Encapsidation of Free Host DNA by Simian Virus 40: A Simian Virus 40 Pseudovirus

Abstract. *Under specified growth conditions, simian virus 40 encapsidated host DNA in a noncircular form free of viral DNA. Two bands of virus particles were present in cesium chloride equilibrium density centrifugation. The host DNA species contained in the upper band was of a lower molecular weight than the DNA present in the mature virus in the lower band.*

The phenomenon of encapsidation of host DNA has been shown to exist in polyoma virus (*1, 2*). Simian virus 40 (*2*), another member of the papovavirus group, has not been shown to contain within the viral capsid host cell DNA unlinked to viral DNA. Aloni *et al.* have presented evidence suggesting that the SV40 genome may contain host DNA sequences as part of the viral DNA (*3*). We now report that a portion of the host DNA becomes encapsidated and that this portion has a lower molecular weight than virus DNA has. Plaque purified, SV40 small plaque (SP) was grown on Vero cells, a continuous green monkey cell line. During the purification of virus passed in Vero cells, two bands became apparent in CsCl equilibrium density centrifugation. The upper virus band, which has a lower density in CsCl, was found to contain host DNA and DNA species of a lower molecular weight than that present in the lower virus band. Additional experiments, in which cell DNA was labeled with [^{32}P]orthophosphate prior to infection, have demonstrated encapsidation of linear host DNA with a sedimentation value of 15.

The SV40 SP was separated from disrupted Vero cells and culture medium by zone sedimentation on to a CsCl cushion (1.40 g/cm^3) followed by two cycles of CsCl equilibrium density centrifugation. At these concentrations CsCl dissociates DNA-protein complexes and leaves intact complete virus stripped of extracapsid DNA. Further, in certain experiments, the virus was treated with pancreatic deoxyribonuclease and ribonuclease after CsCl centrifugation. Virus DNA for immobilization on filters was extracted by lysing the virus in 1 percent SDS at 50°C. Then CsCl was added to precipitate the SDS, the precipitate was removed by centrifugation, and the double-stranded circular DNA-I was separated by equilibrium density centrifugation in CsCl containing ethidium bromide (*4*). The dye was removed by treatment with Dowex-50, and the CsCl was removed by Sephadex G-100 gel filtration. Host DNA was prepared by conventional methods (*5*). DNA-DNA hybridization was performed basically as described by Denhardt (*6*) but on a microscale (*7*). The SV40 DNA-I to be immobilized was first heated at 100°C for 15 minutes in SSC (*2*) to break the circle, denatured with alkali, rapidly diluted in 4 × SSC, neutralized, and finally poured through a nitrocellulose membrane filter, 5.0 cm in diameter. Host cell DNA was similarly treated, but the heating step was omitted. Filters containing DNA and blank control filters, 7 mm in diameter, were cut from the large filters and given preliminary incubation at 68°C under Denhardt's conditions. After this incubation period, samples of fragmented (sheared in a Biosonik II to approximately 400,000 daltons), heat-denatured, and labeled DNA to be tested were introduced into test tubes (10 by 25 mm) with the filters. The final reaction mixture (0.25 ml) contained 1.0*M* NaCl, 0.004*M* TES, *p*H 7.5, and PM (*2*).

Virions containing host DNA, termed SV40 pseudovirions, were observed after passage of plaque-purified SV40 SP in Vero cells (passage numbers 165 to 170) at an input multiplicity of 1 to 10 plaque-forming units per cell. After CsCl equilibrium centrifugation, the virus was distributed into two bands. Although the appearance of the upper band resembled that of defective virus, the band contained host DNA and noncircular DNA. The appearance of DNA in a form other than the double-stranded circle distinguishes these particles from the defective particles described by Yoshiike (*8*).

Vero cells were grown in a half-gallon roller bottle in Eagle's medium with 10 percent fetal bovine serum (FBS). At confluency, the cells were infected, at an input multiplicity of 1, with SV40 SP passed two times in Vero cells. After a 2-hour adsorption period, fresh medium with 5 percent FBS was added. Twenty-four hours after infection, the medium was removed and fresh medium (100 ml) with 2 percent FBS and 250 μc of [^{3}H]thymidine (14 c/mmole) was added. The cells and culture medium were harvested 72 hours later and the virus was purified as described earlier (Fig. 1). The virus shows a peak at 1.348 g/cm^3 with a shoulder at 1.341 g/cm^3. Two bands are clearly apparent on examination of the tube but are only partially resolved on tube puncture and drop collection. The virus particles in both pools (pool 1, fractions 24 to 28; pool 2, fractions 30 to 36) are indistinguishable by negative staining in the electron microscope and are similar in size. As was determined in the Spinco Model E ultracentrifuge the sedimentation coefficient ($s^0_{20,w}$) value of pool 1 is 211; that of pool 2 was 224. However, differences are readily apparent when virus pools are lysed in 0.1*N* NaOH and sedimented in an alkaline sucrose gradient, with an SV40 SP marker made on low-passage Vero cells (passage 40) at an input multiplicity of 0.01 (Fig. 2).

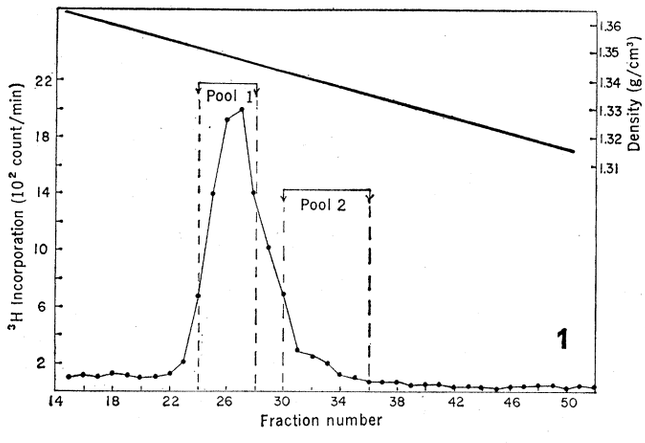
The alkaline sedimentation profile of the DNA from pool 1 closely resembles that of the DNA from the marker virus. Since the double-stranded, closed, circular DNA of the mature virus sediments at 53*S* under alkaline conditions, this DNA form can be readily distinguished from linear and nicked, circular DNA forms that have the same molecular weight but have components sedimenting at 18 and 16*S* under alkaline conditions (*9*). In pool 1, 75 percent of the DNA is in the closed circular form. However, in pool 2, only 50 percent of the DNA is in the closed circular form. The remaining 50 percent is not only in single circles and linear DNA but in even shorter pieces, that is 6*S* or smaller, sedimenting close to the meniscus. The profile of pool 2 further indicates that the DNA in the 53*S* and 18*S* regions has a slightly lower molecular weight than the DNA in the marker virus has. The DNA from pool 2 in these two regions is skewed toward the slower sedimenting side of the 53*S* and 18*S* peaks of the DNA from the marker virus. The lower molecular weights of the DNA's in virus particles of pool 2 account for its lower density, relative to pool 1. The lower molecular weight of the DNA in a virus particle decreases the DNA-protein ratio which

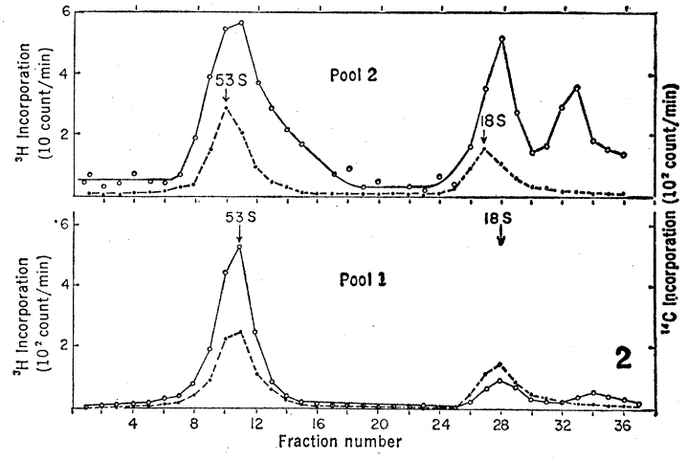
has the effect of lowering the density of the whole particle.

Hybridization of the DNA from the two pools with the host DNA shows that, whereas 3.3 percent from pool 1 hybridizes with host DNA, 13.0 percent from pool 2 hybridizes with host DNA. Since specific activities were approximately equal, the host DNA content of pool 2 is at least four times that of pool 1. In a subsequent experiment, hybridization was performed with material recovered from an alkaline sucrose gradient (as in Fig. 1). From the 18*S* region of pool 2, 16.8 percent of the DNA hybridized with the host DNA, whereas from the 18*S* region of pool 1 only 3.0 percent of the DNA hybridized with the host. The DNA from the 53*S* regions of pools 1 and 2 reacted only with viral DNA. No reaction with host DNA was detected.

To delineate the encapsidation of host DNA by the SV40 virus, Vero cells were grown in three half-gallon roller bottles in phosphate-free Eagle's medium (150 ml) with 3 percent normal FBS, 6 percent FBS dialyzed against 0.15*M* NaCl, and 2.5 mc of carrier-free [^{32}P]orthophosphate. When the cells were confluent, they were washed with complete medium and then exposed to complete medium with 10 percent normal FBS. One day later, the cells were infected at a multiplicity of 5 with SV40 SP that had been passed twice in Vero cells. After a 2-hour adsorption period, fresh complete medium with 2 percent FBS was added. At 96 hours, the cells and culture medium were harvested, and the virus was purified as described, except that an additional CsCl equilibrium centrifugation was used. The two discrete bands were collected separately with a curved needle and syringe rather than by the drop method. The upper band (1.341 g/cm^3) and the lower band (1.348 g/cm^3) were dialyzed and treated with pancreatic deoxyribonuclease and ribonuclease as described in Table 1. A fraction of each band was lysed in NaOH, neutralized with HCl, sonicated, denatured by heat, and then tested by hybridization against Vero and SV40 DNA's. The virus from the upper band is rich in host DNA, whereas the virus from the lower band has mainly viral DNA (Table 1). On the basis of a comparison of these results with the extents of reaction in control experiments (Table 1, numbers 5 and 8), the ^{32}P from the upper band is primarily in host DNA. Because of the size of host DNA and presence of unique sequences, host DNA reacts to a lesser extent than viral DNA.

The virus from the upper and lower bands was lysed in NaOH, and the DNA was analyzed in alkaline sucrose density gradients (Fig. 3). The [^{3}H]-thymidine-labeled DNA from marker virus, grown on Vero cells with seed passed one time in Vero cells, had two peaks, 53*S* and 18*S*, in a 1 : 1 ratio. The 53*S* peak represents the double-stranded intact circle of the mature virus; the 18*S* peak represents the single





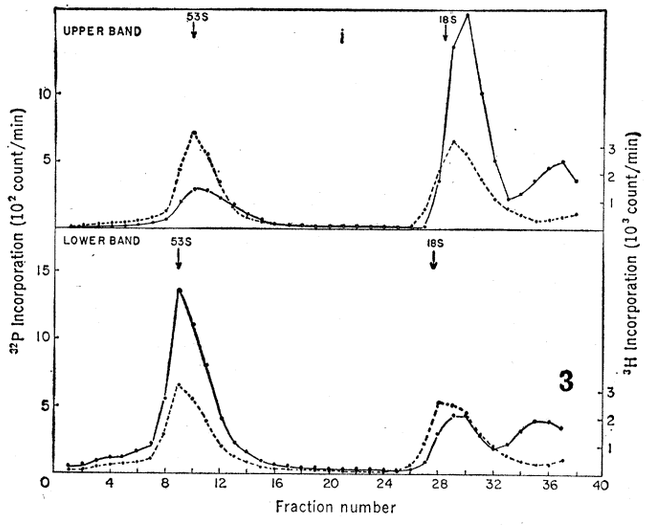


Fig. 1. Purification of SV40 from Vero cells, labeled with [^{3}H]thymidine after infection, in a second CsCl density gradient in the angle-head Spinco No. 65 rotor (35,000 rev/min for 18 hours). Fractions were collected from the bottom of the centrifuge tube, and the radioactive material in a sample of each fraction was determined by precipitating with trichloroacetic acid, collecting on a membrane filter, and counting in a scintillation counter. Fig. 2. Band sedimentation in alkaline sucrose gradients of [^{3}H]thymidine-labeled pools 1 and 2 with [^{14}C]thymidine-labeled marker virus. Virus samples were lysed in 0.1*N* NaOH and sedimented through a sucrose gradient (5 to 20 percent) in 0.30*M* NaOH, 0.70*M* NaCl, 0.01*M* tris-HCl, *p*H 7.5, 0.001*M* EDTA at 40,000 rev/min for 4 hours in the Spinco SW 65 rotor. Fractions were collected from the bottom of the tube, and each fraction was precipitated with trichloroacetic acid, collected on a membrane filter, dried, and counted. Fig. 3. Band sedimentation in alkaline sucrose gradients of upper and lower band virus prepared from ^{32}P-labeled cells with [^{3}H]thymidine–labeled marker virus. Virus samples, lysed in NaOH, were sedimented through an alkaline sucrose gradient (5 to 20 percent) as described for Fig. 2.

Table 1. Hybridization of DNA from upper and lower bands of SV40 with SV40 and Vero cell DNA's. DNA on Vero filter, 10 μg; on SV40 filter, 0.6 μg. Prior to DNA extraction, virus was treated with pancreatic deoxyribonuclease (20 μg/ml) and pancreatic ribonuclease (75 μg/ml) for 1 hour at 25°C in phosphate-buffered saline with 0.004*M* $MgCl_2$.

Reaction No.	DNA		Amounts incubated (count/min)	Specifically bound (% of input)
	On filter	Source in solution		
1	SV40	Upper band	7500 (^{32}P)	16.2
2	Vero*	Upper band	7500 (^{32}P)	17.9
3	SV40	Lower band	9100 (^{32}P)	26.4
4	Vero	Lower band	9100 (^{32}P)	5.3
5	SV40	SV40 DNA-I	2800 (^{14}C, 0.12 μg)	61.9
6	Vero	SV40 DNA-I	2800 (^{14}C, 0.12 μg)	0.76
7	SV40	Vero DNA	28000 (^{14}C, 1.4 μg)	0.03
8	Vero	Vero DNA	28000 (^{14}C, 1.4 μg)	24.1

* As performed, the DNA-DNA hybridization tests between Vero cell DNA and sample measure only the repetitive nucleotide sequences, not the unique sequences. Single copies of SV40 DNA in the host DNA would not have been detected (*11*).

Table 2. DNA-DNA hybridization tests between Vero cell DNA and two components of ^{32}P-labeled SV40 isolated by band sedimentation in alkaline sucrose gradients. DNA on Vero filter, 10 μg; on SV40 filter, 0.6 μg.

Reaction No.	DNA		^{32}P incubated (count/min)	Radioactivity	
	On filter	In solution		Bound to filter (count/min)	% of input
		Upper band virus			
1	SV40	53*S*	960	616	64.2
	None			1	0.1
2	Vero*	53*S*	960	0	0
	None			0	0
3	SV40	18*S*	2300	219	9.5
	None			1	<0.1
4	Vero	18*S*	2300	626	27.2
	None			2	<0.1
		Lower band virus			
5	SV40	53*S*	1675	871	52.0
	None			0	0
6	Vero	53*S*	1675	1	<0.1
	None			0	0
7	SV40	18*S*	920	237	25.8
	None			1	0.1
8	Vero	18*S*	920	186	20.2
	None			0	0

* See footnote to Table 1.

strands of linear and nicked, circular DNA's. The upper band virus harvested from cells previously labeled with ^{32}P yielded 53*S* and 18*S* components in a ratio of 1 to 3.4, whereas the lower band virus yielded 53*S* and 18*S* components in a ratio of 1 to 0.36.

This difference in ratios is a reflection of the differing content of host DNA in the upper and lower bands (Table 1). To use hybridization to identify the slower sedimenting component at 18*S* as the host DNA, sedimentation in the alkaline sucrose gradients was repeated in the absence of marker. The [^{32}P]DNA's from the 53*S* region and from the 18*S* region were collected, dialyzed, sonicated, denatured with heat, and then tested against Vero and SV40 DNA's. The ^{32}P from the 53*S* regions from the upper and lower bands of virus was bound efficiently to the SV40 DNA filter but not at all to the host DNA filter (Table 2). The ^{32}P from the 18*S* regions reacted with both the host and viral DNA filters. However, the extent of reaction with the host filter from upper band is threefold greater than with the viral filter. Comparison of these results with the extents of reaction in control experiments (Table 1, numbers 5 and 8) indicates that the ^{32}P in the 18*S* region of the upper band virus is primarily in host DNA, not in viral DNA. The appearance of ^{32}P in viral DNA in the 53*S* region, the intact double-stranded circle, is presumably a result of either complete breakdown and resynthesis of preexisting DNA or an incomplete elimination of ^{32}P of intracellular pools of nucleotides.

Figure 3 shows, in addition to the 53*S* and 18*S* peaks, a third peak sedimenting at a rate slower than that of 18*S*. This last peak, also evident in Fig. 1, was identified by hybridization as containing both viral and host DNA's. The absence of this peak from the marker virus, lysed and sedimented in an identical manner, suggests that it is not simply degraded DNA.

In order to estimate accurately the size of the host DNA incorporated into the upper band virus, the virus was lysed in NaOH and centrifuged for 8.5 hours in an alkaline sucrose gradient (14.0 ml) with a Spinco SW40 rotor. The 18*S* and 16*S* components of the enzymatically nicked, circular SV40 DNA-II (*9*) served as markers. The major ^{32}P peak was at 15*S*, whereas the single linear strand of the viral DNA had a 16*S* value. In terms of molecular weight, the host strand is 15 percent smaller than the viral strand (*10*).

To test for contamination of the viral preparations with host DNA, reconstruction experiments were performed in which Vero cells labeled with [^{3}H]thymidine were combined with SV40-infected Vero cells and culture medium. Then SV40 virus was purified as described. There were no detectable peaks of ^{3}H in the virus portion of the second CsCl equilibrium centrifugation. Only 30 count/min of 10^6 count/min added were present in the lower density virus band. Further, treatment of the virions and pseudovirions with nucleases did not alter the results.

The physical properties of the encapsidated host DNA of the pseudovirions suggest the presence of a linear DNA molecule somewhat smaller than the DNA of the virus. Hybridization has shown that the intact double-stranded circle contains only viral DNA; there was no host DNA present.

The presence of SV40 pseudovirions containing sufficient host nucleic acid for the coding of four to five host proteins suggests an efficient mechanism for gene transfer from one host cell to another.

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2. Abbreviations: SV40 is simian virus 40; SSC is standard saline-citrate, 0.15*M* NaCl and 0.015*M* trisodium citrate; 4× SSC is quadruple-strength SSC; TES is *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PM (Denhardt's "preincubation" mixture) contains 0.02 percent bovine serum albumin, 0.02 percent Ficoll, and 0.02 percent polyvinylpyrrolidone; SDS is sodium dodecyl sulfate.
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Number Coding in Association Cortex of the Cat

Abstract. *In electrophysiological investigations of single neurons in cortical association response areas of the cat, cells have been encountered that appear to code the property of number. In a sequence of stimulus presentations, these cells characteristically discharge to a particular numbered stimulus in the series. This effect is independent of stimulus modality, intensity, and interstimulus interval; thus, the cells seem to be responding to the number of stimulus presentations.*

Bertrand Russell, in his classic analysis of the concept of number, defined number as "anything which is the number of some class; the number of a class is the class of all those classes that are similar to it" (*1*). Number is a property of stimuli that is independent of all the particular properties of the stimuli and is determined solely by relational class. Results of number perception and memory studies in man suggest that number of objects can be estimated and recalled relatively accurately up to about seven "plus or minus two" (*2*). Animal studies of counting behavior indicate that primates can learn to respond to number of objects in simultaneous presentations (*3*) and that cats can learn to "count" successive stimuli (*4*).

Higher mammals thus appear able to abstract the number of stimuli, independent of the specific aspects of the stimuli; consequently, the brain must in some manner code the number of stimuli. To demonstrate that a neural response is, in fact, coding number, it is necessary to show that the response is at least to some degree independent of particular stimulus characteristics such as quality, intensity, and frequency of presentation. A logical possibility for the locus of this coding process would be higher regions of the brain, where responses to stimuli tend to be somewhat independent of specific stimulus parameters, an example being the polysensory association-response areas of the cerebral cortex (*5*). Several lines of evidence have implicated these regions in "attentive" aspects of behavior, where response is a function of the more abstract aspects of stimuli such as complexity, recency, and "significance" (*6*). Lindsley recently proposed that these areas may subserve the more complex aspects of behavioral alerting and attention (*7*). Counting number of stimuli would seem a relevant aspect of such behavior. In work on properties of polysensory cells in these regions of the cortex in the cat, we have encountered cells that do in fact appear to code number; they behave as though they are counters.

Animals were anesthetized with chloralose (70 mg/kg, intraperitoneally), and isolated single cell activity was recorded by standard techniques with glass-coated tungsten microelectrodes. Stimuli were free field click, binocular light flash, and single shock pulses (0.25 msec duration) to ipsilateral forepaw. The existence of counting cells is revealed when a sequence of stimuli is presented after a period of no stimulation. The cell typically responds to a particular stimulus in the sequence. Such cells also respond occasionally to other stimuli in the sequence, though with a much lower probability, and sometimes to more than one stimulus in the sequence. If the stimulus sequence is continued without interruption, counting cells tend to respond successively at each appropriate stimulus in the sequence, but the pattern is somewhat less clear.

An example of a "number 7" cell (a cell "coding" the concept of number 7) in the association cortex of the cat is shown in Fig. 1A, both in terms of probability of first discharge in the sequence and in terms of total proportion of discharges to each stimulus in the sequence. This discharge pattern differs significantly from a random distribution ($N = 23$, $D = 0.513$, $P < .01$; Kolmogorov-Smirnov one-sample test). Ten separate sequences of ten stimuli (here a trimodal, simultaneous click, flash, and shock) were given with a 2-second interstimulus interval and a 2-minute intersequence interval. The distribution of responses shown in Fig. 1A, particularly the proportion of total responses around the modal seventh stimulus, is strikingly similar to the distribution of behavioral responses in cats trained to respond to a particular stimulus (for example, sixth) in a sequence (*4*).

Responses of counting cells appear to be independent of stimulus modality. An example of a "number 6" cell in the association cortex of the cat is shown in Fig. 1B. In ten sequences of ten stimuli at an interstimulus interval of 1 second, the cell exhibits a clear modal response to the sixth stimulus for both auditory ($N = 14$, $D = 0.429$, $P < .01$) and visual ($N = 13$, $D = 0.423$, $P < .05$) stimulation. Effect of varying the interstimulus interval is also illustrated for this cell in Fig. 1B. The modal value remains the same for an interstimulus interval of 4 seconds and 1 second ($N = 16$, $D = 0.338$, $P < .05$) with an auditory stimulus. Responses of counting cells also appear to be independent of stimulus intensity, at least within certain limits. Although higher stimulus intensities occasionally result in increased overall discharge levels, the modal stimulus number does not shift.

To date we have observed five counting cells in the adult cat, which code the numbers 2, 5, 6 (two cells), and 7. With such a small sample, it is not possible to make a precise estimate of the proportion of cells in nonspecific association response areas of the cortex that "count." However, crude guesses based on the proportion of such cells that we have observed suggest that in the cat about 1 percent of the cells in association areas that respond to stimuli are counting cells (that is, five in a sample of about 500).

The data given above indicate that