*ACTIVATION OF INFECTIOUS SV40 DNA SYNTHESIS IN TRANSFORMED CELLS**

By Saul Kit, T. Kurimura, M. L. Salvi, and D. R. Dubbs

DIVISION OF BIOCHEMICAL VIROLOGY, BAYLOR UNIVERSITY COLLEGE OF MEDICINE, HOUSTON, TEXAS

Communicated by Albert B. Sabin, May 8, 1968

The *entire* SV40 genome can be recovered from at least some SV40-transformed cell lines which do not spontaneously produce this virus. Even after repeated cloning in the presence of SV40-antisera, these cell lines can be activated to produce SV40 if they are cocultivated with susceptible monkey kidney cells. Rescue of SV40 is greatly facilitated when mixtures of susceptible and transformed cells are treated with ultraviolet-inactivated Sendai virus (UV-Sendai), a procedure known to produce heterokaryons.[1-9]

Despite the presence of the entire SV40 genome in transformed cells, Sabin and Koch[1] failed to detect infectious SV40 DNA. The present study, using an infectious DNA assay of greatly increased sensitivity, also shows that superhelical (closed-circular) SV40 DNA molecules are absent from SV40-transformed cells. Infectious SV40 DNA can, however, be detected after susceptible cells and transformed cells are fused. The time required for emergence of infectious SV40 DNA is significant. The data to be presented show that after either fusion of transformed with susceptible cells or productive infection of CV-1 cells, there is about a 16- to 19-hour delay before infectious SV40 DNA is made. Also described are attempts to rescue infectious SV40 DNA from transformed cell lines that have so far never yielded virus and from cell lines that are poor virus yielders.[9]

Materials and Methods.—Cell cultures: CV-1 is an established line of green monkey kidney cells. Transformed mouse kidney lines were obtained by inoculating primary mouse kidney cultures with normal SV40 (mKS-A or mKS-BU100 lines)[6] or with SV40 irradiated with UV light to 10^{-3} to 10^{-5} survival (mKS-U lines).[9] The TSV-5 and the H-50- and 2X-10-transformed hamster cells have been described by Tournier *et al.*[5] and by Melnick *et al.*,[10] respectively. All cell lines were grown with "R5a" medium containing 0.5% lactalbumin hydrolysate and 10% calf serum (Hylands Laboratory, Los Angeles, Calif.).

Cell fusion: Approximately 1–1.7 $\times$ 10^7 CV-1 cells were mixed at 4°C for 15 min with 10^7 transformed cells and 8,000–16,000 hemagglutination units (HAU) of UV-Sendai in a volume of 2 ml. The mixes were shaken for 20 min at 38°C in a water bath, 10 ml of growth medium was added, and the cells were centrifuged and resuspended in 10 ml of growth medium. Two-ml aliquots of cell mixes were seeded in 8-oz prescription bottles, 18 ml of growth medium added, and the cultures were incubated at 37°C for various periods of time.

UV inactivation of Sendai virus: Sendai virus was grown in the allantoic cavity of 10- to 11-day-old eggs for 48–72 hr at 37°C. After the eggs were chilled at 4°C overnight, the allantoic fluid was harvested and clarified by low-speed centrifugation. Sendai virus was pelleted by centrifugation for 30 min at 30,000 rpm (Spinco model L-2, no. 30 rotor), and the pellets were resuspended in phosphate-buffered saline (PBS) solution[11] at 1/10 the original volume and stored in the frozen condition. Aliquots were thawed immediately before use and diluted with 4 vol of PBS to a titer of 5000–8000 HAU per ml, and 1.6-ml aliquots in 60-mm Petri dishes were inactivated by UV irradiation for 5 min at a distance of 25 cm from the light source (G30T8 Sylvania germicidal lamp).

Extraction of DNA: Cell cultures were trypsinized, cell suspensions washed, and the DNA was extracted and purified as previously described.[12] After careful dialysis to remove traces of phenol, the DNA was diluted with PBS solution lacking magnesium and calcium (PBS^-) to a concentration of 20–80 μg/ml and heated for 10 min at 100°C. This heat treatment reduced the viscosity of the solution but did not diminish the infectivity of the SV40 DNA.

Assay of SV40 and infectious DNA: SV40 virus and infectious DNA were assayed on aliquots of the same cell mixtures in the same experiment. For the virus assays, cell suspensions were frozen and thawed, subjected to sonic oscillation, and assayed by plaque formation on monolayer cultures of CV-1 cells.[13] The method of Pagano and coworkers[14, 15] was employed to measure infectious SV40 DNA. Confluent monolayer cultures of CV-1 cells in 60-mm Petri dishes were washed with 3 ml of PBS^- solution and inoculated with 0.2 ml of the DNA sample to be tested. The original DNA solution (and tenfold serial dilutions of this) was dissolved in PBS^- solution containing 1 mg/ml *O*-(diethylaminoethyl)-dextran (DEAE-dextran) (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). After 30 min at 23°C, the monolayers were washed with 5 ml of PBS^- solution and overlaid with 5 ml of a solution consisting of 100 ml of 2.5% Difco agar and 130 ml of double-strength R5a medium with 15% fetal calf serum. After 7 days, a second 5-ml overlay was added (100 ml of 2% Difco agar, 100 ml of double-strength R5a medium with 0.2% yeast extract, 1% lactalbumin hydrolysate, 2% fetal calf serum, and 10 ml of 0.1% neutral red). Plaques were counted 12–18 days after titration. Under the conditions of this assay, SV40 DNA was about 0.01–0.001 as infectious for CV-1 cells as intact virus.

Results.—Absence of infectious SV40 DNA from cell lines before or after incubation with UV-Sendai: DNA was isolated from 10^8 or more cells of the following culture lines: (1) TSV-5; (2) mKS-BU100; (3) mKS-A; (4) CV-1; and (5) these cell lines after each had been treated with UV-Sendai and further incubated for 53–96 hours. In no instance were plaques obtained when the usual amounts of DNA were plated on CV-1 monolayers. It can be estimated conservatively that infectivity would have been detected if 1 cell in 1000 contained an infectious SV40 DNA molecule.

Recovery of infectious DNA from CV-1 cells infected with SV40 at low input multiplicities and from mixtures of infected and transformed cells: Two types of experiments show that failure to isolate infectious DNA directly from SV40-transformed cells cannot be ascribed to the insensitivity of the DNA assay procedure. First, CV-1 cells were infected with SV40 at input multiplicities varying from 0.01 to 10 plaque-forming units (PFU) per cell (Fig. 1). The DNA from the SV40 originally adsorbed by the cells was readily detected three hours after infection, and infectious SV40 DNA was easily detected at each of the later time points studied. Indeed, infectious SV40 DNA has also been detected from 3 to 30 hours after infecting cells at an input multiplicity of 0.001 PFU per cell.

In the second type of experiment, CV-1 cells were infected with SV40 at an input multiplicity of 10 PFU per cell. After 48 hours, infected cells were mixed at a ratio of 1:1000 with uninfected CV-1 cells or with TSV-5 or mKS-BU100 cells. As controls, uninfected CV-1 cells were also mixed with transformed cells. A mixture of 8×10^7 CV-1 and 8×10^4 SV40-infected CV-1 cells yielded a total of 1.5×10^5 PFU of infectious SV40 DNA. The yields from similar mixtures of SV40-infected CV-1 and TSV-5 or mKS-BU100 cells were about 2.6×10^5 PFU of infectious DNA. Mixes of 8×10^4 uninfected

CV-1 cells with either 8×10^7 TSV-5 or mKS-BU100 cells did not yield infectious SV40 DNA.

Abortively infected mouse kidney cells: To learn whether infectious SV40 DNA could be recovered from abortively infected cells, confluent mouse kidney cultures (4×10^6 cells per culture) were infected with SV40 at an input multiplicity of 135 PFU per cell. Three hours after infection, the virus-containing medium was removed and 20 ml fresh growth medium added. At various times from 4 to 50 hours after infection, cultures were trypsinized, and DNA was extracted and assayed for infectivity on CV-1 cells. The infectious SV40 titer remained approximately constant from 4 to 50 hours after infection at a value of about 10^4 PFU per culture. Probably, most of this infectious DNA represents the virus originally added to the cultures. Thus, although infectious SV40 DNA does not increase, it can easily be detected in abortively infected cell cultures for at least 50 hours after infection.

Formation of infectious DNA and virus after fusion of CV-1 with TSV-5 or mKS-BU100 cells: Production of virus and infectious SV40 DNA can be activated by culturing mixtures of SV40-transformed cells with susceptible CV-1 cells (Table 1). Virus yields are enhanced and both virus and infectious

TABLE 1. *Recovery of SV40 virus and infectious SV40 DNA after mixing SV40-transformed with CV-1 cells and UV-irradiated Sendai virus.*

Cells	Hours after mixing	SV40 DNA (PFU/culture) No UV-Sendai	SV40 DNA (PFU/culture) With UV-Sendai	SV40 Virus (PFU/culture) No UV-Sendai	SV40 Virus (PFU/culture) With UV-Sendai
TSV-5 plus CV-1	24	0	1.1×10^2	0	0
	48	1.1×10^2	4.5×10^3	0	8.0×10^3
	70	1.3×10^2	7.2×10^3	0	9.6×10^3
	77	1.1×10^2	2.0×10^4	0	8.4×10^3
mKS-BU100 plus CV-1	24.5	0	1.3×10^2	0	0
	48.5	3.0×10^1	1.2×10^3	0	1.7×10^3
	72.5	9.6×10^1	2.2×10^3	7.2×10^2	7.8×10^3
	96	1.9×10^3	2.1×10^3	1.5×10^3	3.8×10^4

SV40 DNA are synthesized sooner when mixtures of cells are treated with UV-Sendai. Without UV-Sendai treatment, virus was recovered from mKS-BU100 cells at about three days after mixing. In the experiment shown in Table 1, no virus was detected at 77 hours after mixing TSV-5 or CV-1 cells. Usually, however, virus formation has been observed after two to three days in mixes of TSV-5 and CV-1 cells. After UV-Sendai treatment, virus was recovered from either mKS-BU100 or TSV-5 cells 48 hours after fusion. Infectious SV40 DNA was detected earlier than virus, that is, by 24 hours after fusion.

In order to determine the early kinetics of infectious SV40 DNA formation, the experiments given in Table 2 were performed. Infectious DNA was not detectable at 11 hours, but the DNA was made by 22 hours after fusion. In additional experiments, infectious SV40 DNA has been detected 19 hours after fusion of mKS-BU100 or TSV-5 cells with CV-1 cells in the presence of UV-Sendai. The earliest that SV40 virus has been detected is 28 hours after fusion.

The data in Table 2 may be compared with those shown in Figure 1. After *productive* infection of CV-1 cells, infectious DNA does not increase until about

TABLE 2. *Kinetics of infectious SV40 DNA formation after mixing SV40-transformed cells with CV-1 cells and UV-irradiated Sendai virus.*

Hours after mixing	Expt. (a) mKS-BU100 + CV-1 + UV-irradiated Sendai (PFU/culture)	Expt. (b) TSV-5 + CV-1 + UV-irradiated Sendai (PFU/culture)
6	0	0
11	0	0
22	4.7×10^1	1.0×10^2
26	1.5×10^2	4.2×10^2
31	1.7×10^2	1.6×10^3
36	2.2×10^2	1.9×10^3
46	1.3×10^3	4.1×10^3
53	6.0×10^3	1.3×10^4

16 hours after infection (Fig. 1). Thus, the time required to initiate infectious DNA synthesis after fusion of transformed with susceptible cells is almost the same as that after productive SV40 infection of CV-1 cells.

Effect of inhibitors of DNA synthesis on activation of infectious SV40 DNA synthesis: Table 3 shows that treating mixtures of TSV-5 and CV-1 cells with 1-β-D-arabinofuranosylcytosine (ara-C) to inhibit DNA synthesis immediately or six hours after UV-Sendai fusion prevents the activation of infectious SV40 DNA synthesis. If ara-C is removed 24 hours after fusion and the inhibition of DNA synthesis is reversed by deoxycytidine addition, infectious DNA synthesis is initiated and the usual 30-hour yield is obtained at 54 hours after fusion. Also, addition of ara-C 24 hours after fusion halts infectious SV40 DNA synthesis between 24 and 54 hours.

In further experiments, TSV-5 and CV-1 cells were pretreated with 20 μg/ml ara-C for 24 hours. The cells were washed and the following mixes made:

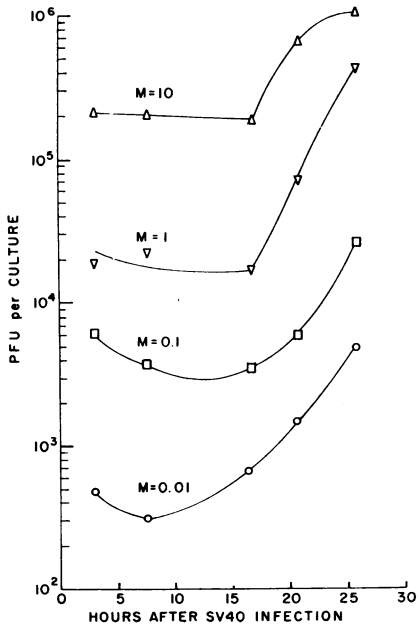

FIG. 1.—Assay of infectious SV40 DNA at various times after infection of CV-1 cells with intact virus. Confluent monolayer cultures were infected with SV40 at input multiplicities of 10, 1, 0.1, or 0.01 PFU/cell. After an adsorption period of 2 hr at 37°C, unadsorbed virus was removed, 20 ml of new growth medium added, and the cultures were further incubated at 37°C. At the times indicated, the cultures were trypsinized and DNA extracts prepared from the resulting cell suspensions.[12]

TABLE 3. *Effect of ara-C (15 μg/ml) on recovery of infectious SV40 DNA from mixtures of TSV-5 and CV-1 cells after treatment with UV-irradiated Sendai virus.*

Treatment	PFU/Culture at Harvest Time: (hr after mixing) 6	24	54
None	0	7.1×10^2	9.1×10^4
Ara-C added at time of mixing	0	0	0
Ara-C added at time of mixing, removed at 24 hr, and 45 μg/ml dC added	...	...	1.9×10^3
Ara-C added 6 hr after mixing	...	...	0
Ara-C added 24 hr after mixing	...	...	1.8×10^2

(1) untreated CV-1 and TSV-5; (2) ara-C pretreated CV-1 and untreated TSV-5; (3) ara-C pretreated TSV-5 and untreated CV-1; and (4) ara-C pretreated CV-1 and pretreated TSV-5 cells. All cell mixtures were treated with UV-Sendai and the cultures were incubated for 53 hours at 37°C. At the time of harvest, the titer of infectious SV40 DNA was 1.5×10^4 PFU per culture for the untreated cultures and varied from 1.5×10^4 to 3×10^4 in the cultures made with ara-C pretreated cells. Thus, pretreatment of either the susceptible or the transformed cells or both before fusion to inhibit DNA synthesis does not prevent the formation of infectious SV40 DNA after fusion.

Similar pre- and posttreatment experiments were performed with the thymidine analogue, dBU. Pretreatment of CV-1 or TSV-5 or both with 25 μg/ml dBU for 24 hours did not reduce the subsequent formation of infectious DNA after fusion. However, addition of dBU to cultures immediately after fusion prevented the formation of infectious SV40 DNA from 24 to 72 hours after fusion.

Experiments on the recovery of infectious SV40 DNA from transformed cell lines that are poor virus yielders or yield no virus: In contrast to results with TSV-5 and mKS BU100 cell lines,[5, 6] SV40 virus has not been recovered from mixtures of monkey kidney and the H-50 or 2X-10 lines of transformed hamster cells.[10, 16] Many lines of mouse kidney cells transformed by UV-irradiated SV40 yielded either little virus, compared with TSV-5 or mKS-BU100 cells, or no virus at all, and clone 4 of mKS-A-transformed cells failed to yield virus in mixed culture (no UV-Sendai treatment), although parental mKS-A and other clones of mKS-A cells did so.[6] All of the above-mentioned cell lines contained the SV40 T-antigen.

In order to learn whether infectious SV40 DNA and virus could be rescued from the preceding transformed cell lines, the experiments given in Table 4 were carried out. All cell mixtures were treated with UV-Sendai virus and the incubation time after fusion was in some cases increased to seven days.

Infectious SV40 DNA and virus were recovered from parental mKS-A cells and from secondary clones of mKS-A clone 4 cells, although the yields were less than those from mKS-BU100 or TSV-5 cells. The mKS-A (TU-1) cells, a transformed subline that had grown as a tumor in mice and was then propagated again in tissue culture, also yielded infectious SV40 DNA and virus. Two of the lines transformed by UV-irradiated SV40 (mKS-U1 and mKS-U13)

TABLE 4. *Experiments on the recovery of SV40 virus and infectious SV40 DNA from mixtures of CV-1 and various transformed murine or hamster cell lines.*

Transformed cells	Recovery of SV40 DNA on: Day 3 (PFU/culture)	Recovery of SV40 DNA on: Day 7 (PFU/culture)	Recovery of SV40 virus on day 7 (PFU/culture)
H-50	0	0	0
2X-10	0	0	0
mKS-A (parental)	0.5×10^1	...	1.6×10^2
mKS-A Cl 4-1	0.3×10^1	...	1.6×10^3
mKS-A Cl 4-8	1.7×10^1	...	6.6×10^2
mKS-A Cl 4-10	1.0×10^1	...	1.6×10^2
mKS-A (TU-1)*	...	4.7×10^2	8.4×10^4
mKS-U1	0	2.9×10^2	9.3×10^3
mKS-U13	3.5×10^1	5.3×10^4	3.9×10^5
mKS-U7	1.8×10^1	0	0
mKS-U14	1.8×10^1	3.5×10^1	0
mKS-U22	0	2.6×10^1	0
mKS-U26	0	2.6×10^1	0
mKS-U3, U5, U12, U16, U17, U25, U27, U30	0	0	0
mKS-BU100	1.2×10^2	1.3×10^5	$>10^5$

* mKS-A (TU-1) was isolated from a mouse tumor produced by inoculating BALB/c mice with 4×10^6 mKS-A cells (passage 71). The tumor cells were trypsinized and propagated in tissue culture for 7 passages.

yielded infectious SV40 DNA and virus after fusion. Four of the mKS-U lines, i.e., mKS-U7, mKS-U14, mKS-U22, and mKS-U26, have occasionally yielded low levels of SV40 DNA, but have so far not yielded SV40 virus. Neither infectious SV40 DNA nor virus was recovered from mixtures of susceptible cells with H-50, 2X-10, or several other lines of mKS-U cells.

Discussion.—Studies on the incorporation of H^3-thymidine into DNA have previously shown that: (1) DNA synthesis is stimulated in CV-1 cells productively infected with SV40, beginning at about 16 hours after infection; (2) SV40 DNA is not made 2–12 hours after infection; and (3) H^3-thymidine-labeled SV40 DNA is not detected in mouse kidney cells abortively infected with SV40 or in SV40-transformed mouse kidney cells.[12, 13] The present study shows that: (1) infectious SV40 DNA increases, beginning at about 16 hours after productive infection of CV-1 cells, but does not increase after abortive infection of mouse kidney cells; and (2) less than one in a thousand SV40-transformed hamster or transformed mouse kidney cells contain infectious SV40 DNA. Since this and previous studies demonstrate that the entire SV40 genome can be rescued from transformed cell lines that do not spontaneously produce the virus, it is reasonable to conclude that SV40 DNA exists in transformed cells in a noninfectious form, perhaps as a linear duplex.[17] We suggest that during transformation SV40 DNA becomes integrated at a cellular *site*, and that this process changes the conformation of the superhelical molecules so that they are noninfectious. The nature of the *site* could perhaps be the nuclear membrane,[18] or a structure of the type postulated for the attachment of *E. coli* DNA,[19] or the replicative form of phage ϕX174 DNA.[20] We do not exclude the possibility that attach-

ment to an essential *site* may favor subsequent recombinational events leading to the insertion of the SV40 DNA into a cellular chromosome, or that the *site* may be a linear insert in a chromosome, *per se*.

A salient fact concerning the rescue of infectious SV40 DNA from mixtures of transformed and susceptible cells is that 1–10 per cent of TSV-5 cells and up to 1 per cent of mKS-BU100 cells acquire the capacity after fusion of forming infectious centers when plated with monkey kidney cells.[5, 9] The magnitude of this number suggests that rare recombinational events involving either several partial SV40 DNA molecules or two mutated SV40 DNA molecules within a single cell do not explain SV40 DNA rescue. It suggests rather that one of the following models may prevail: (1) SV40 DNA is replicated semiconservatively at the attachment site and the progeny DNA are subsequently converted to closed-circular infectious molecules; or (2) SV40 DNA is easily detached from the site, converted to closed-circular DNA, and then undergoes autonomous replication.

After fusion, about 19 hours elapse before infectious SV40 DNA can be detected. This lag time is so similar to the time required for SV40 DNA replication in productively infected cells as to suggest that the events after fusion are equivalent, with minor variations, to those which ensue after productive infection. The experiments with ara-C and dBU show that continuous SV40 DNA synthesis occurs after fusion, but that DNA synthesis is not needed prior to fusion. This contraindicates the possibility that infectious SV40 DNA arises from the closure into circular molecules of DNA pre-existing in the cell.

Some of the transformed lines used in this study were "poor yielders" or defective lysogens transformed by UV-irradiated SV40.[9] These lines produced less infectious DNA and virus than did TSV-5 and mKS-BU100 cells, or none at all. We propose the following hypothesis for the "poor yielders" and the defective lysogens. The entire nucleotide contour length of SV40 DNA is integrated in these transformed lines, but the DNA contains mutational lesions at sites essential for virus replication and/or detachment from an integrated *site*. It is also possible to envision mutations in SV40 genes that are translated into surface changes on the transformed cells, thereby reducing fusion with susceptible monkey cells. Provided that fusion does occur, SV40 replication may be activated and superhelical SV40 DNA may be produced. However, the latter DNA would form fewer plaques or no plaques at all on CV-1 cells when assayed by the infectious DNA method. The hypothesis predicts that viral DNA synthesis could be detected after fusion of defective lysogens with susceptible cells by induction of tumors in hamsters, transformation of cells in culture, or by hybridization with DNA from SV40 particles. These possibilities are currently being studied.

Summary.—Infectious SV40 DNA was readily detected when extracts were prepared from about 10^8 monkey kidney (CV-1) cells productively infected with SV40 at input multiplicities varying from 0.001 to 10 PFU per cell, or from SV40-infected CV-1 and transformed cells mixed in a ratio of 1:1000. Extracts of about 10^8 transformed murine (mKS-BU100) or hamster (TSV-5) cells did not yield infectious SV40 DNA or virus. Nevertheless, both virus and infectious

DNA were recovered after mixtures of transformed cell lines and susceptible CV-1 cells were treated with UV-inactivated Sendai virus. The time required to initiate infectious SV40 DNA synthesis after fusion was almost the same as that after productive SV40 infection of CV-1 cells. Infectious SV40 DNA was not detected during the first 11 hours, but was observed at 19 hours after fusion and then rapidly increased. Addition of ara-C or dBU to cultures at the time of fusion prevented infectious DNA synthesis. The ara-C inhibition was reversed by removing the drug at 24 hours and adding deoxycytidine. However, pretreatment of either transformed or CV-1 cells, or both, with ara-C or dBU for 24 hours before fusion did not reduce the subsequent formation of infectious SV40 DNA after fusion. A number of transformed cell lines, particularly those transformed by UV-inactivated SV40, produced less infectious DNA, or none at all, after fusion.

* This investigation was aided by grants from the American Cancer Society (E-291), the National Science Foundation (GB-5917), the Robert A. Welch Foundation (Q-163), and by U.S. Public Health Service grants CA-06656, 1-K6-AI2352, and 5-K3-CA-25,797. We thank Marjorie Johnson, Carolyn Smith, Judith Rotbein, and Sun Ock Yim for able technical assistance.

[1] Sabin, A. B., and M. A. Koch, these PROCEEDINGS, **50,** 407 (1963); Sabin, A. B., in *Recent Results in Cancer Research,* ed. W. H. Kirsten (New York: Springer Pub., Inc., 1966), p. 164.

[2] Gerber, P., and R. L. Kirschstein, *Virology,* **18,** 582 (1962).

[3] Gerber, P., *Virology,* **28,** 501 (1966).

[4] Black, P. H., *J. Natl. Cancer Inst.,* **37,** 487 (1966).

[5] Tournier, P., R. Cassingena, R. Wicker, J. Coppey, and H. Suarez, *Int. J. Cancer,* **2,** 117 (1967).

[6] Dubbs, D. R., S. Kit, R. A. de Torres, and M. Anken, *J. Virol.,* **1,** 968 (1967).

[7] Koprowski, H., F. C. Jensen, and Z. Steplewski, these PROCEEDINGS, **58,** 127 (1967).

[8] Watkins, J. F., and R. Dulbecco, these PROCEEDINGS, **58,** 1396 (1967).

[9] Dubbs, D. R., and S. Kit, *Bacteriol. Proc.,* 177 (1968).

[10] Melnick, J. L., K. S. Khera, and F. Rapp, *Virology,* **23,** 430 (1964).

[11] Merchant, D. J., R. H. Kahn, and W. H. Murphy, Jr., *Handbook of Cell and Organ Culture* (Minneapolis, Minnesota: Burgess Pub. Co., 1961).

[12] Kit, S., R. A. de Torres, D. R. Dubbs, and M. L. Salvi, *J. Virol.,* **1,** 738 (1967).

[13] Kit, S., D. R. Dubbs, P. M. Frearson, and J. L. Melnick, *Virology,* **29,** 69 (1966).

[14] Pagano, J. S., J. H. McCutchan, and A. Vaheri, *J. Virol.,* **1,** 891 (1967).

[15] McCutchan, J. H., and J. S. Pagano, *Bacteriol. Proc.,* 143 (1967).

[16] Sabin, A. B., and M. A. Koch, these PROCEEDINGS, **52,** 1131 (1964).

[17] Bourgaux, P., D. Bourgaux-Ramoisy, and M. Stoker, *Virology,* **25,** 364 (1965).

[18] Sved, J. A., *Genetics,* **53,** 747 (1966).

[19] Chai, N.-C., and K. G. Lark, *J. Bacteriol.,* **94,** 415 (1967).

[20] Sinsheimer, R. L., C. A. Hutchison, III, and B. H. Lindqvist, in *The Molecular Biology of Viruses,* ed. J. S. Colter and W. Paranchych (New York: Academic Press, Inc., 1967), p. 175.