with 10<sup>8.2</sup> TCID of E46<sup>-</sup>. The cultures were harvested 4 days later, when CPE were complete.

Virus titrations were done in HEK cultures, using tubes held for at least 28 days, or plaque

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dishes, using a procedure described to us by Dr. R. M. McAllister and Dr. C. R. Goodheart; details of the plaque procedure are given in the accompanying paper (6).

*Tissue Culture Assays for Neoantigen Induction.*—For routine assay of the induction of immunofluorescent-stainable antigens, primary cultures of HEK cells were trypsinized and seeded into 60 mm plastic Petri dishes containing four 11 × 22 mm glass coverslips; each dish received  $5 \times 10^5$  cells in 3.5 ml of growth medium consisting of 10 per cent fetal bovine serum in Eagle's basal medium with penicillin, streptomycin, and glutamine (BME). The cultures were held at 37°C in 5 per cent CO<sub>2</sub> atmosphere. When the monolayer was confluent, the cultures were washed twice and the medium changed to 2 per cent agammaglobulinic calf serum in BME, and the cultures inoculated with 0.1 ml (in some experiments 0.2 ml or more) of appropriate virus dilution. At various times (21 to 24 hours for routine first cycle antigen assays) coverslips were removed, rinsed, and fixed in cold acetone.

Since SV40 virus does not induce antigen efficiently in HEK cultures, assays of the SV40-Ad. 7 mixed infection harvest were done in AGMK coverslip dishes, using the same procedure.

To assess possible helper effect of adenovirus on SV40 neoantigen induction, some materials were assayed in the presence of added adenovirus. E46<sup>-</sup> or Ad. 2 was mixed with the sample to be assayed, and 0.2 ml inoculated per dish. The E46<sup>-</sup> virus was used at a multiplicity of 5 TCID<sub>50</sub> per cell, and induced Ad. 7 neoantigen in 40 to 60 per cent of cells in 21 to 24 hours. The Ad. 2 was used at a multiplicity of about 10, and as described below, did not induce neoantigen stainable with the standard Ad. 12 serum.

*FUDR Inhibition Tests.*—Experimental and control cultures were rinsed twice and maintained in medium prepared with serum dialysed against 0.85 per cent NaCl. At various times before or after virus inoculation the medium was replaced with the same medium containing 5-fluorouracil deoxyriboside (FUDR).

*Fluorescent Antibody Staining.*—Procedures for fixing, staining, and reading FA tests have been described previously (2, 7, 8). Tests with hamster sera were done by the indirect procedure, and with rabbit serum by the direct test.

SV40-tumored hamster serum was from animals bearing transplants of the THK-1 line of SV40-transformed hamster kidney cells (9). The specificity of the SV40 neoantigen staining has been discussed before (2).

Hamster serum for staining adenovirus neoantigens was a pool of sera from animals with transplants of Ad. 12 (Huie strain) hamster tumors. Sera were selected for having high titer complement-fixing (CF) antibody to both Ad. 12 tumor and viral preparations. Although type-specific in CF, such serum pools react in the FA test with a neoantigen shared by a number of adenoviruses (8); the serum pool (Pool A) used here stained fleck-shaped antigens in nuclei of HEK cells infected with adenovirus types 12, 3, 4, 7, 8, and a simian adenovirus, but not with type 2. A more recent serum pool stained similar antigens in HEK cells infected with adenovirus types 1 through 18. Pool A stained fleck antigens in Ad. 12 hamster tumor cells, but was negative with Ad. 7 tumor cells. It was also negative with cells infected with SV40, mouse adenovirus, Gallus adenovirus-like virus, herpes simplex, vaccinia, Coxsackie A9, and reovirus 3.

These findings confirm our previous interpretation (8) that the antigen (or antigens) detected in Ad. 7 infected cells by staining with Ad. 12-tumored hamster serum is a virus-specific, group-reactive antigen which is apparently different from the ones in tumor and standard viral antigen preparations which react with the Ad. 12 serum in CF tests (10).

Detection of Ad. 7 viral antigen was done with standard rabbit anti-Ad. 7 typing serum. A pool of sera from 8 hamsters with Ad. 7-induced tumors was used in one experiment.<sup>1</sup>

*Heat Inactivation Tests.*—One ml samples of clarified virus stocks were sealed in 2 ml glass

<sup>1</sup> These sera were kindly provided by Dr. R. J. Huebner.

ampoules and submerged in a 50°C water-bath. Vials were removed at intervals and cooled by plunging into an ice-bath.

RESULTS

*Quantitation of Adenovirus and SV40 Antigen-Inducing Particles by Immunofluorescence.*—In the preceding report (2) data were presented which indicated that the E46<sup>+</sup> particles inducing SV40 neoantigen could be quantitated by immunofluorescent staining of cells late in the first cycle of infection. Since Ad. 7 infection also induces a neoantigen, detectable by staining with Ad. 12-tumored hamster serum (8), studies were done to determine if this antigen

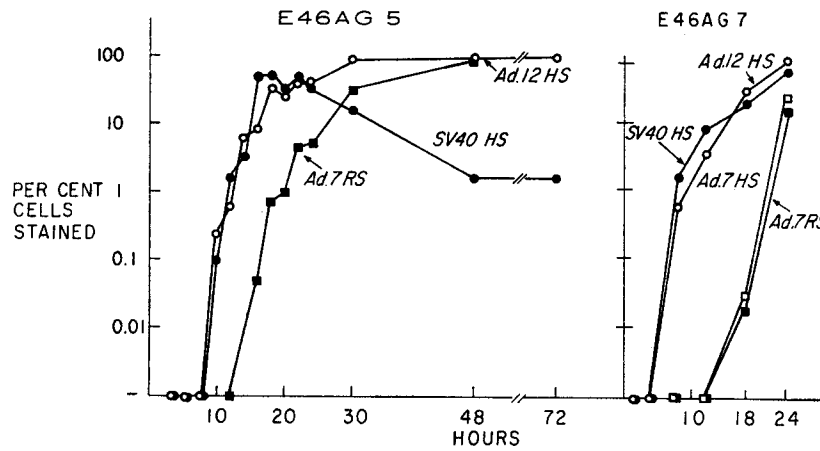


FIG. 1. Time course of development of FA-stainable antigens in HEK cells infected with E46<sup>+</sup>. HS, tumored hamster serum; RS, hyperimmune rabbit antiserum. The passage level beyond the E46 pool is identified by "AG" followed by the number of passages.

could be used to assay the number of particles carrying adenovirus genetic material.

First, the latent period for development of this adenovirus neoantigen, as well as other antigens, was determined. HEK cells were infected with AGMK-passed E46<sup>+</sup> virus at a multiplicity of about 2, and coverslips removed at various times and portions stained with the various antisera (Fig. 1). The first experiment is the same as that for which the SV40 neoantigen curve was presented in Fig. 3 of reference 2. In both experiments the latent periods for the SV40 and Ad. 7 neoantigens were identical: 9 to 10 hours in the first experiment, and about 6 hours in the second. In contrast, Ad. 7 viral antigen appeared 6 to 12 hours after the neoantigens, but increased at a comparable rate once initiated. Surprisingly, antigen detected with serum from hamsters carrying Ad. 7-induced tumors stained identically with that seen with Ad. 7

rabbit antiserum, both with regard to time course and the distribution of antigen in the nucleus.

Although all of the antigens studied were almost exclusively restricted to the nucleus, each showed a unique morphology. As described previously (7, 11), the SV40 neoantigen typically filled the nucleus, except for nucleoli, as homogeneously staining material; occasionally, the antigen was of more granular appearance. The Ad. 7 neoantigen presented a sequence of forms. In the earliest stages of high multiplicity infection, the antigen was seen as a few small bright dots (Stage 1); within several hours the nuclear antigen increased markedly and presented the characteristic fleck-shaped appearance (8) (Stage 2). By 24 to 30 hours the antigen was often reduced in amount and appeared to have condensed into thick strands, amorphous material, or ring-shaped masses (Stage 3). At 48 hours there were again large numbers of cells with Stage 2 staining, presumably representing second cycle infections. From experiments with various multiplicities of infection it appeared that Stage 2 and its Stage 3 sequel occurred during the first cycle chiefly with the higher multiplicities, and that cells infected with only one adenovirus particle showed only Stage 1 staining. Thus, it appeared that the amount of antigen induced was a function of input multiplicity. Two other staining patterns occasionally were seen with the Ad. 12 hamster serum, but could not be clearly associated with time or multiplicity; in one pattern the nucleus was filled with brightly staining granules, sometimes containing flecks also, while a rare pattern was a homogeneously stained nucleus, with nucleolar sparing, resembling SV40 neoantigen distribution.

The third class of antigen, Ad. 7 viral antigen, appeared as a small number of inclusion-like masses, generally on a homogeneously stained background.

The proportional relationship between dose of virus and percentage of cells developing the various types of antigen in the first cycle of infection is illustrated in Table I; the SV40 neoantigen data from this experiment were presented previously (2).

Since each Petri dish contained approximately  $10^6$  cells, the titer ( $\log_{10}$ ) of antigen-inducing particles could be calculated by adding 6.0 to the log of the proportion of cells stained plus the log of the dilution factor (Table I). Percentages greater than 50 per cent could be used only to establish a minimum titer estimate because of the plateau effect at high dosage. The titers of Ad. 7 and SV40 neoantigen-inducing particles were of comparable order of magnitude, while that of particles inducing Ad. 7 viral antigen at 20 to 24 hours was lower, as expected from its later development.

The adenovirus titer obtained by first cycle neoantigen induction was generally 0.5 to 1  $\log_{10}$  lower than the infectivity titer determined in HEK tubes or plaque dishes. This titer relationship was found also with E46<sup>-</sup>, Gomen, and Pinckney strains of Ad. 7.

These findings thus provided the basis for rapid, simple, and relatively precise quantitation, *i.e.*, by fixing a coverslip at 21 to 24 hours and staining one half with Ad. 12 hamster serum and the other half with SV40 hamster serum it was possible to quantitate both adenovirus and SV40 genetic carriers and to determine the relative proportion of each. In particular, the ratio of adenovirus to SV40-staining percentages provided an extremely sensitive method for evaluating attempts to separate the carriers of their genetic information. In general, E46<sup>+</sup> virus grown in AGMK cultures gave Ad./SV40 staining ratios of 1:2 to 2:1, while virus grown in HEK cells gave ratios of 2:1 to 3:1. The ratios, as well as titers, varied between tests, but were highly reproducible within tests.

TABLE I  
*Use of First Cycle Antigen Induction to Assay Titer of Particles Carrying Various Genetic Material in E46<sup>+</sup>\**

Dilution	Per cent of cells stained			Titer estimate (log <sub>10</sub> )		
	SV40 HS‡	Ad. 12 HS	Ad. 7 RS§	SV40 HS	Ad. 12 HS	Ad. 7 RS
10 <sup>0</sup>	52			5.72		
10 <sup>-0.5</sup>	17.7	10.5	2.0	5.75	5.52	4.80
10 <sup>-1.0</sup>	7.1			5.85		
10 <sup>-1.5</sup>	1.5	1.2	0.22	5.68	5.58	4.84
10 <sup>-2.0</sup>	0.75			5.88		
10 <sup>-2.5</sup>	0.33	0.18	0.04	6.02	5.76	(5.10)

\* 0.2 ml of dilutions of E46AG5 (the fifth AGMK passage beyond pool E46) were added per Petri dish of HEK cells, and the cells fixed at 20 hours. This virus titered 10<sup>6.8</sup> TCID<sub>50</sub> per 0.2 ml in HEK tube cultures.

‡ HS, tumored hamster serum.

§ RS, rabbit antiviral serum.

*Effect of FUDR on Antigen Induction.*—Further insight into the nature of the induction of adenovirus and SV40 neoantigens by E46<sup>+</sup> was obtained from inhibitor experiments. Rapp *et al.* (12) have shown that SV40 neoantigen induction by either SV40 or E46<sup>+</sup> is unaffected by addition of cytosine arabinoside to cultures 1 hour after infection, while formation of SV40 viral antigen is markedly inhibited; and Gildea *et al.* (13) showed that pretreatment with FUDR had a similar effect. Similarly, FUDR treatment had no effect on induction of either adenovirus or SV40 neoantigens by E46<sup>+</sup> (Table II), even when cells were pretreated with 10<sup>-4.1</sup> M concentration. In contrast, Ad. 7 viral antigen staining was completely prevented. It is of interest that FUDR did not inhibit development of CPE.

In view of these findings, together with evidence presented above that the

TABLE II  
Effect of FUDR on Formation of Adenovirus and SV40 Antigens in HEK cells Infected with E46+

Exp. No.	Virus dilution	FUDR		21 hours			48 hours					
		Concentration	Time added hours	CPE	Staining			CPE	Staining			
					Ad. 12 HS per cent	SV40 HS per cent	Ad. 7 RS per cent		Ad. 12 HS per cent	SV40 HS per cent	Ad. 7 RS per cent	
1	10 <sup>0</sup>	None	4	±	18	17	0.34					
		10 <sup>-4.5M</sup>		±	22	14	<0.01					
2	10 <sup>0</sup>	None	-1	+	(80)*	(85)		++	(80)	(100)	(50)	<0.01
		10 <sup>-4.5M</sup>		+ to ++	(90)	(95)		+++	(100)	(98)		
	10 <sup>-1</sup>	None	-1	±	36	19		+ to ++	29	15	(30)	<0.01
		10 <sup>-4.5M</sup>		± to +	27	22		++ to +++	61	50		
10 <sup>-2.3</sup>	None	10 <sup>-4.5M</sup>	-1	±	1.6	1.3		±	7.6	2.7	3.9	
				±	2.5	0.9		±	6.1	3.6	<0.01	

\* Figures in parentheses are estimated percentages.

amount of Ad. 7 neoantigen per nucleus was a function of virus dose, it seems probable, as pointed out by Gilden *et al.* (13), that the neoantigens are coded for by input virus DNA, while viral antigen requires new DNA synthesis.

It should be noted that the adenovirus neoantigen in FUDR-treated cultures did not show the transition from Stage 2 to Stage 3 morphology, suggesting that an event occurring during DNA synthesis or viral antigen formation is responsible for the change in pattern.

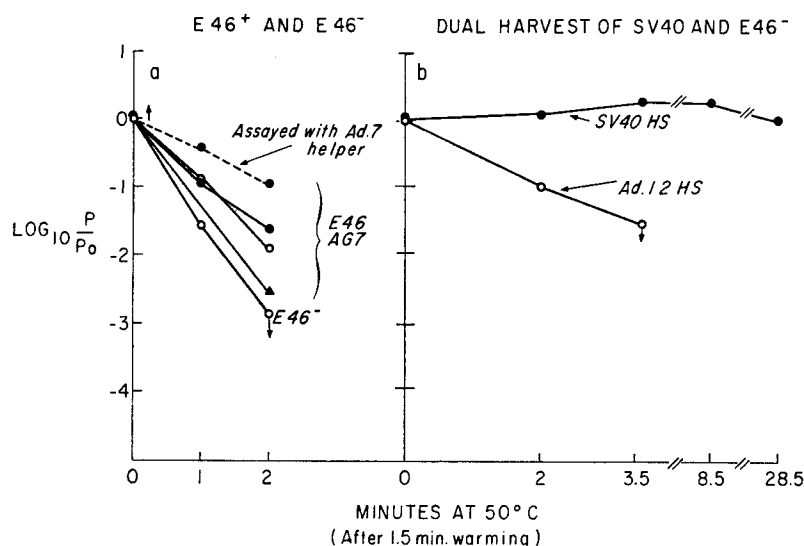


FIG. 2. Heat inactivation of E46<sup>+</sup>, E46<sup>-</sup>, and Ad. 7-SV40 mixed infection preparations, assayed by first cycle neoantigen induction. P/P<sub>0</sub> is the surviving fraction. ▲, infectivity titer; HS, tumored hamster serum. The passage level beyond the E46 pool is identified by "AG" followed by the number of passages.

*Attempts to Dissociate Adenovirus and SV40 Neoantigen-Induction by Heat and Centrifugation.*—In the previous report (2) we showed that SV40 neoantigen induction by E46<sup>+</sup> was abolished by heating at 56°C for 10 minutes, suggesting adenovirus rather than SV40 heat stability characteristics. To obtain more precise information, studies were done with 50°C heating; inactivation curves were studied of standard E46<sup>+</sup> and E46<sup>-</sup> preparations (AGMK and HEK grown, respectively) (Fig. 2 a), and E46<sup>+</sup>, E46<sup>-</sup>, and Gomen strain Ad. 7 grown in the same batch of HEK cultures to minimize the effect of medium variation on inactivation rate (Fig. 3). Also, the mixed infection harvest of E46<sup>-</sup> and SV40 was studied (Fig. 2 b).

As seen from the figures, the inactivation rates of the adenovirus and SV40 antigen-inducing components in the mixed harvest were markedly different,

while in E46<sup>+</sup> preparations they were indistinguishable. In view of the possibility that the identity of the inactivation curves for SV40 and Ad. 7 neoantigen could be due to the adenovirus being necessary to provide a helper effect for SV40 neoantigen induction, the heated E46<sup>+</sup> materials were also assayed in the presence of unheated adenovirus. In Fig. 2 *a*, E46<sup>-</sup> was used as helper; a 3- to 4-fold increase in SV40-staining percentage was found, but as far as could be determined the inactivation rate was not affected. In Fig. 3, Ad. 2 was used because the pool of Ad. 12 hamster serum then in use did not stain Ad. 2 neoantigen, and it was thus possible to measure if this helper phenomenon affected only SV40 carriers or adenovirus carriers as well. As seen from Fig. 3

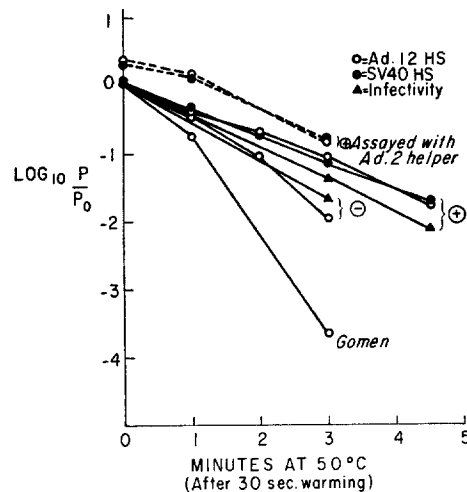


FIG. 3. Heat inactivation of Ad. 7 viruses, and effect of Ad. 2 helper on adenovirus and SV40 neoantigen induction by E46<sup>+</sup>. HS, tumored hamster serum.

there was again a helper effect, but of equal magnitude for both SV40 and Ad. 7 neoantigen induction and without effect on the slope of the inactivation curve.

It can thus be concluded that the SV40 and Ad. 7 neoantigen-inducing particles in E46<sup>+</sup> preparations are inactivated at identical rates at 50°C. This finding is similar to that of Easton *et al.* (14), who showed that SV40 infectivity in a possibly hybridized Ad. 4 preparation is inactivated with adenovirus kinetics.

Several additional points in Figs. 2 *a* and 3 are noteworthy. In both experiments, as well as in another test not shown here, E46<sup>-</sup> virus was inactivated more rapidly than E46<sup>+</sup>; Gomen strain was much more heat sensitive. Also, the infectivity of the E46<sup>+</sup> preparations was lost at a somewhat faster rate



than antigen induction, while with the E46<sup>-</sup> virus the rates were the same. The reasons for these differences are not known.

Attempts to dissociate Ad. 7 and SV40 neoantigen-inducing particles by isopycnic equilibrium density gradient or simple ultracentrifugation were also unsuccessful.

For centrifugation testing the starting materials were treated with chloroform (20 per cent, 4°C 2 hours), clarified, and then trypsin-treated (15) (0.01 per cent trypsin, 37°C 30 minutes) to break up possible aggregates. Fig. 4 shows

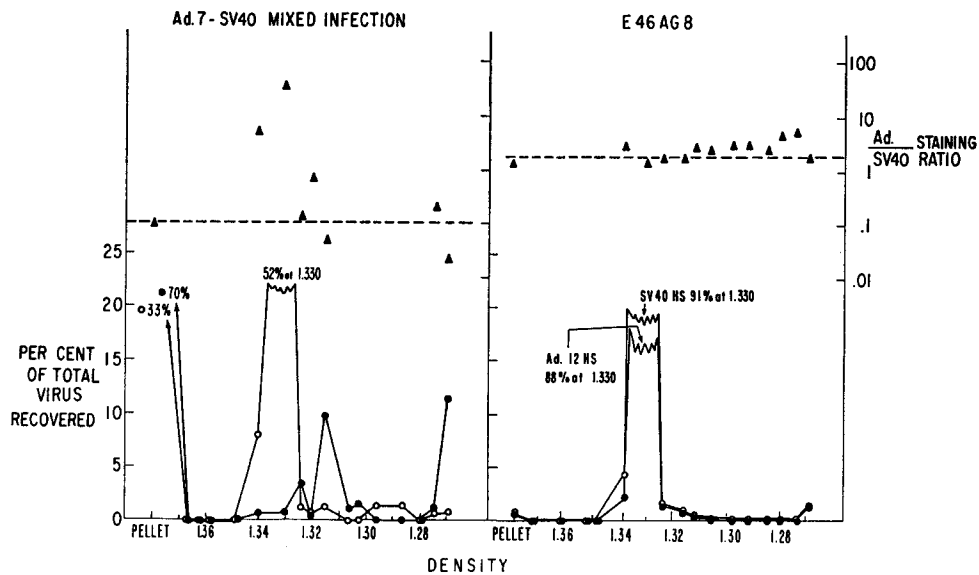


FIG. 4. Equilibrium density gradient centrifugation of E46<sup>+</sup> and Ad. 7-SV40 mixed infection preparations, assayed by first cycle neoantigen induction. O, Ad. 12-tumored hamster serum; ●, SV40-tumored hamster serum; ▲, Staining ratio. The dashed line is the staining ratio of the starting material. The passage level beyond the E46 pool is identified by "AG" followed by the number of passages.

results of centrifuging E46<sup>+</sup> and the Ad. 7-SV40 mixed infection viruses in a RbCl equilibrium density gradient run (SW39 Spinco rotor, 100,000 g average, 44 hours). The gradient readily resolved SV40 and adenovirus particles in the mixed infection harvest, but gave no indication of separation with the E46<sup>+</sup> virus, both activities banding at 1.330; an identical result was obtained in a second run with a different E46<sup>+</sup> preparation.

The contrast between the two preparations is clearly shown by the Ad./SV40-staining per cent ratios, shown at the top of Fig. 4. With the mixed virus harvest, the ratios in the gradient fractions ranged from 36:1 to 1:42,

a 1500-fold range, while with E46<sup>+</sup> the range was only from 5.5:1 to 1.5:1, or 3.7-fold.

In all of the gradient tests the Ad. 7 and SV40 antigen-inducing particles in the starting material were quantitatively recovered in the gradient fractions.

Likewise, simple ultracentrifugation at 10,000 g for 1 hour (Fig. 5) clearly separated adenovirus and SV40 in the dual infection preparation, but gave identical sedimentation of the Ad. 7 and SV40 neoantigen-inducing particles in E46<sup>+</sup>; again assay with E46<sup>-</sup> helper showed a slight helper effect, but did not change the pattern of sedimentation.

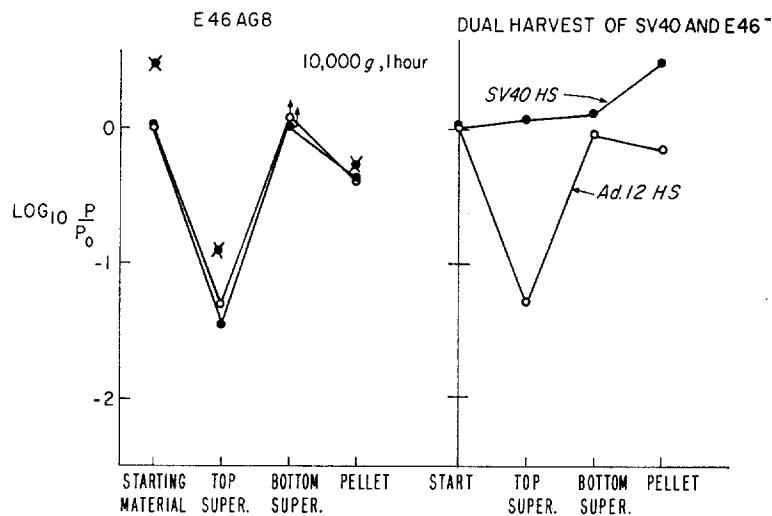


FIG. 5. Ultracentrifugation of E46<sup>+</sup> and Ad. 7-SV40 mixed infection preparations, assayed by first cycle neoantigen induction.  $\times$  indicates SV40 neoantigen staining of fractions assayed with E46<sup>-</sup> helper. HS, tumored hamster serum. The passage level beyond the E46 pool is identified by "AG" followed by the number of passages.

#### DISCUSSION

Since the quantitative assay procedure described here is central to our elucidation of E46 virus, it is important to examine the inference that it is giving direct information on the genetic content of the virus population. On the most important question, "is the visualization of antigen a direct indicator of activity of a specific viral DNA," there seems to be little doubt. The specificity of the Ad. 12- and SV40-tumored hamster serum staining is well established. That the portion of the virus which induces neoantigen formation is DNA is indicated by the findings that SV40 DNA induces neoantigen (16) and that ultraviolet irradiation of E46<sup>+</sup> preparations decreases Ad. 7 and SV40 neoantigen-inducing titers at the same rate as viral infectivity (17).

A second important question is whether the Ad./SV40-staining ratio can be taken as the actual ratio of the respective genomes in the preparation, *i.e.*, are the two antigens induced with equal efficiency. To some extent this cannot be precisely correct, since the ratios differ (through about a 4-fold range) in different lots of HEK cultures; however, this variation is relatively minor, and like the variations in antigen-induction titers between tests, may only reflect differences in latent period of antigen induction, as seen in Fig. 1.

Together with the previous evidence (1-3), the quantitative studies on heat-stability and centrifugation of E46<sup>+</sup> reported here leave no alternative but that the SV40 genetic portion is carried in an adenovirus capsid. Although a slight helper effect of adenovirus on SV40 neoantigen induction was observed, this could not explain the dependence of SV40 antigen induction on adenovirus infectivity, for the following reasons. The helper effect appeared to be equally operative on adenovirus particles, as indicated by the increased adenovirus-staining percentage; the heat inactivation rate and centrifugation characteristics of SV40 antigen-inducing particles were not altered by assay with helper; and the SV40 antigen-inducing activity was quantitatively recovered in the centrifugation experiments, showing that its association with adenovirus activity could not be due to the adenovirus being the limiting factor in the assay, with the SV40 material being distributed in other fractions.

Although we have referred to the E46 particles carrying SV40 genetic material as hybrids, the word must be considered in a qualified sense until it can be established whether or not the SV40 and Ad. 7 genetic materials are actually in the same capsid.

#### SUMMARY

The titers of adenovirus and SV40 genetic carriers in hybrid preparations (E46<sup>+</sup>) can be quantitated by determining the percentage of cells showing neoantigens stainable with fluorescent antibody (FA) at 21 to 24 hours; both titers can be obtained with a single coverslip. The adenovirus and SV40 antigen-inducing titers so obtained are of the same order of magnitude in stock preparations of E46<sup>+</sup> grown in either African green monkey kidney (AGMK) or human embryonic kidney tissue culture (HEK), and are generally within one log of the infectivity titer.

Quantitative studies of 50°C heat inactivation, ultracentrifugation, and equilibrium density gradient centrifugation of E46<sup>+</sup> gave no indication that SV40 neoantigen induction could be dissociated from adenovirus, whereas adenovirus and SV40 virus grown as a mixed infection were readily dissociated by these procedures.

Pretreatment of HEK cells with a medium containing 5-fluorouracil desoxyriboside (FUDR) did not affect induction of either adenovirus or SV40 neoantigen or development of cytopathic effects after infection with E46<sup>+</sup>, but did prevent formation of Ad. 7 viral antigen.

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