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Inactivation of T Antigen-Forming Capacities of Simian Virus 40 and Adenovirus 12 by Ultraviolet Irradiation

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Methods to measure T antigen-forming capacities of simian virus 40 (SV40) and adenovirus 12 (Ad12) were investigated, and a method to measure the capacity in terms of T antigen-forming units was employed by the use of cytosine arabinoside. Plaque-forming units and T antigen-forming units of SV40, SV40 deoxyribonucleic acid, or Ad12 were inactivated by ultraviolet (UV) irradiation at the same rate, roughly following a single-hit curve. T-antigen formation by UV-irradiated SV40 and Ad12 was enhanced in cells multiply infected and in cells in a growing state. These observations showed that it was difficult or impossible to estimate the size of the gene for T antigen by UV inactivation.

The transforming capacities of polyoma virus and adenovirus are more resistant to inactivation by irradiation than are their infectivities, suggesting that the target sizes of the genome required for transformation may be smaller than those which are essential for viral replication (2, 4, 6, 8, 9, 13). In accord with these findings, it has been reported that the capacities of simian virus 40 (SV40) and adenovirus 12 (Ad12) to induce T antigen were more resistant to inactivation by irradiation than their infectivities (4, 7, 8, 11, 13, 19). However, zur Hausen (23) reported that the infectivity and T antigen-forming capacity of Ad12 were inactivated at the same rate by ultraviolet (UV) irradiation. Strohl (19) reported that T antigen-forming capacity of Ad12 was a little more resistant to UV inactivation than infectivity.

The present communication describes the UV inactivation of T antigen-forming capacities of SV40 and Ad12, showing that the capacities were inactivated at the same rate as their infectivities when the capacity was measured in nongrowing cells and that T-antigen formation was enhanced in cells multiply infected and in cells in a growing state. Reasons for the discrepancies among reports hitherto published are discussed.

MATERIALS AND METHODS

Cell cultures and viruses. Primary or secondary cultures of green monkey kidney cells (GMK) and human embryonic kidney cells (HEK) were used for SV40 and Ad12, respectively. SV40 Conn II strain, which was isolated in this laboratory, and Ad12 Huie strain were used. Crude virus fluid was prepared by sonic treatment of suspensions of cells showing cytopathic effect in Eagle's minimal essential medium (MEM) without serum and by centrifugation at 3,000 rev/min (1,500 × *g*) for 30 min. The infectivity of virus was titrated by plaque assay in GMK and HEK as described (22) and expressed in plaque-forming units (PFU).

Preparation of SV40 DNA. The purification of SV40 virions and the extraction of deoxyribonucleic acid (DNA) therefrom were carried out in a similar manner to that applied to adenovirus (18). Virions were purified by isopycnic sedimentation in CsCl solution, and DNA was extracted from the purified virions (density 1.34) by treatment with sodium dodecyl sulfate (0.5%) and phenol extraction. Phenol extraction was repeated, and the final aqueous layer containing DNA was dialyzed against phosphate-buffered saline (PBS).

Inactivation of virus by UV irradiation. A 1-ml amount of virus stock was spread in a thin layer in a petri dish (10 cm in diameter) and irradiated by a germicidal lamp (10 w) at a distance of 30 cm for the time indicated in results. Water lost by evaporation during irradiation was replaced by sterile water, which was used to rinse the dish after irradiation. Although irradiation was carried out mostly with crude virus fluid, it was confirmed that the same inactivation kinetics as that with virions purified by isopycnic sedimentation in CsCl solution (density 1.34) could be obtained, possibly due to the absence of serum in the suspending medium.

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Measurement of the T antigen-forming capacity. T antigen-forming units (TFU) described by Uchida et al. (21) to measure the capacity of SV40 to induce T antigen were used with a technical modification. Details of the immunofluorescence technique to examine Ad12 T antigen have been described (17). Narrow-reacting serum was used to avoid the involvement of virion antigens. The same technique was used to stain SV40 T antigen with anti-SV40 T conjugate prepared from sera of hamsters transplanted with SV40 tumors. It was confirmed that these sera did not contain antibody against SV40 virion antigen. A monolayer of GMK or HEK on a cover slip was inoculated with 0.2 ml of fourfold serial dilutions of virus. After adsorption at 36 C for 2 hr, the cover slips were incubated at 36 C for 72 hr (SV40) or 48 hr (Ad12 and SV40 DNA) in Eagle's MEM containing cytosine arabinoside (AraC, 10 μg per ml) to avoid secondary infection. The cover slips were rinsed in PBS, dried, treated with CCl_4, and stained. The numbers of cells with and without specific fluorescence were counted under a fluorescence microscope. Preparations in which less than 10% of cells showed specific fluorescence were selected and used for the calculation of TFU for the reason described below. Two to three preparations were used for the determination of TFU. Microscopic observation at a high magnification was necessary for Ad12 so as not to miss faint fluorescent flecks. Therefore, TFU of Ad12 were calculated by the percentage of T antigen-positive cells among the cells on a cover slip. The original method of Uchida et al. (21) was also used, in which 80 to 100 neutralizing units of rabbit antiserum, instead of AraC, was added to avoid the secondary infection. Induction of T antigen in growing cells was examined as follows. After adsorption of virus for 2 hr, cells were resuspended, diluted 10 times in growth medium containing 80 to 100 neutralizing units of anti-SV40 serum, and plated onto cover slips in small bottles. After incubation for the indicated time, T antigen-positive cells were examined. TFU were calculated by the number of T antigen-positive cells at each dilution.

Determination of PFU and TFU of SV40 DNA. PFU and TFU of SV40 DNA were measured by the method of McCutchan and Pagano (15). GMK monolayers were washed twice with a mixture of Eagle's MEM and PBS in equal amounts, containing neither antibiotics nor sodium bicarbonate. A 0.2-ml amount of DNA, diluted with the same medium containing diethylaminoethyl dextran (Pharmacia, Uppsala; molecular weight, 3×10^6) at a concentration of 500 μg/ml, was inoculated in a bottle or on a cover slip. After adsorption at room temperature for 30 min, cells were washed with Eagle's MEM. Further procedures were the same as described above.

RESULTS

Time course of T-antigen synthesis. Time course of T-antigen synthesis by UV-irradiated or unirradiated SV40 and Ad12 was tested. The appearance of T antigen-positive cells was slower in cultures infected with UV-irradiated SV40 and Ad12 than with unirradiated virus. Percentage of T antigen-positive cells in GMK infected with UV-irradiated and unirradiated SV40 increased up to 72 hr postinfection (pi) and then remained unchanged. Therefore, 72 hr pi was adopted as the time to examine SV40 T antigen. Percentage of T antigen-positive cells in HEK infected with UV-irradiated and unirradiated Ad12 increased up to 48 hr; thus, 48 hr pi was adopted as the time to examine Ad12 T antigen. Forty-eight hours pi was adopted as the time to examine T antigen induced by SV40 DNA, since the decrease in the percentage of T antigen-positive cells was observed from 48 to 72 hr pi.

Dose response of T antigen-forming capacity. The relationship between the percentages of T antigen-positive cells and the dilutions of virus is shown in Fig. 1 and 2. A linear relationship was always observed in repeated experiments when the percentage of T antigen-positive cells was less than 10%. The responses deviated from the linear relationship in the range in which more cells showed specific fluorescence. When similar tests were carried out with UV-irradiated SV40 and Ad12, the percentage of T antigen-positive cells was obtained as expected by virus dilution in the range in which less than a few per cent of cells showed specific fluorescence. In contrast to the unirradiated virus, UV-irradiated SV40 and Ad12 induced T antigen in more cells than expected when cells were infected with lower dilutions of

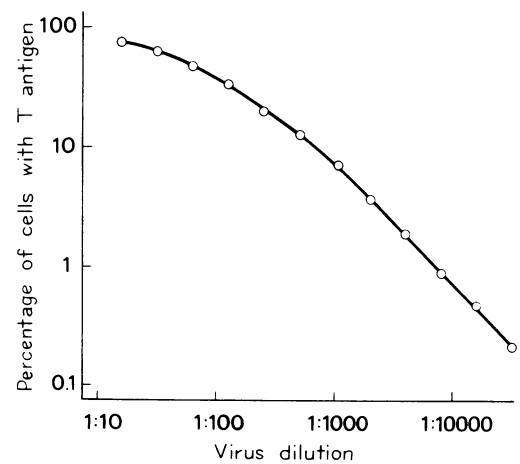

FIG. 1. *Dose-response curve of T antigen-forming capacity of Ad12. HEK cells on a cover slip, infected with twofold serial dilutions of Ad12 virus, were incubated at 36 C for 48 hr in medium containing AraC. Cells were stained as described. The percentages of cells with specific fluorescence were counted under a fluorescence microscope.*

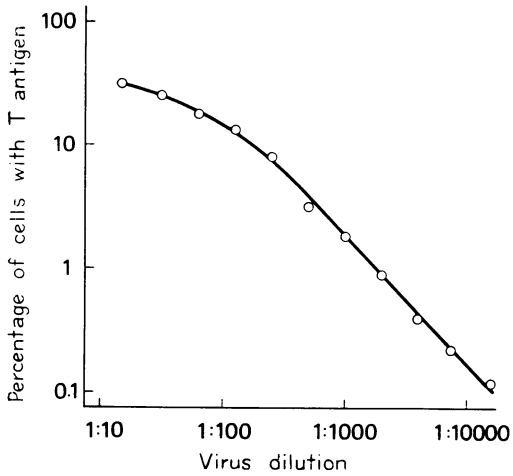

FIG. 2. *Dose-response curve of T antigen-forming capacity of SV40. GMK cells were infected with SV40 and expressed in the same manner as in Fig. 1.*

TABLE 1. *Induction of T antigen by UV-irradiated Ad12 and SV40*[a]

Inocula		Percentage of cells with T antigen		Ratio (O/E)
Virus	Dilution	Observed value (O)	Expected value (E)[b]	
		%	%	
Ad12 (UV-irradiated for 15 min)	1:1	71.7	21.76	3.29
	1:2	28.6	10.88	2.62
	1:4	10.7	5.44	1.97
	1:8	4.1	2.72	1.50
	1:16	1.35	1.36	0.99
	1:32	0.68	0.68	1.00
SV40 (UV-irradiated for 27 min)	1:1	18.8	6.56	2.87
	1:2	5.9	3.28	1.80
	1:4	1.43	1.64	0.87
	1:8	0.68	0.82	0.83
	1:16	0.41	0.41	1.00

[a] HEK or GMK, on a cover slip, infected with UV-irradiated Ad12 or SV40, was maintained in medium containing AraC. After incubation at 36 C for 48 hr (Ad12) or 72 hr (SV40), T antigen in cells was stained. The number of cells with T antigen was counted under a fluorescence microscope, and the percentage of cells with T antigen was calculated. The standard deviation was approximately ±15% of the observed value at each dilution.

[b] The expected value was calculated from the percentage of T antigen-positive cells at the highest dilution in the table.

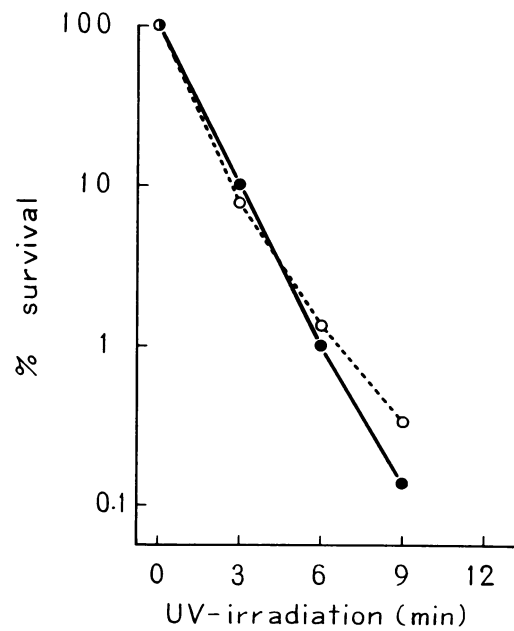

FIG. 3. *Inactivation of Ad12 PFU and TFU by UV irradiation. After UV irradiation of Ad12 for the time indicated, PFU and TFU were measured. Survivals of PFU (●) and TFU (○) relative to unirradiated control are depicted.*

virus (Table 1), suggesting that multiplicity reactivation may have occurred in cells infected with UV-irradiated virus at a higher multiplicity.

A linear dose response of SV40 DNA, unirradiated and UV-irradiated for 6 min, was observed in the range in which less than 0.5% of cells were T antigen-positive. The dose response of SV40 DNA, UV-irradiated for 12 min, could not be confirmed. Therefore, TFU of SV40 DNA, UV-irradiated for 12 min, were calculated from preparations which showed less than 10 T antigen-positive cells in the whole area.

Inactivation of SV40 and Ad12 in PFU and TFU. The rate of inactivation of PFU and TFU of SV40 or Ad12 after UV irradiation is shown in Fig. 3 and 4. PFU and TFU of SV40 or Ad12 were inactivated at the same rate, roughly following a single-hit curve. The relative survivals of SV40 DNA in terms of PFU and TFU are shown in Fig. 5. PFU and TFU of SV40 DNA were inactivated at the same rate, in accord with the UV inactivation of SV40 virions. This observation indicates that UV inactivation of virions resulted from damage to DNA and not by other factors, such as inefficient adsorption or uncoating of irradiated virions.

Formation of T antigen in growing cells. Secondary cultures of GMK monolayers were in-

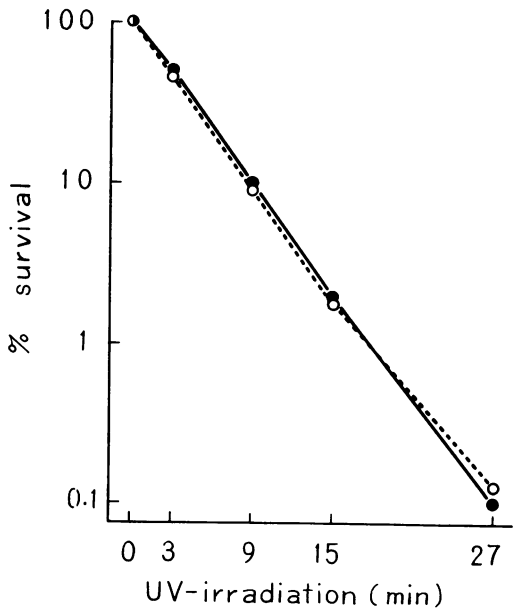

FIG. 4. *Inactivation of SV40 PFU and TFU by UV irradiation. Procedure and symbols are the same as in Fig. 3.*

fected with SV40 that had been UV-irradiated for 22 and 45 min (reduction of PFU about 10^{-2} and 10^{-5}, respectively). After adsorption, cells were subcultured on a cover slip and incubated at 36 C in a CO_2 incubator. T antigen-positive cells were examined at 72, 96, and 120 hr pi. Aggregates of more than 2 T antigen-positive cells appeared 96 hr pi, possibly due to cell-to-cell infection in medium containing antiserum. Therefore, an aggregate was counted as a T antigen-positive cell. At the same time, PFU and TFU (in media containing either AraC or antiserum) were measured (Table 2). As shown in the table, T antigen-positive cells increased in growing cultures, suggesting that T-antigen formation by UV-irradiated SV40 may have been enhanced in growing cells. It was noticed that TFU measured in media containing antiserum gave a slightly higher value than that in media containing AraC. A similar test was carried out with HEK cells, infected with Ad12, unirradiated and UV-irradiated for 10 min. However, TFU of UV-irradiated Ad12 in growing cells gave variable results and are not included in the table. It was also confirmed that TFU measured in media containing antiserum gave a slightly higher value than those in media containing AraC.

Then, semiconfluent monolayers of HEK were infected with Ad12, UV-irradiated for 15 min. After adsorption, half of the cultures were maintained in growth medium containing antiserum, and the other half was maintained in the same medium containing AraC instead of antiserum and incubated at 36 C. The numbers of cells in cultures and the percentages of T antigen-positive cells were counted at the times indicated (Fig. 6). It was shown that T antigen-positive cells increased in parallel with the growth of cells. Thus, it was suggested that the induction of T antigen may be enhanced in growing cells.

DISCUSSION

PFU are calculated from the number of plaques produced by diluted virus under conditions in which it is improbable for a single cell to receive more than one virion. To compare PFU to the other biological capacities of viruses, measurements must be made under the same condition. Thus, TFU titration described by Uchida et al. (21) was measured with a modified method in which AraC was used instead of antiserum. Examination of the incorporation of ^{3}H-thymidine in autoradiograms confirmed that DNA synthesis, either cellular or viral, was completely inhibited in cells maintained in media containing AraC and no serum. Thus, cells were maintained in a nongrowing state, and the secondary infection by replicating virus was prevented.

The measurement of TFU in medium con-

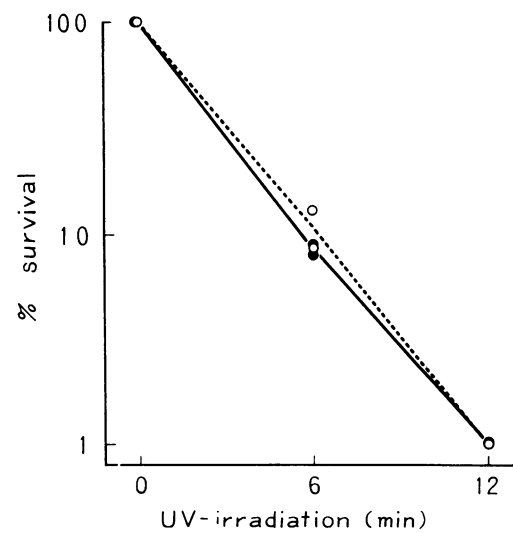

FIG. 5. *Inactivation of SV40 DNA PFU and TFU by UV irradiation. SV40 DNA was irradiated by UV in the same manner as virus for 6 and 12 min. After irradiation, PFU and TFU of unirradiated DNA and UV-irradiated DNA were determined. Survivals of PFU (●) and TFU (○) relative to the unirradiated control are depicted.*

TABLE 2. *Induction of T antigen in cells under various conditions*[a]

Determination	Unirradiated	UV-irradiated[b]		Titer reduction	
		A	B	A	B
SV40					
PFU	$10^{7.2}$	$10^{5.0}$	$10^{2.1}$	$10^{-2.2}$	$10^{-5.1}$
TFU (AraC)[c]	$10^{7.5}$	$10^{5.1}$	$10^{2.7}$	$10^{-2.4}$	$10^{-4.8}$
TFU (antiserum)[c]	$10^{7.3}$	NT[d]	$10^{3.2}$	NT	$10^{-4.1}$
TFU in growing cells					
72 hr pi	$10^{7.4}$	$10^{5.6}$	$10^{3.8}$	$10^{-1.8}$	$10^{-3.6}$
96 hr pi	$10^{7.6}$	$10^{6.2}$	$10^{4.4}$	$10^{-1.4}$	$10^{-3.2}$
120 hr pi	$10^{7.6}$	NT	$10^{4.4}$	NT	$10^{-3.2}$
Ad12					
PFU	$10^{6.0}$	$10^{3.0}$		$10^{-3.0}$	
TFU (AraC)[c]	$10^{6.5}$	$10^{3.7}$		$10^{-2.7}$	
TFU (antiserum)[c]	$10^{6.1}$	$10^{5.0}$		$10^{-1.1}$	

[a] GMK or HEK were infected with SV40 or Ad12, respectively; PFU and TFU were determined as described in text and expressed in PFU or TFU per 0.2 ml.

[b] UV irradiation of SV40 for 22 min (A) and 45 min (B) and of Ad12 for 10 min.

[c] Measured in cells maintained in media containing AraC. Measured in cells maintained in media containing 80 to 100 neutralizing units of antiserum.

[d] Not tested.

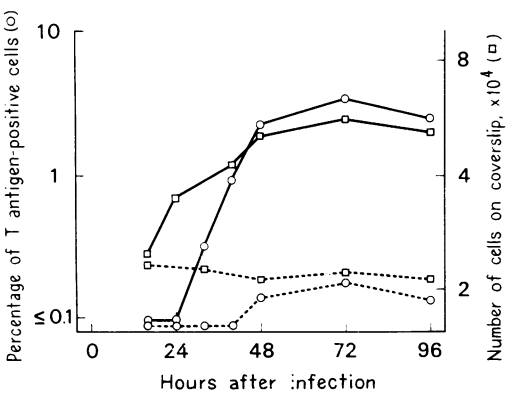

FIG. 6. *Time course of T-antigen synthesis by UV-irradiated Ad12. After infection of semiconfluent monolayers of HEK with Ad12 UV-irradiated for 15 min, the cultures were incubated in media containing either AraC or antiserum. T antigen-positive cells were examined at the indicated time. At the same time, the number of cells on a cover slip was measured. Solid lines represent the percentage of T antigen-positive cells (○) and the number of growing cells maintained in media containing antiserum on a cover slip (□). Broken lines show the percentage of T antigen-positive cells (○) and the number of nongrowing cells in media containing AraC on a cover slip (□).*

taining AraC became feasible, since a linear relationship between the number of T antigen-positive cells and virus dilutions was confirmed in cells inoculated with diluted virus. Deviations from the linear dose response were observed when cells were infected at higher multiplicities, probably due to Poisson distribution of unirradiated virions and to multiplicity reactivation of UV-irradiated virions.

PFU and TFU of SV40 or Ad12 were inactivated at the same rate, roughly following a single-hit curve. A similar result was obtained when Ad12 was inactivated by electron beam irradiation, although the inactivation curve deviated somewhat from a single-hit curve (data are not included). Although the present results and those reported by zur Hausen (23) showed the same inactivation rate of the capacity to induce T antigen and the infectivity by UV irradiation, many investigators have reported that the former is far more resistant than the latter (5, 7, 8, 11). However, in these studies (5, 7, 8, 11), the capacity to induce T antigen was measured in the range in which more than 10% of cells induced T antigen. The present study suggested that multiplicity reactivation of irradiated virus in T-antigen formation may have occurred in cells in which more than a few per cent of cells became T antigen-positive. It is, therefore, suggested that the marked difference in sensitivity to UV inactivation suggested between plaque-forming and T antigen-forming capacities in other reports (5, 7, 8, 11) may be due to multiplicity reactivation. The multiplicity reactivation of UV-irradiated SV40 and Ad12 was also confirmed in studies of plaque-forming and infective center-forming capacities (Yamamoto, *unpublished data*).

Strohl (19) reported that T antigen-forming capacity of Ad12 was a little more resistant to UV inactivation than infectivity. Uchida (*per-*

sonal communication) also obtained a similar result with SV40. In these studies, T antigen-forming capacity was measured under conditions in which multiplicity reactivation could be ruled out. However, cells were maintained in media without AraC, and a small portion of cells could grow, in which T antigen-formation may be enhanced. Thus, it is conceivable that the measurement of T antigen-forming capacity may vary, depending upon the experimental conditions employed. At one extreme, TFU may be equal to PFU in nongrowing cells, such as cells maintained in media containing AraC. On the other hand, T antigen-forming capacity may be four times more resistant than infectivity when measured by transformation (22). Measurement of TFU in growing cells and in cells maintained in media containing antiserum should result in values between the two extremes (Fig. 7), and discrepancies among reports may be due to reactivation of irradiated virus in cells multiply infected and to the enhancement of T-antigen formation in growing cells.

The UV inactivation of Ad12 or SV40 PFU and TFU activity was due neither to inefficient adsorption nor to uncoating of UV-irradiated virions but may have resulted from damage to viral DNA induced by UV irradiation, since PFU and TFU of SV40 DNA were inactivated at the same rate. It is suggested that similar damage to Ad12 DNA can be induced by UV irradiation, since UV-irradiated Ad5 was uncoated at the same rate as the unirradiated control (14). The above observation, however, cannot be interpreted to show the relative target size of genome loci responsible for the induction of T antigen, since many observations indicate that the gene for T antigen synthesis must be a part of the whole viral genome. Especially, estimation of the portion of the viral genome transcribed in SV40 or Ad2-transformed cells (1, 10, 16) clearly showed that the gene for T antigen is only a part of the viral genome. In accord with these estimations, the gene for transformation by SV40 was estimated to be about one-fourth of the whole viral genome by UV inactivation and that for tumor-production of Ad12 was 5 to 7% of the viral genome; all the transformed cells were T antigen-positive (22).

It has been suggested that T-antigen formation may be enhanced in growing cells. Ben-Bassat et al. demonstrated that the synthesis of T antigen was enhanced in replicating 3T3 cells infected with SV40 (3). The presence of AraC in medium that inhibited cellular DNA synthesis may contribute to the inefficient formation of T antigen and may have resulted in the same rate of UV inactivation in PFU and TFU. Although multiplicity reactivation and enhancement of T-antigen formation in growing cells were only suggested and could not be completely proven, it was concluded that the size of the gene for T antigen could not be estimated by UV inactivation.

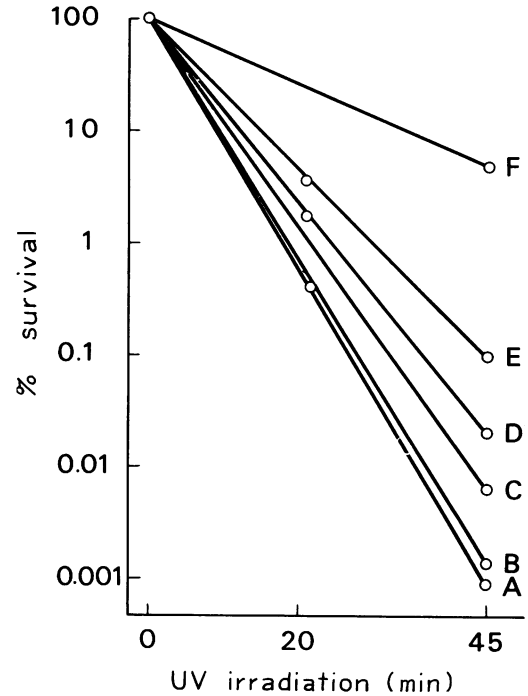

FIG. 7. *Inactivation of SV40 T antigen-forming capacity by UV irradiation, measured under various conditions. After UV irradiation of SV40 for the time indicated, TFU were measured under various conditions, and survival relative to unirradiated control is depicted. Inactivation of (A) PFU, (B) TFU in AraC, (C) TFU in antiserum, (D) TFU in growing cells measured at 72 hr pi, or (E) TFU in growing cells measured at 96 hr pi. (F) UV inactivation curve of transforming capacity of SV40, as described elsewhere (22).*

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