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Analysis of Minimal Functions of Simian Virus 40

II. Enhancement of Oncogenic Transformation in Vitro by UV Irradiation

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After *light* UV irradiation (5,000 to 10,000 ergs/mm^2) "complete" and "defective" simian virus 40 (SV40) showed an enhancement of oncogenic transformation capacity in Syrian hamster kidney cells in vitro up to 180 and 270% of the controls, respectively. Simultaneously with the enhancement of transformation, an increase in T-antigen induction was observed in CV-1 cells infected with light UV-irradiated SV40; infectivity, however, was correspondingly reduced by 1 $\log_{10}$. After strong UV irradiation (10,000 to 80,000 ergs/mm^2) of "complete" and "defective" SV40, transformation capacity in vitro proved to be the most resistant viral function. It was only slightly reduced in comparison with a 4 to 5 $\log_{10}$ reduction of infectivity. T-antigen induction of SV40 was also equally resistant to strong UV irradiation. We found no evidence of "multiplicity reactivation" involved in the high resistance of transformation capacity of SV40 after UV irradiation. Syrian hamster kidney cells transformed in vitro by UV-irradiated SV40 contained the SV40-specific T-antigen and showed the same morphology and growth characteristics as cells transformed by non-irradiated "complete" or "defective" SV40. They induced malignant tumors after subcutaneous inoculation into Syrian hamsters.

Progressive inactivation of oncogenic viruses by gamma or ultraviolet (UV) irradiation has been used to determine the survival rate of various viral functions and to clarify the minimal function or target size of the genome compatible with the phenotypic expression of oncogenicity (3, 4, 6, 17, 27, 28, 29). Results of inactivation of Papova viruses in different laboratories agree on the relative rate of inactivation of some functions of SV40 and polyoma such as the induction of T-antigen and cell DNA synthesis and virus replication (6, 7, 27, 35). As a result of physical inactivation of SV40 and polyoma virus, some investigators found a reduced capacity for transformation in vitro (3, 4, 17, 35), whereas—paradoxically—we observed an increased capacity for oncogenicity in vivo at a certain level of irradiation (6). We also found, after strong UV irradiation, a high resistance of viral transformation capacity of SV40 (Strain 777) in Syrian hamster kidney cells in vitro and of its tumorigenicity (26, 27).

In this paper we report the expression and dissociation of different functions of "complete" and "defective" SV40 after UV irradiation. These functions include the induction of T-antigen and DNA synthesis and their correlation to the transformation capacity of Syrian hamster kidney cells in vitro, with special emphasis on the phenomenon of enhancement of oncogenic transformation in vitro.

MATERIALS AND METHODS

Virus. SV40 (strain RH 911) (11) was grown in CV-1 cells (15). Two types of pools, "complete" and "defective," were produced according to the method of Uchida et al. (31, 32). Two "complete" pools were prepared at a multiplicity of infection (MOI) of 0.01 mean infective dose (ID_{50}) and had a titer of $10^{8.2}$ and $10^{8.4}$ mean tissue culture infective dose ($TCID_{50}$)/ml. The "defective" pool was prepared by infection at a MOI of 10 ID_{50}. This procedure was repeated three times, and the third passage pool had a titer of $10^{6.2}$ $TCID_{50}$/ml. The infectivity of these pools correspond well to that obtained by Uchida and Watanabe (30) under similar conditions of infection. The "defective" pool had a ratio of T-antigen inducing unit to infec-

tious units of 20:1, similar to that reported by Uchida et al. (32) in their preparation. The virus pools were treated with trypsin (0.01%) and sodium deoxycholate (1%) at 37 C for 30 min, and prior to infection, each sample was sonically treated three times for 15 s in a Branson sonifier (Heat Systems Co., Melville L.I., N.Y.).

The infectivity titer was estimated in CV-1 cells by using the method of Reed and Muench (20).

UV light irradiation of SV40. UV irradiation was done with a Westinghouse sterile lamp (7820L-20) that delivered 95% of energy at 2,537 nm. A 5-ml portion of SV40 in a petri dish (diameter of 91 mm) was placed 14 cm from the lamp and continuously agitated. At this distance the energy reaching the surface was 5×10^3 ergs per mm^2 per min as measured with a germicidal-erythemal photometer (IL 254; International Light Inc., Newburyport, Mass.). After UV irradiation the SV40 samples were kept in the dark and frozen at −25 C.

Cell cultures. Preparation and cultivation of primary Syrian hamster kidney cultures have been described previously (24). CV-1 cells were grown in Eagle minimal essential medium (MEM) supplemented with 8% fetal calf serum, whereas maintenance medium contained 2% fetal calf serum. In some experiments the medium contained 0.25% of anti-SV40 serum as reported in detail under Results. The anti-SV40 hyperimmune serum of rabbit was a gift from B. C. Del Villano, Jr., and neutralized 100 ID_{50} of SV40 at a dilution of 1.2×10^6.

Transformation assay. The transformation assay of Syrian hamster kidney cells has been previously described (24, 25). Syrian hamster kidney cells were infected with SV40 at a MOI of approximately 1,000 ID_{50} or ID_{50} for the "complete" or "defective" SV40 pool, respectively. The transformed colonies were scored at 16 to 18 days postinfection (p.i.).

The frequency of transformation amounted to 100/10^6 cells with the non-irradiated, complete SV40 and to 20/10^6 cells with the "defective" SV40 pool at the indicated MOI.

Transformed colonies were identified under a microscope on the basis of morphological characteristics and tested for the presence of SV40 T-antigen.

Immunofluorescence technique. The indirect immunofluorescence method was used for the detection of T-antigen positive cells (27). The percentage of positive cells was determined on 1,000 to 2,500 cells.

Test for oncogenicity. The tumorigenicity of in vitro transformed Syrian hamster kidney cells was tested by subcutaneous inoculation into weanling Syrian hamsters (27).

RESULTS

Inactivation of infectivity. Figure 1 presents typical survival curves of infectivity of "complete" and "defective" SV40 after different periods of UV irradiation. Inactivation is a single-hit exponential process, but these survival curves consist of two sections, and they do not follow a simple first-order kinetic; the change in slopes for both "complete" and "defective" SV40 virus occurs at a similar irradiation dose between 5,000 and 10,000 ergs/mm^2.

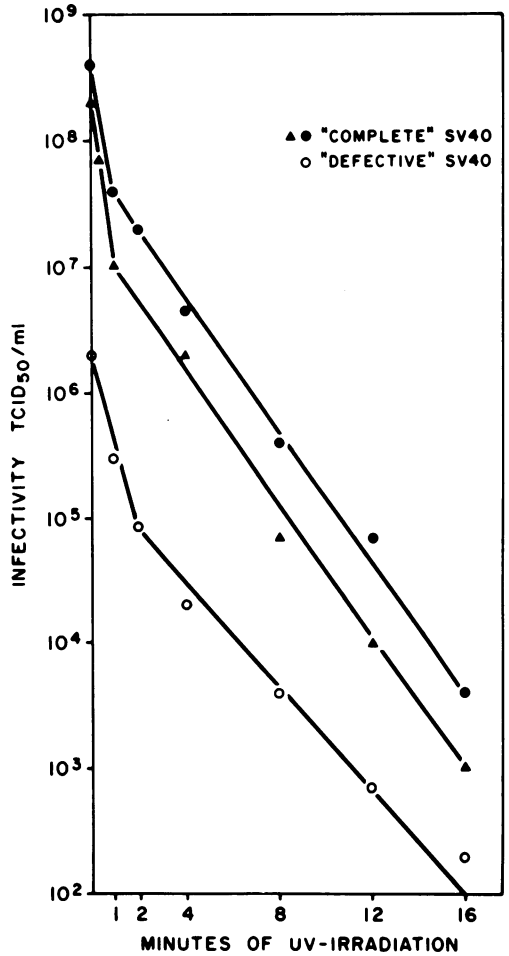

FIG. 1. *Reduction of infectivity by UV irradiation of "complete" and "defective" SV40. The virus samples were irradiated with Westinghouse germicidal lamp 782-L-20 (95% of energy emission at 2,537 nm), and the energy reaching the surface was 5×10^3 erg per mm^2 per min. Infectivity was measured in CV-1 cells.*

In the first part of the survival curve infectivity is reduced by 1 $\log_{10}$ per min, corresponding to a UV irradiation of nearly 5,000 erg/mm^2. In the second part, the reduction of infectivity of 1 $\log_{10}$ step requires at least 4 min, corresponding to a UV irradiation dose of 20,000 ergs/mm^2. Changes in slopes of survival curves after UV irradiation have been reported for different viruses (2, 33).

The flattening of the SV40 survival curves

after UV irradiation may reflect a virus heterogeneity (20), the participation of a "host cell repair mechanism," or the result of "multiplicity reactivation" (36). The similarity between survival curves of "complete" and "defective" SV40 after UV irradiation, however, suggests that heterogeneity, which should be more prevalent in "defective" SV40 pools, is not the main reason for the change in slope.

Inactivation of the capacity for T-antigen induction. The inactivation of the capacity to induce T-antigen in CV-1 cells after infection at a MOI of 300 and 3 for "complete" and "defective" SV40, respectively, was measured at 48 h p.i. In some experiments anti-SV40 serum was incorporated into the medium to prevent possible reinfection with progeny virus. In several experiments, an example of which is illustrated in Fig. 2a and b, the inactivation of T-antigen induction decreased exponentially as a function of the time of irradiation at a rate of intermediate value between the rates of loss of infectivity and transformation. The curves of inactivation however, differed when anti-SV40 serum was present in the medium. In the absence of anti-SV40 serum, the survival curve for T-antigen induction was a straight line in a semilogarithmic scale as previously reported (7). But in the presence of antiserum, the survival curve for T-antigen induction had different component parts, with both "complete" and "defective" virus. Thus, with the 1′ and 2′ UV samples, the proportion of T-antigen positive cells was higher than with the 0′ sample, and this difference was more evident with the "defective" virus. Therefore, the results of the experiments in the presence of antiserum are more representative of the actual T-antigen inducing capacity, as was suggested by Aaronson (1).

Survival of transforming capacity. Of all functions measured, the transformation capac-

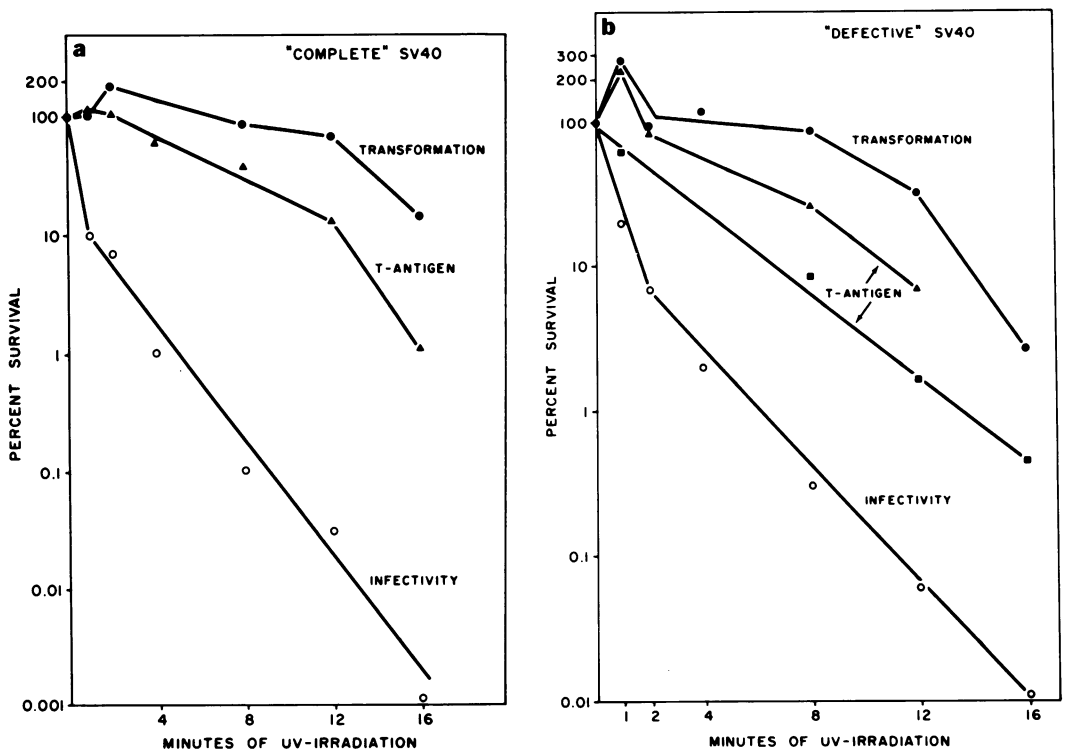

FIG. 2. *Effect of progressive UV irradiation on transforming capacity, induction of T-antigen, and infectivity of "complete" and "defective" SV40. Infectivity and T-antigen induction were measured by immunofluorescence 48 h postinfection on CV-1 cells. Cultures were kept in the presence (▲) or absence (■) of 0.25% anti-SV40 serum in the medium. With the 0′ UV "defective" SV40 in the absence of anti-SV40 serum, the percentage of T positive cells was 38% in the presence of 7.0% antiserum; with the 0′ UV "complete" SV40 in the presence of anti-SV40 serum, the percentage was 50%. Transforming capacity was determined in Syrian hamster kidney cells infected at a MOI of 1,000 for "complete" and 10 for "defective" SV40 of the 0′ UV-time (see Materials and Methods).*

ity was the most resistant to UV inactivation when tested in Syrian hamster kidney cells (Fig. 2a and b). The rate of inactivation of transformation did not follow a single-hit inactivation curve in this system. At a low UV dose (5,000 to 10,000 ergs/mm^2) when the infectivity was reduced by approximately 1 log, the transformation frequency of both "complete" and "defective" virus was increased to 180 and 270% of the control, respectively. At higher doses (10,000 to 60,000 ergs/mm^2) when infectivity had decreased by more than 3.5 log, the transformation frequency was reduced to only 70 and 30% of the control. With additional irradiation, the rate of decrease of transformation frequency was more rapid, even though at a dose of 80,000 ergs (16' UV), 2 and 5% of transformation capacity remained when infectivity was reduced by 4 and 5 log, respectively.

The absolute number of transformed colonies is illustrated in Fig. 3. It appears that the ratio of transformation efficiency of the "complete" versus "defective" pool is 5:1, whereas the ratio of infectivity is slightly higher than 100:1. Uchida and Watanabe (30) also observed that the transforming capacity for 3T3 cells by a "defective" SV40 pool was much higher than that expected from its plaque-forming activity and paralleled its T-antigen inductive activity.

Because a very high number of virus particles per cell is necessary to induce one transformation event in vitro, the relatively high resistance of transformation ability of SV40 to UV irradiation can be the result of a "multiplicity reactivation." Yamamoto and Shimojo (36) reported evidence of "multiplicity reactivation" in the recovery of infectivity and ability to induce T-antigen in African green monkey kidney cells infected with UV-irradiated SV40. In order to test this possibility we compared the transformation activity (Fig. 4 and 5) of undiluted and 1 and 2 $\log_{10}$ diluted "complete" and "defective" SV40 before and after UV irradiation. The slope of the survival curves of transformation (Fig. 4) remained unchanged suggesting that "multiplicity reactivation" does not play a significant role. It is striking that a proportional increase in transformation frequency of "complete" and "defective" SV40 after short-time UV irradiation was also demonstrable after 1 and 2 $\log_{10}$ dilutions (Fig. 5).

The properties of the Syrian hamster kidney cells transformed in vitro by UV-irradiated SV40 even at the highest doses of treatment were similar to those of cells transformed by

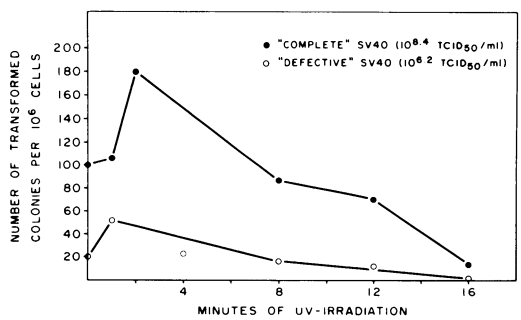

FIG. 3. *Effect of UV on the transforming capability of SV40. The number of transformed colonies per 10^6 Syrian hamster kidney cells after infection with "complete" and "defective" SV40, non-irradiated (MOI 1,000 and 10, respectively) and UV-irradiated for different periods of time. The results represent the average of two separate experiments.*

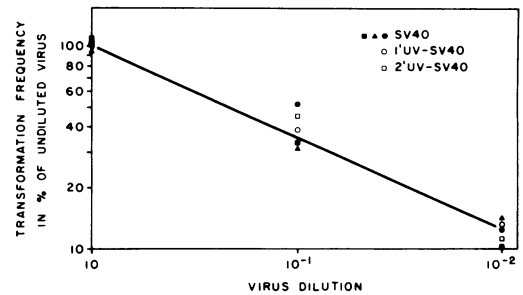

FIG. 4. *Relative transformation frequency of undiluted and diluted non-irradiated and UV-irradiated "complete" SV40 in Syrian hamster kidney cells in vitro.*

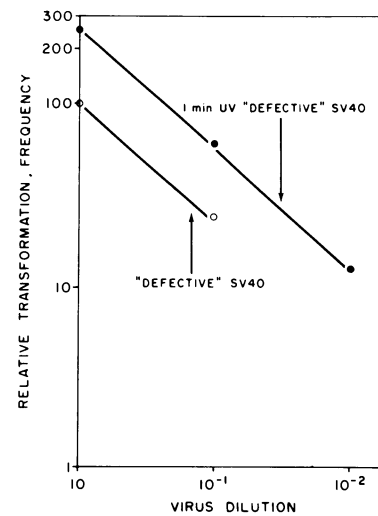

FIG. 5. *Enhancement of transformation capacity of 1-min UV-irradiated "defective" SV40 in comparison with non-irradiated SV40 in Syrian hamster kidney cells after 10- and 100-fold virus dilutions.*

untreated virus (Table 1). There were no morphological differences between cell lines induced by "complete" and "defective" untreated or irradiated SV40: All cell lines contained SV40-specific T-antigen, and they showed similar growth characteristics. In addition, all of the cell lines were oncogenic at a dosage of inoculum between 10^5 and 10^6 cells per adult Syrian hamster, and the tumors had the same typical morphology as that of tumors induced by kidney cells transformed by non-inactivated SV40. No infectious virus could be recovered from these lines.

TABLE 1. Properties of Syrian hamster kidney cells transformed by UV-irradiated and non-irradiated SV40

SV40		Cell line	Cell tumorigenicity[a]
Complete (CP) or defective (DF)	UV light irradiation (min)		
CP	0	10-1	9/10 (1.8×10^5) 6/6 (1.5×10^6)
CP	0	11-2	3/4 (1.5×10^5)
CP	0	13-1	2/3 (2.4×10^5)
CP	0	13-3	6/6 (6×10^5)
CP	8	40-54	4/4 (9×10^5)
CP	8	40-58	7/8 (7×10^5)
CP	12	47-23	6/6 (7×10^5)
CP	16	62-31	6/6 (1.4×10^6)
CP	16	62-32	5/5 (7×10^5)
CP	16	62-38	4/6 (4×10^5)
DF	0	64-5	5/6 (3.6×10^6)
DF	0	64-9	6/6 (3×10^6)

[a] Incidence of animals with tumors and, in parentheses, number of cells inoculated per animal.

DISCUSSION

Our results show a dissociation of different SV40 functions after progressive doses of UV irradiation and confirm reports from this and other laboratories (6, 7, 26, 27, 35, 36). However, of considerable significance are the following observations: (i) an increase of transforming capacity in vitro of SV40 after low UV irradiation (5,000 to 10,000 ergs/mm^2) that causes a 1 $\log_{10}$ reduction in infectivity; and (ii) a high resistance of transforming capacity to a high dose of UV irradiation up to 60,000 or more ergs/mm^2, corresponding to a 4 to 5 $\log_{10}$ reduction in infectivity. These results were obtained with both "complete" and "defective" virus pools. The enhancement of in vitro transforming capacity of SV40 by low UV irradiation confirms the observed increase of oncogenicity in vivo (6, 7, 34). Recently, Duff et al. (8) reported an increase of transforming capacity of adeno-7-SV40 hybrid virus for Syrian hamster embryo fibroblasts in vitro after a short period of UV irradiation. When the phenomenon was first reported several explanations were suggested. Most plausible among them was the possibility that inactivated virus could induce tumors defective for transplantation antigen, therefore increasing the probability of progressive growth of each initially transformed cell. Kleitmann and Seemayer (16) showed that hamster cells transformed by UV-irradiated or photodynamically inactivated SV40 did not express specific surface antigen. However, in several experiments with numerous tumors induced by UV-inactivated SV40, only a few were found to be defective for immunogenicity and immunosensitivity for the SV40-induced specific transplantation antigen (V. Defendi, unpublished data). The present observation that enhancement occurs also in an in vitro system makes an immunological explanation improbable.

Enhancement of transformation in vitro after irradiation of SV40 or polyoma virus has not been reported in publications from other laboratories (3, 4, 17, 35). It should be noted, however, that the host cells in these studies were different, and that different cells vary in the capacity for mechanism of "repair" (9, 19). Furthermore, the amount of irradiation used by other authors (in part deduced from their losses of infectivity) was in most cases higher than that at which we observed enhancement. The fact that oncogenicity of SV40 polyoma and adeno-7-SV40 hybrid is increased after cobalt 60 and UV irradiation and of SV40 after β-propiolactone treatment (29) indicates that the phenomenon is not specific for the method of inactivation. Theoretically we may assume that enhancement results after the triggering of some process important for cell transformation or by elimination of a "repressor." No evidence for either is available.

Precedents for enhancement effect by low doses of irradiation in other biological systems, however, exist. For example, the transducing capacity of the bacteriophage P22 in *Salmonella typhimurium* (10) is increased after low UV irradiation. The transducing capacity for different markers was also resistant to a dose of UV light that was sufficient to reduce lytic activity by a factor of 10^8. Genetic recombination of phage lambda is also increased after a small dose of UV irradiation (14), and the integration of SV40 DNA into the host cell DNA shows several features similar to recombination (12).

In agreement with reports of other authors (3, 35), we found no indication that "multiplicity reactivation" plays a major role in the oncogenic transformation in vitro of "complete" and "defective" SV40 after light and strong UV irradiation. No evidence of "multiplicity reactivation" was also found in the process of transformation in vitro by photodynamically inactivated SV40 (27).

The proportional "enhancement" in transformation capacity of SV40 after low UV irradiation was also demonstrable after serial virus dilutions and must be related to the intrinsic properties of the viral population at the time of infection.

A possible explanation for the general phenomenon of enhancement of various functions after low UV and X irradiation may be inferred from the recent work of Comorosan et al. (5). These authors showed that a substrate irradiated with low doses of UV light or X rays becomes energized to a metastable state and that this stimulation is transferred from an irradiated to a non-irradiated substrate. This stimulation was limited to a certain dosage range. Such a phenomenon was also demonstrated in complex cellular processes; for example, there was a clear stimulation of growth of *Escherichia coli* after uptake of irradiated (10 R) methionine (5). Our results of enhancement of transformation, T-antigen induction, and DNA synthesis after low UV irradiation of "complete" and "defective" SV40 could be explained on the basis of an "energizing" effect on the substrate viral DNA with a consecutive stimulation of enzymes involved in the expression of these functions.

The high resistance of transforming capacity of "complete" and "defective" SV40 after stronger UV irradiation (10,000 to 80,000 ergs/mm^2) contrasts to the remarkable reduction of infectivity and again indicates that only a fraction of the viral genome is responsible for oncogenic transformation. As our results show, oncogenicity of cells transformed in vitro by UV-inactivated SV40 was fully expressed, resulting in induction of malignant tumors after inoculation into Syrian hamsters.

As indicated above, the enhancement and high resistance of transformation capacity of UV-inactivated SV40 are reminescent of the increase and slight reduction of transducing capacity of bacteriophages after UV irradiation (10); this effect is modified by bacterial host cell factors (18, 22, 23). The analogy between these observations is further supported by the fact that we have obtained good evidence that a caffeine-sensitive host cell repair mechanism is involved in the transformation phenomenon as well as in the expression of other SV40 functions after UV irradiation of the virus (N. H. Seemayer and V. Defendi: manuscripts in preparation).

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