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Cell Biology

Efficient infection of monkey cells with DNA of simian virus 40

(double-label immunofluorescence/tumor antigen/DNA transfection)

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ABSTRACT **With standard protocols for DNA infection, only a small fraction (about 4%) of monkey cells exposed to purified DNA of simian virus 40 (SV40) exhibits signs of infection. We have devised a protocol by which we can extend the time of exposure of BSC-1 cells to DNA in the presence of low concentrations of DEAE-dextran. The efficiency of infection is proportional to the time of exposure. With an 8-hr exposure, we are reproducibly able to infect 25% of the cells, and we have been able to achieve levels of infection as high as 50% with a 16-hr exposure. The percentage of cells infected was measured either by scoring for nuclei positive for SV40 tumor antigen or by an infectious centers assay. We also report the use of ethidium bromide as a nonspecific nuclear counterstain in the immunofluorescence assay for SV40 tumor antigen.**

When a monolayer of monkey cells is infected at high multiplicity with particles of simian virus 40 (SV40), virtually all the cells become positive for SV40 tumor (T) antigen and exhibit cytopathic effects. In contrast, several investigators have found that when the same cells are incubated with high concentrations of SV40 DNA, even in the presence of a facilitator such as DEAE-dextran (1), only a small fraction (1–6%) of the cells exhibits signs of infection (2, 3). This low efficiency of DNA infection has frequently made interesting experiments arduous if not impossible. For example, it is difficult to study the biological properties of many potentially interesting viral mutants because one must begin with an inefficient DNA infection. Similarly, the inefficiency of DNA infection has limited the usefulness of SV40 DNA as a cloning vehicle in mammalian cells. For this reason, we decided to investigate ways to increase the efficiency of SV40 DNA infection.

Two models have been proposed to explain the low efficiency of infection of viral DNA. In the first, a small subset of cells is thought to be competent to respond to a DNA infection because the cells are sensitive during a limited portion of the cell cycle (4). Because only a few cells in a randomly growing population would be in a susceptible phase of the cell cycle at any one time, this model predicts that the percentage of cells infected should be proportional to the time of exposure to DNA. In a second model, the fraction of cells that can be infected with DNA is thought to be controlled by "self-interference" in a way that would be independent of the cell cycle (2). This type of interference might occur, for example, because of competition between DNA molecules for a limited supply of cellular factors required for a successful infection. If limiting factors in the cell become available with time, one might predict that longer exposures of cells to DNA should increase the efficiency of infection.

Both models suggest that the efficiency of infection with SV40 DNA could be increased by lengthening the period during which cells are exposed to DNA. To this end, we have devised a protocol in which monkey cells can be incubated for several hours at 37°C with saturating amounts of SV40 DNA in the presence of low concentrations of DEAE-dextran. With this procedure we have been able to infect as many as 50% of the cells in a monolayer.

MATERIALS AND METHODS

Virus and Cells. For our experiments, we used SV40 wild-type strain 776 and BSC-1 monkey cells grown in Dulbecco's modified Eagle's medium (DME medium) supplemented with 7% calf serum.

SV40 DNA. We extracted SV40 DNA from infected cells by the Hirt procedure (5) and then purified form I DNA (covalently closed circular, supercoiled) by phenol extraction, ethanol precipitation, and centrifugation in a cesium chloride/ethidium bromide equilibrium gradient. We extracted the DNA three times with isopropyl alcohol to remove the ethidium bromide and dialyzed it extensively against 15 mM NaCl/1.5 mM sodium citrate to remove the cesium chloride.

DNA Infection. For all DNA infections we used DEAE-dextran as a facilitator in a modification of the method of McCutchan and Pagano (1). BSC-1 cells were plated onto 35-mm dishes to be slightly subconfluent at the time of infection. DEAE-dextran (molecular weight, 2×10^6) was dissolved in DME medium/0.05 M Tris·HCl (pH 7.3). For infections at room temperature, we washed the cells two times with serum-free DME medium and incubated them with 0.7 ml of SV40 DNA I in DME medium/Tris/DEAE-dextran, the DEAE-dextran concentration being 500 μg/ml. The dishes were kept in a laminar-flow hood for 30 min. For incubations at 37°C, we washed the cells twice with serum-free DME medium (an important step) and incubated them with 0.7 ml of SV40 form I DNA in DME medium/Tris/DEAE-dextran at 37°C in a CO_2 incubator. The concentrations of DEAE-dextran are given in the figure legends. After all infections, we washed the cells once with serum-free DME medium and fed them with DME medium supplemented with 7% calf serum.

Assay for SV40 T Antigen. Infected cells growing on coverslips in 35-mm dishes were fixed with acetone (−70°C) and incubated with hamster anti-SV40-tumor serum for 30 min in a humid 37°C incubator. After washing the coverslips with phosphate-buffered saline, we incubated them with fluorescein-conjugated goat anti-hamster-IgG serum together with ethidium bromide at a final concentration of 2 μg/ml for 30 min at 37°C. After washing the coverslips twice more in phosphate-buffered saline and once in distilled water, we inverted them onto a drop of glycerol/phosphate-buffered saline (2:1, vol/vol) on a glass slide and examined them microscopically under ultraviolet illumination. We scored approximately 200 T-antigen-positive nuclei for each data point.

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Abbreviations: SV40, simian virus 40; T antigen, tumor antigen; DME medium, Dulbecco's modified Eagle's medium.

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Infectious Centers Assay. Cells that had been infected with either SV40 DNA or virions were treated with trypsin 1 day after infection, diluted, and replated onto subconfluent monolayers of BSC-1 cells such that each 60-mm dish received a sufficient number of cells to produce about 50 plaques. The next day the monolayers were overlaid with agar and treated as a standard plaque assay.

RESULTS

DNA infections in which DEAE-dextran is used as a facilitator are normally carried out at room temperature for a relatively short period of time, usually about 30 min (1). Fig. 1 shows that with this standard protocol no more than about 3.5% of BSC-1 cells in a monolayer can be infected, even with saturating amounts of SV40 DNA ($\geq$0.1 μg per 10^5 cells).

To try to increase the efficiency of DNA infection, we extended the period of exposure of cells to SV40 DNA. Exposures longer than about 30 min have not previously been used because the DEAE-dextran at the high concentrations used in standard procedures can be toxic to cells (1). These standard protocols were designed to maximize the specific infectivity of DNA (plaque-forming units/μg of DNA, an important consideration when only small amounts of DNA are available), rather than the percentage of cells infected. In our protocol, BSC-1 cells are exposed to SV40 DNA in the presence of relatively low concentrations of DEAE-dextran under conditions that approximate those normally used for growing the cells, namely incubation at 37°C in a CO_2 incubator rather than at room temperature in a laminar-flow hood.

Because the cells are sensitive to DEAE-dextran, we performed pilot experiments to determine concentrations of this facilitator that would not be toxic, even during long incubation periods (Table 1). During an 8-hr exposure, cell survival was significantly lowered for concentrations of DEAE-dextran $\geq$400 μg/ml, whereas for concentrations $\leq$200 μg/ml, the number of cells remaining viable was virtually the same as in control cultures not exposed to the facilitator. Because the efficiency of infection was reduced with DEAE-dextran concentrations below about 200 μg/ml, we chose 200 μg/ml as the optimal concentration for our experiments with BSC-1 cells.

Table 1. Optimizing the DEAE-dextran concentration for an 8-hr exposure

DEAE-dextran, μg/ml	% of cells surviving exposure*	% T^+ cells†
0	100	0
100	110	9
200	90	22
400	70	20
800	50	21

* Twenty-four hours after plating BSC-1 cells at a density of about 3 $\times$ 10^5 per 35-mm dish, we exposed duplicate cultures to DEAE-dextran at various concentrations for 8 hr at 37°C. To ensure that we would score only viable cells remaining after exposure, we replated the cells from each 35-mm dish into a 60-mm dish the next day. Twenty-four hours later, we determined the number of cells per dish and calculated the percentage of cells surviving exposure as (average no. of cells in a culture/average no. of cells in control cultures *not* treated with DEAE-dextran) $\times$ 100.

† BSC-1 cells were plated on coverslips and exposed to DEAE-dextran as for the survival measurement, except that 0.1 μg of SV40 form I DNA was included for each culture. At 36 hr after incubation, we determined the percentage of cells positive for T antigen by indirect immunofluorescence.

We compared the standard protocol, using a 30-min incubation at 23°C, with our protocol, using a 37°C incubation. The efficiency of infection for these 30-min incubations was the same at both temperatures (about 4%) as measured either by an infectious centers assay or by the immunofluorescence assay for SV40 T antigen. By extending the incubation time at 37°C to 8 hr (about one-quarter of the generation time for BSC-1 cells), we were able to infect about 25% of the cells (Fig. 2). With a 16-hr incubation, we have sometimes been able to infect about 50% of the cells, but the reproducibility of this result has been poor, probably because of the sensitivity of BSC-1 cells to DEAE-dextran.

The difference between the 30-min and 8-hr incubations is vividly illustrated in Fig. 3. Here, we used indirect immunofluorescence to assay the percentage of cells positive for SV40 T antigen. In these experiments, we used ethidium bromide as a nonspecific counterstain, an innovation that makes the determination much easier, because nuclei positive for SV40 T

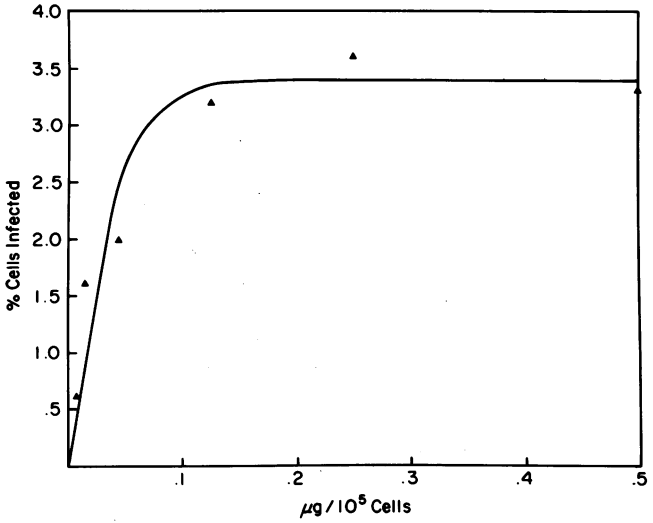

FIG. 1. Dishes of BSC-1 cells were infected at room temperature for 30 min with various amounts of SV40 form I DNA in the presence of DEAE-dextran (500 μg/ml). The percentage of cells infected was determined by an infectious centers assay. The values have been normalized to 100% for a high-multiplicity infection with virions, included in the assay as a control.

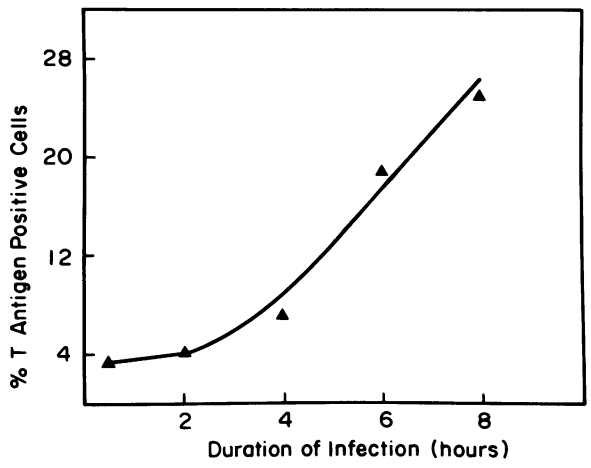

FIG. 2. Dishes of BSC-1 cells were infected with saturating amounts of SV40 form I DNA (1.5 μg per 10^5 cells) in the presence of DEAE-dextran (200 μg/ml) for different lengths of time at 37°C. The fraction of cells infected was determined by indirect immunofluorescence with anti-SV40-tumor serum at about 36 hr after infection. Loss of cells at each time point was negligible (see Table 1).

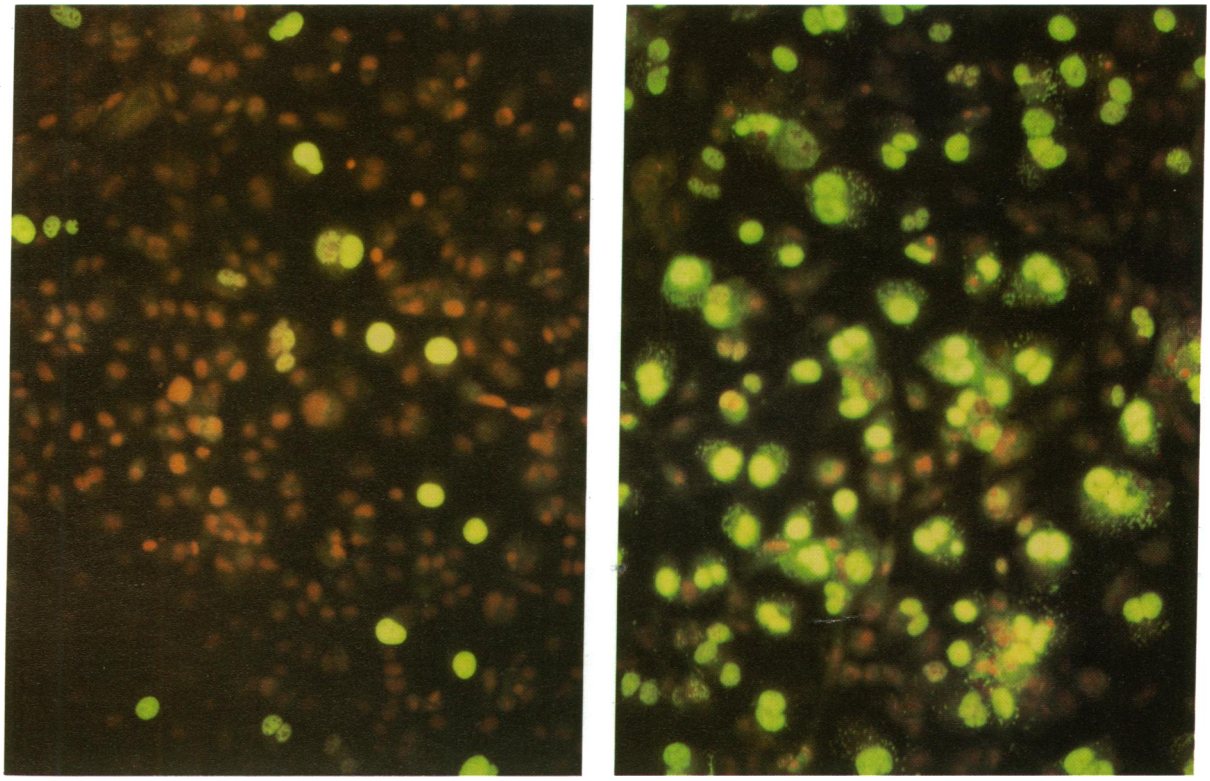

FIG. 3. Dishes of BSC-1 cells were infected with saturating amounts of SV40 form I DNA (1.5 μg per 10^5 cells) in the presence of DEAE-dextran. The fraction of cells infected was determined by indirect immunofluorescence, with ethidium bromide as a counterstain. Nuclei positive for SV40 T antigen are green; negative nuclei are orange. (*Left*) Cells infected at room temperature for 30 min with the DEAE-dextran at 500 μg/ml. (*Right*) Cells infected for 8 hr at 37°C, with the DEAE-dextran at 200 μg/ml.

antigen are green (fluorescein stain) and those negative for T antigen are orange (ethidium bromide).

To determine whether a saturation effect is observed with our protocol, we incubated BSC-1 cells for 8 hr at 37°C with various amounts of SV40 DNA. We observed that a plateau of cells infected did occur, but at about 22% (Fig. 4) rather than the 3.5% observed with a 30-min incubation (Fig. 1). That the saturating level of input DNA was the same for the two experiments underscores the importance of time of exposure.

DISCUSSION

In this paper we report a significant improvement in the efficiency of DNA infection as well as a useful nuclear counterstain for the immunofluorescence assay. The use of ethidium bromide as a nonspecific nuclear stain greatly facilitates the determination of the percentage of nuclei positive for SV40 T antigen (Fig. 3). Because a low concentration of orange ethidium bromide does not quench the green fluorescence of fluorescein, the two stains can be viewed with the same filters, thus eliminating the need to switch from ultraviolet to visible illumination to estimate the total number of nuclei in a field.

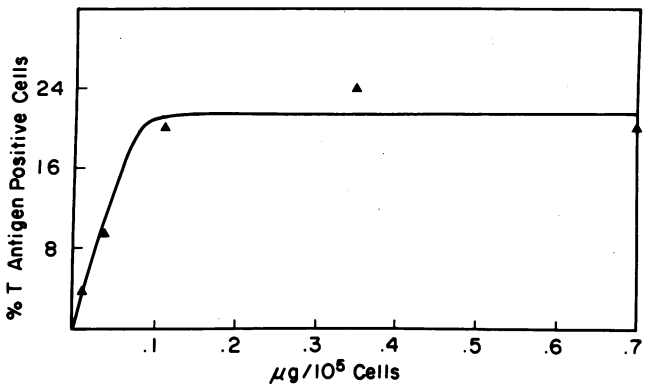

FIG. 4. Dishes of BSC-1 cells were infected for 8 hr at 37°C with various amounts of SV40 form I DNA in the presence of DEAE-dextran at 200 μg/ml. The percentage of cells positive for SV40 T antigen was determined by indirect immunofluorescence at about 36 hr after infection.

Using the protocol we have developed, we are reproducibly able to infect about 25% of the cells rather than the 3.5–4% we obtain with the standard protocol. This significant improvement should make possible many experiments that have previously not been feasible because of the inefficiency of DNA infection. Because the percentage of cells infected is proportional to time of exposure, as shown in Fig. 2, we infer that if we were able to expose BSC-1 cells to SV40 DNA for about 30 hr we should be able to infect all of them. However, the toxicity of DEAE-dextran prevents routine use of exposures longer than about 8 hr.

Parker and Stark (3) have reported that CV-1 monkey cells can be infected with SV40 DNA with an efficiency of about 6% when calcium phosphate is used as a facilitator (6). Our protocol should be particularly useful for cells such as BSC-1 that do *not* survive even short exposures to calcium phosphate. Also of interest are the observations that with the calcium phosphate technique, the efficiency of DNA infection (6) and the efficiency of "transformation" of cells with the herpes virus thymidine kinase gene (7) are both increased with longer incubation periods.

Our results do not serve to differentiate between the two models that have been proposed to explain the low infectivity of SV40 DNA. However, the correlation between the fraction of the generation time over which the incubation is carried out

and the percentage of cells infected (Fig. 2) is suggestive of a cell-cycle dependence.

Though we developed our protocol specifically for infecting BSC-1 cells with SV40 DNA, we can think of no reason that our protocol cannot be used for other DNAs with the same cells or be adapted for use with other cell lines.

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