Infectious SV40 and SV40 DNA: Rapid Fluorescent Focus Assay (34738)

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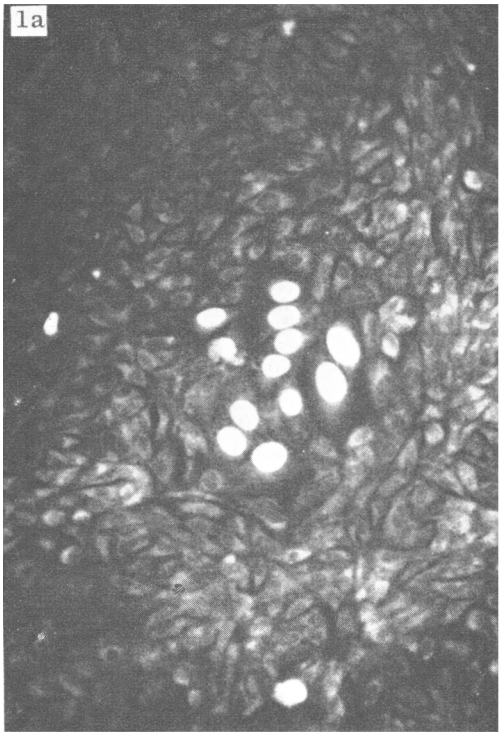
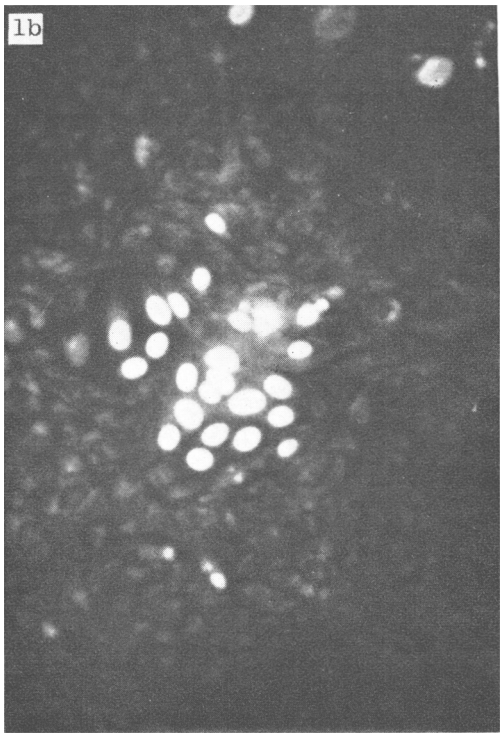
SV40 is a small DNA virus that grows vegetatively in monkey kidney cells; in cells such as the mouse, it produces *in vitro* neoplastic transformation without virus replication (1, 2). Assays for both infectivity (3) and transforming ability (4) have been described. A practical limitation of the infectivity assay is the time required for plaque formation. This is a particular problem with the minute plaque variant (the most efficient transforming SV40), where 18–20 days are required to complete an assay (3). In the present report, we describe a more rapid method using the indirect immunofluorescent technique to detect the virion (V) antigen. Foci of V-antigen containing cells are counted at 6 days after infection. The method has a sensitivity comparable to that of the standard plaque-forming assay and has been used to titrate infectious DNA as well as whole virus.

Methods. Plaque-purified stocks of large (SV-L), small (SV-S), and minute (SV-M) variants of SV40 were used. DNA isolated from large and small plaque virus, as previously described (5), was also tested. Primary cultures of GMK, grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Colorado Serum Co.), were seeded onto 50-mm plastic petri dishes containing three 11 × 22-mm coverslips. Confluent cell layers were exposed to virus for 3 hr or to SV40 DNA for 30 min. Plaque assays were carried out according to methods of Takemoto *et al.* for whole virus (3) and McCutchen and Pagano for viral DNA (6). In the fluorescent antibody (FA) focus assay 0.5% rabbit SV40 antiserum (Grand Island Biological Co., Grand Island, New York) was added to all cultures after the adsorption period to minimize secondary infection. This concentration of antiserum was capable of neutralizing over 10^5 infectious units of SV40; it, however, did not prevent spread of virus from cell to adjacent cell in confluent GMK cultures.

For the T-antigen assay, hamster anti-T-antibody obtained as serum from hamsters with a transplantable SV40 tumor was supplied by Dr. W. Rowe (NIH). For V-antigen staining, hamster antivirion (V) antibody was supplied by Dr. K. Takemoto (NIH). Commercial preparations of SV40 V antibody were also available from Flow Laboratories, Rockville, Maryland. Goat antihamster fluorescein conjugated globulin was obtained from the Resources and Logistics Section, National Cancer Institute, but can also be obtained from several companies (*e.g.*, Roboz Surgical Instrument Co., Washington, D. C.; Antibodies, Inc., Davis, Calif.; and Sylvana Co., Millburn, N. J.) The quality of individual batches of the commercially available fluorescein conjugates varied. Some did not work at all, and of the others, each had to be tested at a variety of dilutions to find the proper dilution for optimal staining. Following the fluorescent antibody method of Pope and Rowe (7) coverslips were fixed in acetone at —20° for 10 min and then incubated for 30 min at 37° with either the anti-T or anti-V antiserum. After three washes in phosphate buffered saline the cells were incubated with the fluorescein conjugated hamster antiserum for another 30 min. The counterstain, rhodamine, obtained from the Resources and Logistics Section, was added at a final dilution of 1:60 to the fluorescein conjugates. Rhodamine reduced the level of nonspecific binding of the fluorescein conjugate to the cells making it easier to score the T- or V-antigen-containing nuclei.





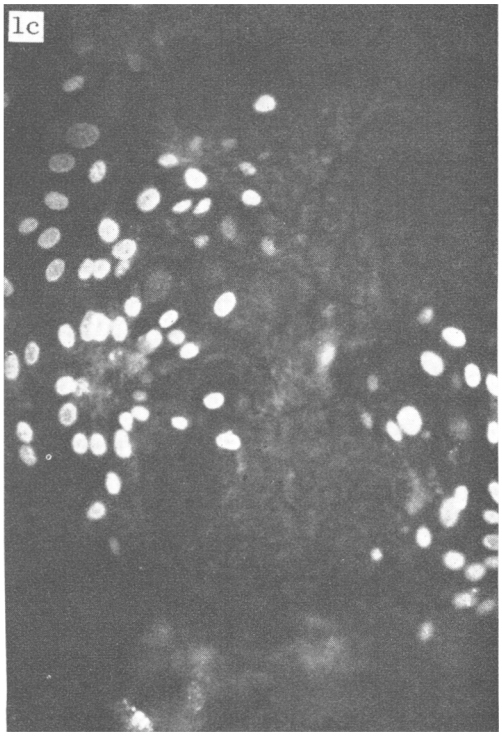


FIG. 1. Indirect fluorescent antibody technique showing intranuclear localization of SV40 virion antigen × 420: (a focus containing 12 cells with positive nuclei stained 4 days after infection; (b) focus containing 24 cells with positive nuclei stained 6 days after injection; and (c) two foci approaching each other 8 days after infection.

The coverslips were washed in PBS, inverted, and mounted onto slides with a drop of glycerol. Slides could be stored at —20° for several weeks without significant loss of fluorescence.

Results. Figure 1 shows typical foci at several time intervals after infection. The number of foci per coverslip did not increase between days 3 and 8; day 6 was chosen for the assay. At this time, the foci were large enough to score easily and yet it was still possible to see up to 100 discrete focal areas per coverslip. Occasionally at 6 days a single V-antigen positive cell was seen; these, however, were not scored. Whether they were the result of abortive infection or the occasional spread of virus through the medium is not known. At a time later than 6 days the foci

TABLE I. Titration of SV40 DNA by the FA Focus Assay and the Plaque Assay.

Source of DNA	Dilution ($-\log_{10}$)	FA foci/coverslip[a]	Plaques/petri dish[b]
SV-L	1	TMC, TMC, TMC[c]	NT[d]
	2	21, 25, 29	TMC, TMC
	3	3, 4, 6	21, 24, 38
	4	0, 0, 1	1, 2, 1
	5	NT	0, 0, 0
SV-S	1	TMC, TMC, TMC	NT
	2	67, 75, 81	TMC, TMC
	3	7, 8, 11	53, 56, 60
	4	1, 0, 0	2, 4, 7
	5	NT	0, 0, 0

[a] Three coverslips at each dilution were examined in their entirety for the number of FA-positive foci at 6 days. The surface area of each coverslip was 2.4 cm^2; the surface area of the petri dish was 20 cm^2, a factor of 8.33 times greater.

[b] Plaque assay performed on three petri dishes at each dilution. Final count at 15 days for SV-L DNA and 17 days for SV-S DNA.

[c] TMC = too many to count.

[d] NT = not tested.

had enlarged sufficiently so that they often merged with one another (Fig. 1C).

Virus or viral DNA was inoculated at a series of dilutions, and those dilutions where there were discrete foci were counted. Table I shows the results obtained by the plaque method as compared to the FA focus method for DNA extracted from SV-S and SV-L plaque mutants. It can be calculated from the data in Table I that the number of foci/coverslip multiplied by a factor accounting for the smaller surface area of the coverslip compared to the petri dish closely correlated with the number of plaques scored in the standard assay. The titration pattern by both FA focus and plaque assays was linear for each DNA preparation.

With certain virus stocks an even quicker method was used. This was possible because the preparations contained relatively little "defective" virus. The virus was grown by continually plaque-purifying the stock, thereby reducing the number of defective (T-antigen inducing but noninfectious) virus particles (8). If each T-antigen containing monkey kidney cell were to go on to lysis then the titer could be obtained by determining the percentage of T-antigen positive cells and multiplying by the dilution factor and the total number of cells per plate. At 48 hr after infection maximal numbers of infected cells produced T-antigen as a result of the initial infection. At this time the majority of T-antigen positive cells were single and randomly distributed. In the occasional case where two adjacent cells were both T-antigen positive, it was assumed that this resulted either from cell division or secondary infection. In this situation only one of the positive cells was counted. A number of virus and DNA preparations were titrated by the standard plaque assay and by the 2-day T-antigen and 6-day V-antigen FA assay. Table II shows the results obtained using each method.

One of the great difficulties in working with SV40 minute plaque variant is that it takes a relatively long time to complete a plaque assay. In addition, many primary GMK cultures contain contaminating viruses (9); some of these produce plaques that can be confused with SV40 minute plaques. The FA test avoids this complication. The number

TABLE II. Comparison of Infectivity of SV40 as Determined by FA Focus Assay and Plaque Assay.

	Plaque-forming activity (on GMK)[a]	FA titer at 2 days[b]	FA titer at 6 days[c]
Virus			
SV-S	2.0×10^8	2.5×10^8	1.0×10^8
SV-M	8.0×10^7	7.7×10^7	3.3×10^7
DNA			
SV-S	2.2×10^6	1.7×10^6	2.6×10^6
SV-S Type I	1.6×10^5	1.7×10^5	1.0×10^5
SV-S Type II	2.0×10^5	8.3×10^4	8.5×10^4

[a] Plaque assay on GMK scored at 17 days for SV-S and 20 days for SV-M.

[b] Titer estimate at 2 days = % positive cells × dilution factor × cells/plate; for confluent GMK the number of cells/plate was 2×10^6.

[c] Titer estimate at 6 days = number of foci/coverslip × dilution factor × 8.33, where 8.33 corrects for the greater number of cells used in the plaque assay as compared to the FA focus assay.

of cells per focus using the minute plaque virus was smaller at 6 days (4–10 positives) than the number per focus in comparable small plaque virus infected cultures (10–50 positives).

Discussion. The FA focus assay for the titration of SV40 virus and viral DNA gives titers that are comparable to those obtained by the standard plaque method. Whereas the plaque assay requires a minimum of 15–20 days, by the FA method the titration can be completed in 6 days. In addition, for preparations that are known to contain little or no defective virus, good titer estimates can be made at 2 days. Antivirion antiserum is essential in the assay since in the absence of an agar overlay, there is considerable secondary infection. While the above studies have used primary monkey kidney cells, there are available continuous monkey cell lines (10) that have retained sensitivity to SV40 that also work in the FA focus assay.

The recognition of the particular plaque type by the FA focus assay is reliable only with the minute plaque variant where the number of positive cells per focus at 6 days was consistently lower than the number seen with small and large plaque viruses. The difference at 6 days between the size of foci with small and large plaque variants was not a reliable indicator of plaque type. The large plaque virus, however, is temperature sensitive (3); thus if cells were maintained at 40°, the two plaque types could be distinguished.

Summary. A method utilizing the indirect immunofluorescent technique is described that permits a more rapid assay for the infectivity of the oncogenic virus, SV40. With this method both large and small plaque viruses as well as the DNA extracted from them give titer estimates that are comparable to those obtained in the standard plaque method.

This work was supported by Contract No. PH-43-65-641 from the National Cancer Institute. We would like to thank Claire Weaver and Elaine Rands for excellent technical assistance.

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Received Jan. 6, 1970. P.S.E.B.M., 1970, Vol. 134.