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Abstract

The correlation of infectivity to the length of SV 40 DNA by dilute and undiluted passage was described. DNA was extracted from purified virions by Marmur's method, and propagation of virus was done using VERO cells. The infectivity of SV 40 (simian virus 40) ran parallel with the length of its DNA. Undiluted passage caused shortening of DNA and decrease in infectivity, but when these undiluted group was passaged dilutely, length of DNA approached the original length and the infectivity recovered. In undiluted passage group small circular DNAs under 1.0,11_, so far not reported in the SV 40 DNA, were found in low frequency. Replicating form and dimers were also noted from virions and the nuclei of SV 40 infected VERO cells.

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RELATIONSHIP BETWEEN MOLECULAR LENGTH AND BIOLOGICAL ACTIVITY OF SV40 DNA

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It was reported that successive undiluted passage of simian virus 40 (SV 40) in African green monkey kidney cells caused a gradual decrease in the production of plaque forming virus (UCHIDA *et al.* 1966 (1)). Undiluted passage yields contained a large number of defective virus particles of different classes, designated as T and V particles (UCHIDA *et al.* 1968 (2)). SV 40 virions prepared by undiluted passages consisted mainly of defective particles with lower densities and DNA from light virions were shorter than reference molecules by 15 % (YOSHIIKE, 1968 (3)). The present work confirmed the previous observation that DNA molecules extracted from virions of undiluted passage were shorter than those of dilute passage and that molecular length of SV 40 DNA ran parallel with infectivity of virions, and newly demonstrated the existence of small circular DNA molecules shorter than 1 μ from virions prepared by undiluted passages and dimers as well as replicating form of defective SV 40 DNA from both purified virion and the nuclei of SV 40 infected VERO cells. The implications of these findings are discussed.

MATERIALS AND METHODS

Virus

SV 40 virus strain 777, small plaque type (plaque size 1—2 mm), kindly supplied from Dr. K. TOYOSHIMA at Research Institute of Microbiology, Osaka University, was used in the present studies. Virus stocks were prepared by dilute and undiluted passages. In the case of undiluted passage, 5 ml of supernatant fluid of infected VERO cells with complete cytopathic effect was transferred succesively to monolayer cultures in dilution bottle (55×120 mm), while 5 ml of 1/10,000 diluted virus fluid was transferred to new cultures in the case of dilute passage.

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Tissue culture

VERO cells, an established cell line from an African green monkey kidney cell (YASUMURA *et al.* 1963 (4)), obtained through the courtesy of Dr. ITO of Toshiba Chemical Industry, were used for virus propagation and infectivity assays. The cells were grown in medium YLE containing 0.1 % PVP, 2 % heat-inactivated bovine serum. Medium YLE consisted of Earle's balanced salt solution, 0.1 % yeast extract, 0.5 % lactalbumin hydrolyzate, penicillin 100 units/ml, and streptomycin 100 μg/ml.

Production of virus

Dilution bottle cultures with confluent cell sheets (approximately 10^7 cells/bottle) were washed with phosphate buffered saline (PBS(-)). Virus was inoculated at 37°C for 2 hours. After adsorption the fluid was discarded and then the cell sheets were washed with PBS (-) and replaced with 10 ml culture medium. On reaching maximum CPE (4+), generally at the 10-14th day, the cultures were harvested by rubber policeman and frozen and thawed in dry ice acetone three times. Infected cells were homogenized by Potter homogenizer and centrifuged at 10,000×g for 30 minutes. The supernatant fluid was collected and ultracentrifuged at 80,000×g for 2 hours. The pellet was resuspended in PBS(-) and treated with 0.01 % trypsin and 1 % sodium deoxycholate for 30 minutes at 37°C. The suspension was then centrifuged at 10,000×g for 30 minutes, and the pellet was removed (BLACK *et al.* 1964 (5)).

CsCl density gradient analysis of virion

Part of virions purified above from infected cells with undiluted passage was analyzed in CsCl density gradients by centrifugation in an SW 39 rotor of Spinco Model L at 35,000 rpm for 20 hours (YOSHIIKE, 1968 (3)). Seven drops fractions were collected from the tube bottom by puncturing with a needle. 0.5ml PBS (-) were added to each fraction, after which optical density at 260 mμ was determined and the samples were used for the extraction of DNA.

Virus assay

Infectivity of virions was determined by test tube method and expressed as TCID 50/ml. Monolayer culture of VERO cells in test tube was infected with 1 ml of virus supensions successively diluted from 10^{-1} to 10^{-10}, adsorbed at 37°C for 2 hours, washed with PBS (-), and then supplemented by 1 ml of culture medium. CPE appeared usually at 7-10th day and TCID 50/ml was determined at the 10th day after infection.

Extraction of DNA

The extraction of SV 40 DNA was done according to the method of MARMUR (1961 (6)). The SV 40 virus infected VERO cells and purified virion were suspended in saline-EDTA, pH 8.0. Lysis was effected by the addition of equal volume of 2 % SLS (sodium lauryl sulfate) and the mixture was placed in a 60°C water bath for 10 minutes, then cooled to room temperature. 5M sodium perchlorate was added to a final concentration of 1 M to the viscous, lysed suspension and the whole mixture shaken with an equal volume of chloroform-isoamyl alcohol (24 : 1) for 30 minutes. The resulting emulsion was separated into three layers by centrifugation at 3,000 rpm for 30 minutes. The upper aqueous phase was further

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added with equal volume of chloroform-isoamylalcohol, shaken and centrifuged again. The final aqueous layer was carefully pipetted off into a narrow flask. The nucleic acid was precipitated by gently layering 2 volumes of ethyl alcohol on the aqueous phase. DNA fiber was spooled off by a stirring rod, which consisted of cellular DNA and viral DNA. DNA fiber and precipitate were transferred to 1/10 SSC (0.015 M NaCl+0.0015 M Na-citrate), and gently shaken until dispersion was complete. The solution was adjusted to standard saline-citrate concentration (0.15 M NaCl+0.015 M Na-citrate) by adding 10 SSC (1.5 M NaCl+0.15 M Na-citrate).

Electron microscopy

For the determination of the length of SV 40 DNA strands protein monolayer technique of KLEINSCHMIDT *et al.* (1961 (7)) was used. DNA at a concentration of 10 μg/ml and 0.01 % cytochrome c and 0.5 % formalin in 2 M ammonium acetate were spread on hypophase containing 0.3 M ammonium acetate and 0.5 % formalin. The rotary shadowed specimens were examined in a Hitachi 11 D at an instrumental magnification of 10,000 x. Magnification calibration of the electron microscope was performed with carbon grating replicas. Contour length measurement of two dimensional images of DNA were carried out with a calibrated map ruler. The virus particles were prepared for electron microscopy by placing a drop of virus on a carbon coated collodion covered specimen grid, allowed to settle for a few seconds, drained with filter paper. The grid was then inverted onto a drop of 1 % potassium phosphotungstate (PTA), floated for 60 seconds, and drained with filter paper and dried in the air. Electron micrographs were made at an instrumental magnification of 50,000 x.

RESULTS

Relationship between infectivity and length of DNA

Virus was separated into three groups (Fig. 1). Group I is high-titered original virus having 10^7 TCID50/ml, group II is undiluted passaged virus of group I, the titer being 10^4TCID50/ml, and group III is dilute passaged virus of group II and regained the titer of 10^6 TCID50/ml. Mean length of DNA molecules in each group was 1.50 ± 0.29 μ (64 molecules), 1.40 ± 0.17 μ (179 molecules) and 1.47 ± 0.14 μ (30 molecules) respectively. The correlation between molecular length and infectivity of virions indicated that undiluted passage caused shortening of DNA and defective virus was produced, but if defective virus group received dilute passage again, infectivity was recovered again.

Small circular DNA

One thing which was characteristics of group II was that there were small circular DNAs shorter than 1.0 μ (Fig. 2), which was not detected either in group I or III. In the case of group II (mean length 1.40 μ) these small circular DNAs had 72—30 % deleted from defective virus DNA.

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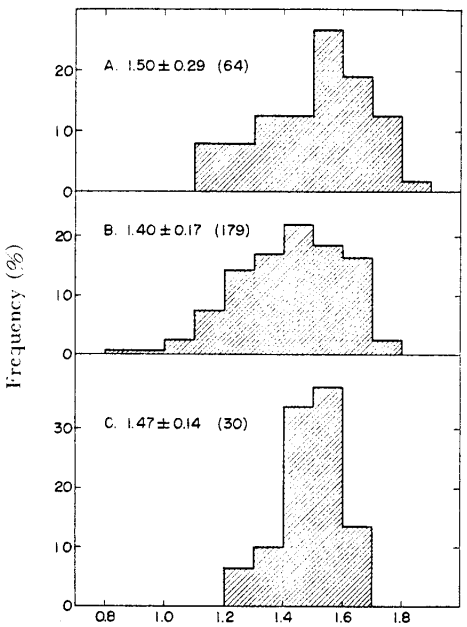


Fig. 1 Contour length distribution of SV 40 DNA
(A) original virus (10^7 TCID50/ml) designated as group I
(B) undiluted passaged virus (10^4 TCID50/ml) designated as group II
(C) dilute passaged virus of group II (10^6 TCID50/ml) designated as group III

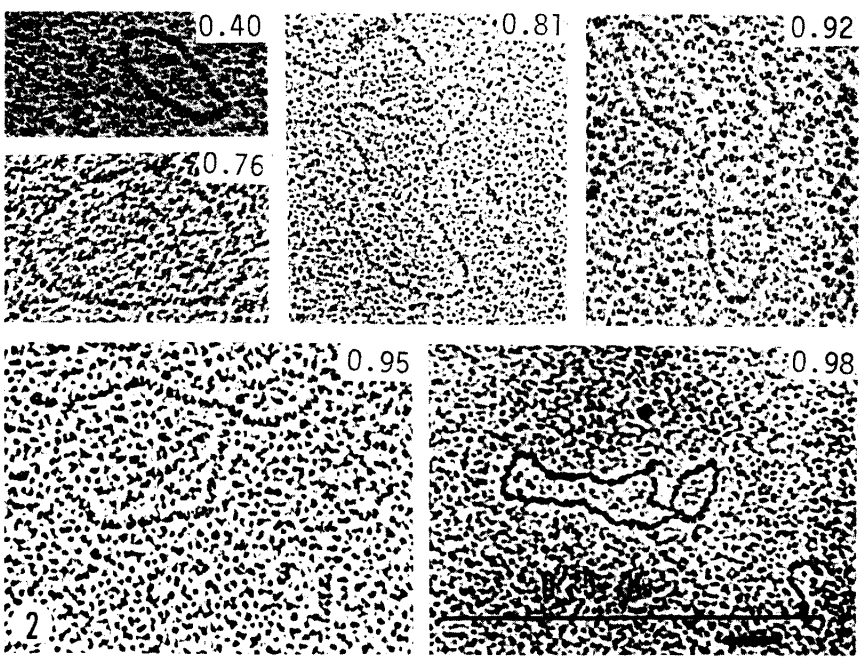


Fig. 2 Small circular DNA from undiluted passaged SV 40 virus. rotary shadowed with platinum palladium. magnification 100,000 x.

These short molecules were found in extremley low frequency, so biological activities of them were undetermined, .

Replicating form and dimer

Group II contained various sizes of DNA molecules from 0.81 to 1.70 μ (Fig. 3), but besides these molecules, replicating form (Fig. 4) and dimer (Fig. 5) were also found in low frequency from virions and the nuclei of SV 40 infected VERO cells. Fig. 4 a, b, and c would reveal the beginning

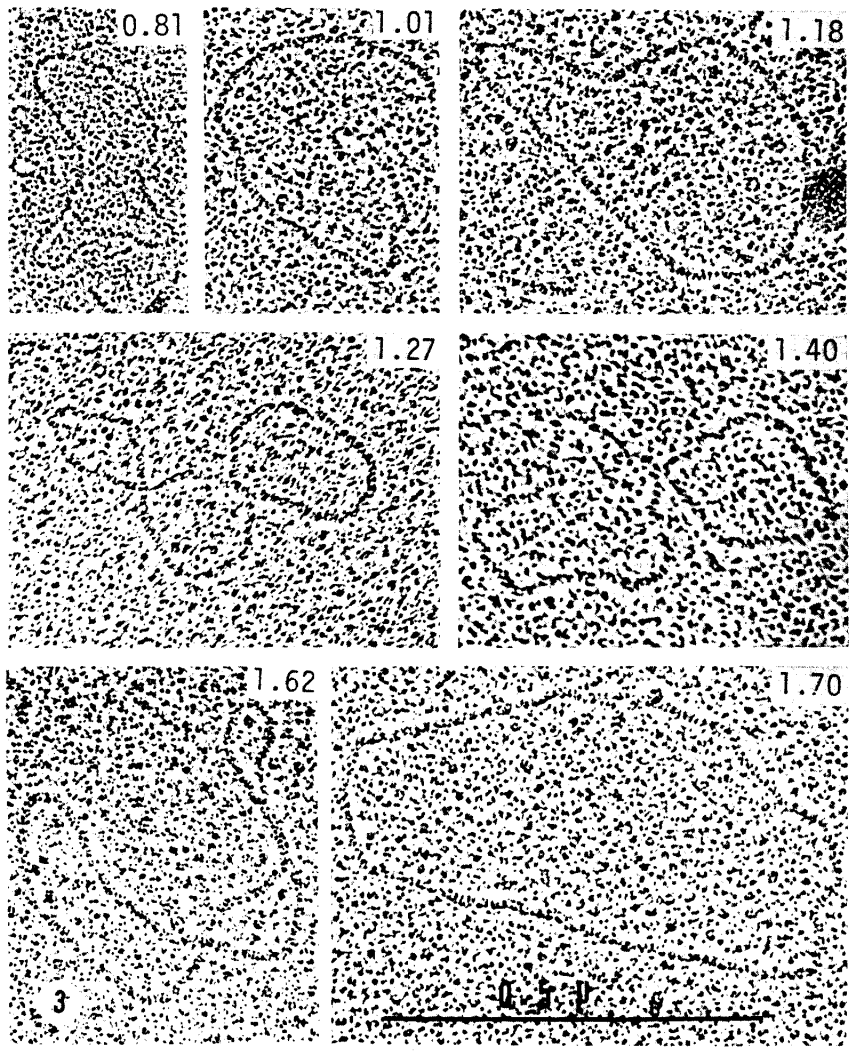


Fig. 3 Various length of DNA extracted from undiluted passaged virus. Magnification 100,000 x.

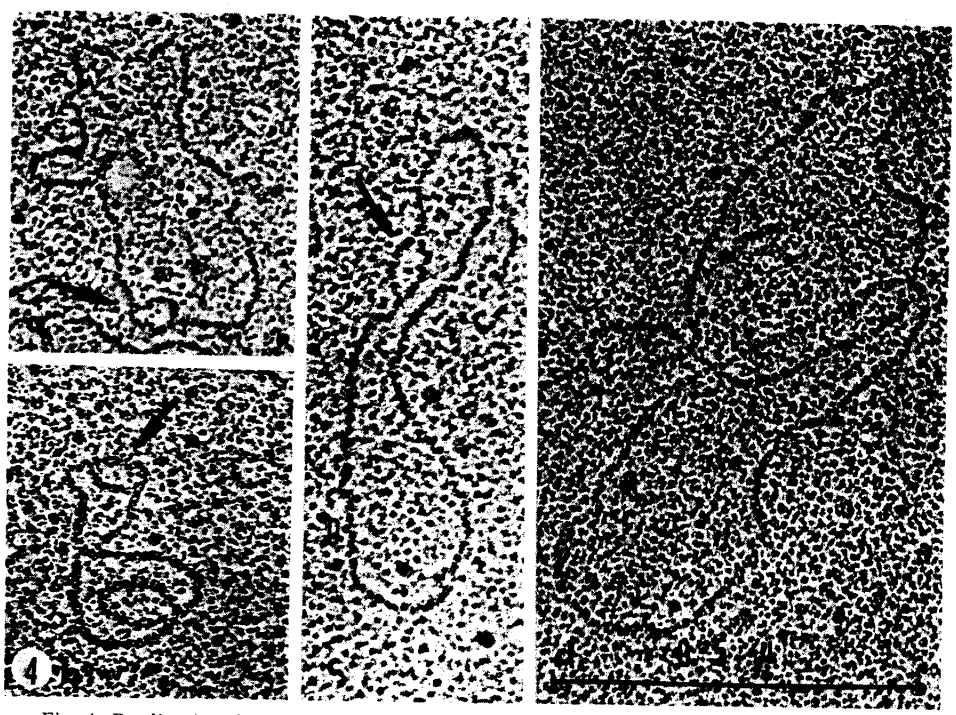


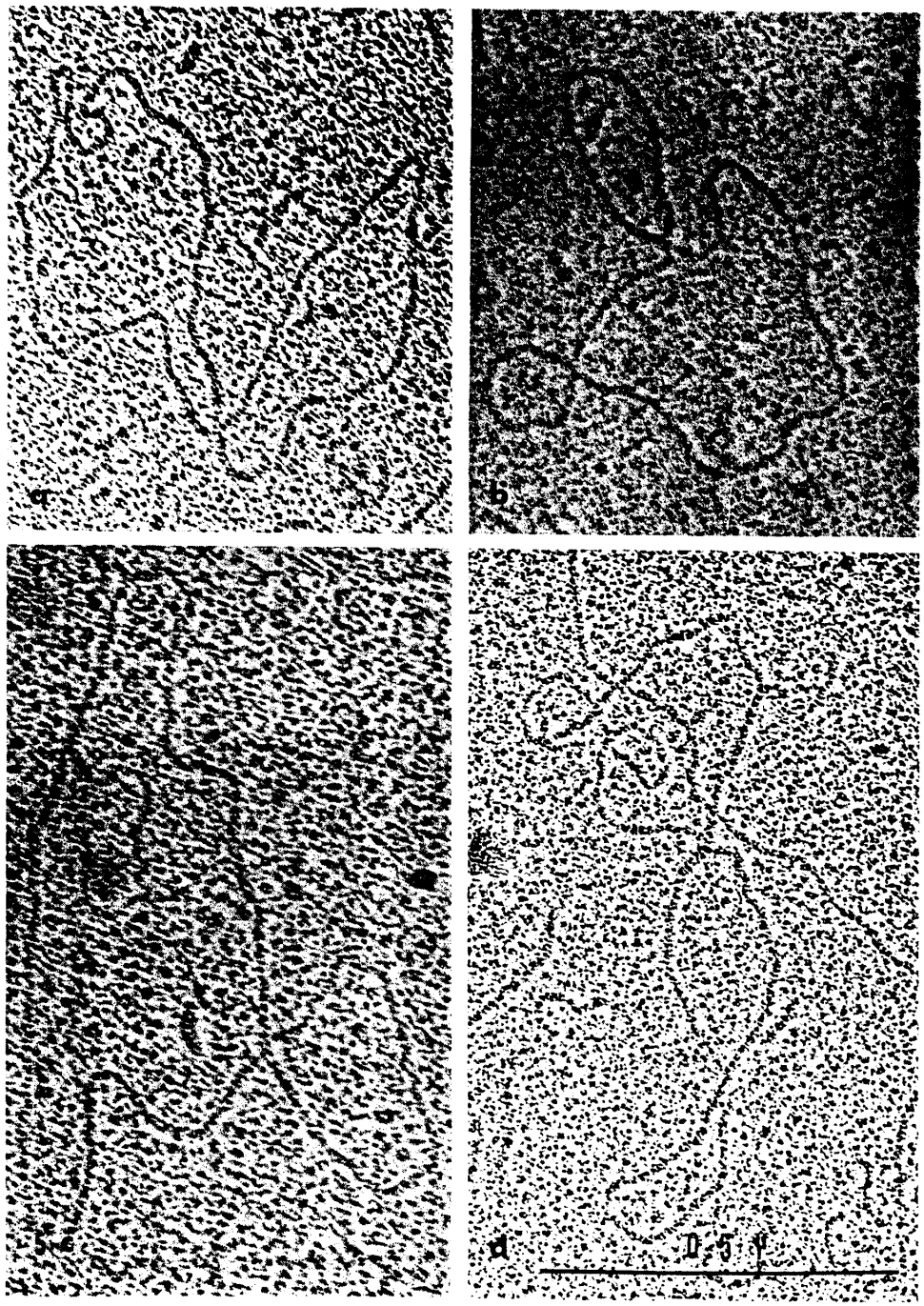
Fig. 4 Replicating form of SV 40 DNA. Arrow in photos (a, b, and c) indicates the site of replication and presumably reveals the beginning of replication. It is thought to be the final form of replication. As a and b are of small circular form, they are thought to be replicating form of defective SV 40 virus DNA. Magnification 100,000 x.

of DNA replication, and d the end of replication. As a and b in Fig. 4 showed the length less than 1.2 μ, it might be said that defective SV 40 virus DNA could replicate. Replication of SV 40 DNA was said to begin at 24—48 hours after infection (BUTEL *et al.* 1968 (8)), so these replicating form was extractd mainly from SV 40 infected VERO cells at the beginning of CPE appearance. Dimers shown in Fig. 5 a, b, and c would reveal those of defective virus DNA.

CsCl density gradient centrifugation

As shown in Fig. 6, virus II could be separated into two peaks, fraction 30 and 40. Fraction 30 contained mainly "full particles" and most of fraction 40 consisted of "empty particles" (Figs. 7 and 8). From each fraction DNA was extracted and was examined by electron microscopy. Contour length of DNA molecules extracted from fraction 30 and 40 was

Fig. 5 Dimers of SV 40 DNA. a, b and c are thought to be of defective SV 40 DNA. magnification 100,000 x.


a
b
c
d
0.5 μ

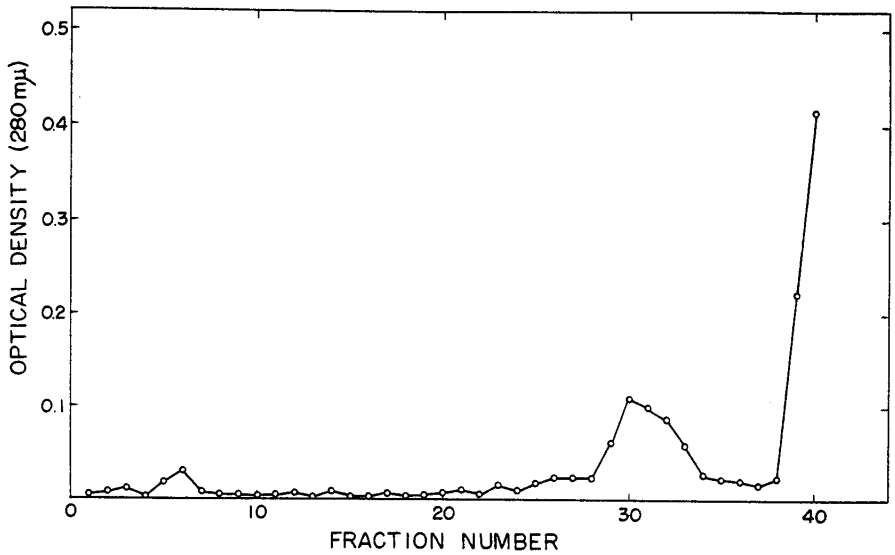


Fig. 6 CsCl density gradient centrigugation of undiluted passaged SV 40 virus. purified virus was centrifuged in an SW 39 rotor of Spinco Model L at 37,000 rpm for 40 hours, mean density 1.38 g/ml. optical density at 280 mμ.

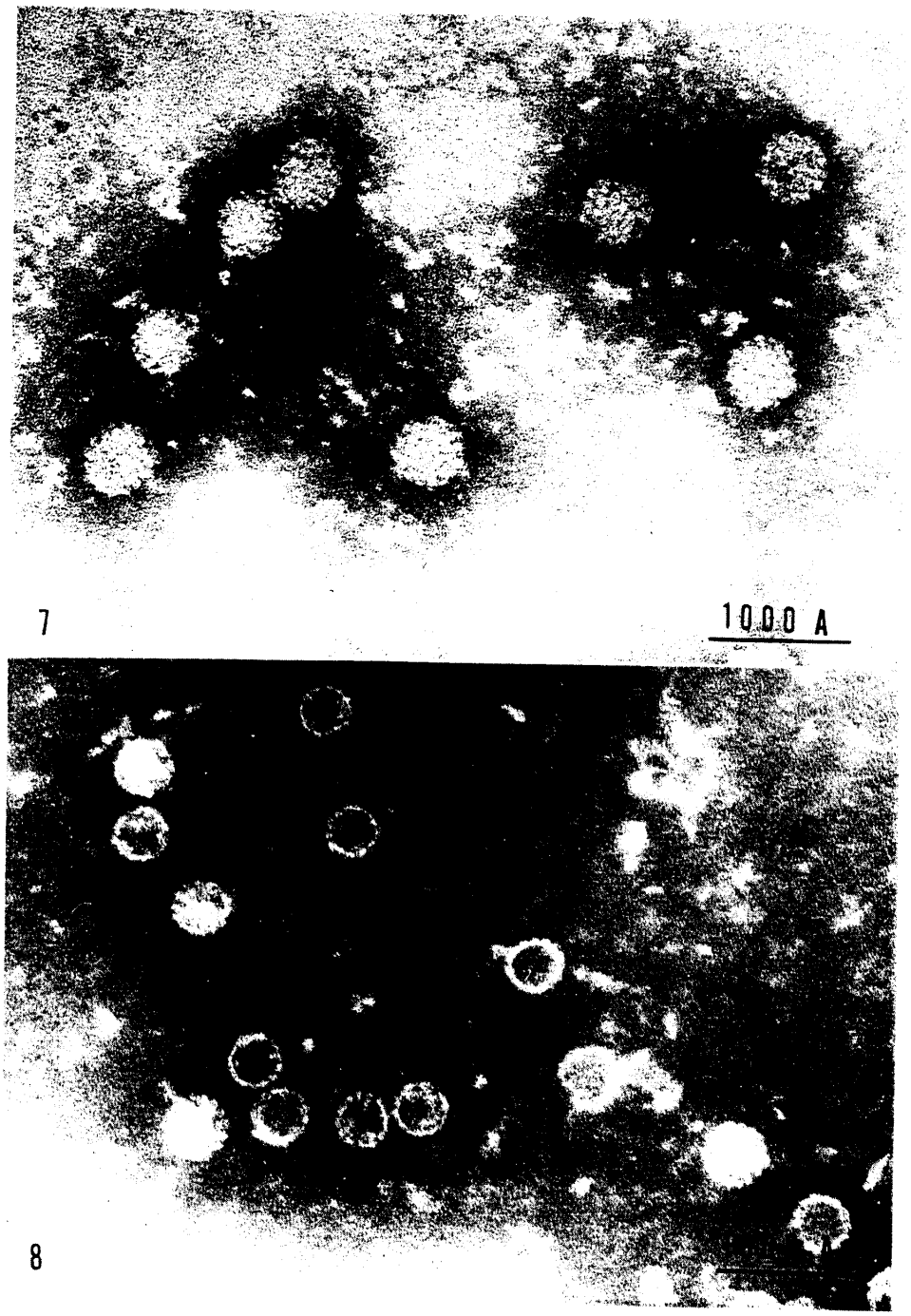
$1.34 \pm 0.13\ \mu$ (17 molecules) and $1.19 \pm 0.15\ \mu$ (10 molecules) respectively (Figs. 9 and 10). Fraction 30 contained twisted or tangled molecules as major components and less often extended circles as minor components and vice versa as to fraction 40.

DISCUSSION

YOSHIIKE (1968 (3)) reported the relation between biological activities of defective virion and length of DNA, stating that the reference plaque formers had the mean length of $1.76 \pm 0.09\ \mu$, that of defective virions being $1.50 \pm 0.12\ \mu$, and PFU was 150×10^1/particles and 1.0×10^4/particles respectively. Contour length of DNA from defective virion was 15 % shorter than plaque formers. ANDERER *et al.* (1967(9)) reported the size of DNA in which open circular form and noncircular form had mean length of 1.6 μ and 1.4 μ respectively at pH 6; the value had broad maximum at 1.65—1.69 μ for the open circular form and 1.45—1.6 μ for the noncircular form at pH 8. In our observation of SV 40 strain 777,

Fig. 7 Negatively stained virus of fraction 30. "Full paricles" are noted. Magnification 500,000 x.

Ftig. 8 Negatively stained virus of fraction 40. "Empty particles" are predominant. Magnification 500,000 x.


7
1000 A
8

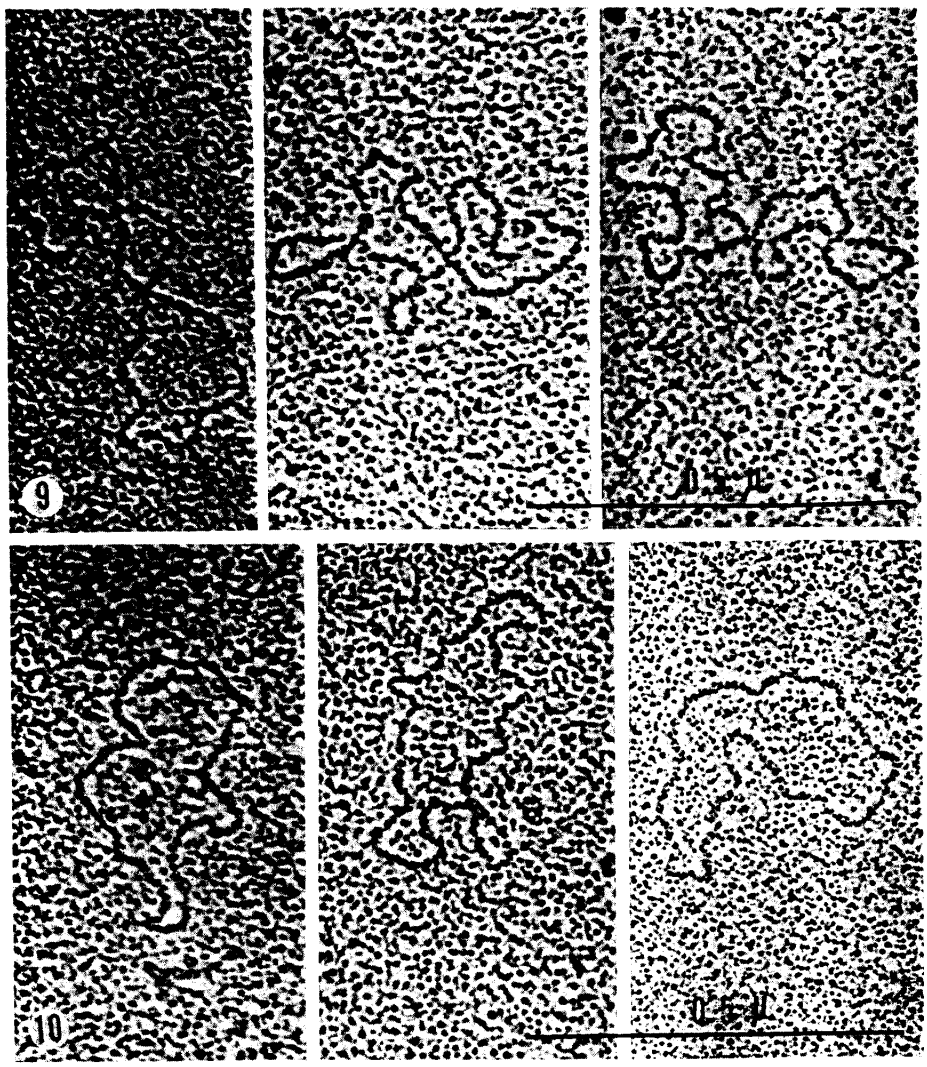


Fig. 9 Electron micrographs of DNA from fraction 30. Mean length 1.34 ± 0.13 μ. Magnification 100,000 x.

Fig. 10 Electron micrographs of DNA from fraction 40. Mean length 1.19 ± 0.15 μ. Magnification 100,000 x.

original virus (group I) was somewhat smaller than those reported by YOSHIIKE or ANDERER *et al.* Defective particles could also be found in the materials prepared by starting from the plaque-purified virus (UCHIDA *et al.* 1968 (2)). In serial undiluted passages, the yield of infective virus decreased markedly, but the yield of virus associated CF antigen was only

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slightly reduced (UCHIDA *et al.* 1966 (1)) and these T and V particles had no difference in tumorigenicity in hamsters, regardless of the length of circular DNA molecule contained therein (UCHIDA *et al.* 1968 (2)). In our experiences low titered virus could develop tumor to new born hamsters injected, as well as by high titered virus (unpublished data). In undiluted passage i-factor which interfered with the replication of virus when mixed with dilute seed were found (UCHIDA *et al.* 1966 (1)), but the recovery of infectivity of group III by dilute passage would indicate a reduction in the concentration of the i-factor from defective virus group. KOCH *et al.* (1967 (10)) reported the molecular weight of SV 40 17.3×10^6, and MAYOR *et al.* (1963 (11)) 44×10^6. The diameter of full particles was reported to be 45.0 ± 1.7 mμ and that of empty particles 42.0 ± 1.7 mμ (10), but in our observation they had no difference in diameter between them.

Defective virus DNA was thought to be due to the deletion of DNA of plaque formers, which occurred during replication of the virus (YOSHIIKE, I968 (3)). Molecular weight of SV 40 virus was reported to be 17.3×10^6 (KOCH *et al.* 1967 (11)) and that of slow component (16.1S) DNA and fast component (21.2 S) DNA was calculated to be 3.2×10^6 and 7×10^6 respectively (CRAWFORD *et al.* 1964 (12)). The number of deleted nucleotides could be estimated directly by electron microscopy, assuming that a single DNA fragment of 750 nucleotide pairs was deleted by a single step at a random site in a ring molecule of 5000 nucleotide pairs, the T-antigen forming activity would decrease to about 35 % of that of plaque formers (YOSHIIKE, 1968 (3)). Defective DNA molecules extracted from small plaque type and a large plaque variant was 15 %, 12 % shorter than those from plaque formers (YOSHIIKE, 1968 (13)). Supposing that 1.50 μ were the mean length of plaque formers in group I, DNA shorter than 1.32 μ would become defective. Small circular DNAs shorter than 1.0 μ were inevitably of defective virus. From previous papers about the length of SV 40 DNA, such short DNA molecules could not be detected. Since such a small circular DNA was very few, it was impossible to collect them specifically by CsCl density gradient and detect the biological activities of them, but these DNAs were obtained from purified virion, so the origin of small circular DNA must necessarily be SV 40 virus. Small circular DNA was also observed from Hela cell (RADLOFF *et al.* 1967 (14)., OMURA *et al.* 1968 (15)), human cancer cell mitochondria (ODA, 1967, 1968 (17), ODA, 1968 (18), ODA, 1969 (3)), and chemical carcinogen-induced and virus-induced tumor cell mitochondria and nuclei (INABA, 1967 (20), ODA, 1967, 1968 (17), ODA, 1968 (18), ODA *et al.* 1969 (21)). The significance of the small circular DNA found in oncogenic virus and cancer cell remains

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unclarified.

Replication of SV 40 virus was noted at 48 hours after infection (BUTEL *et al.* 1968 (8)), and replicating form was mainly extracted from VERO cells 3—4 days after infection. Whether these molecules are really replicating form or denatured form could be easily differentiated. Mitochondrial DNA of lambda phage was denatured by heating at 100°C and fixed by formalin at the same time, which caused separation of double stranded DNA into two single stranded DNAs (NASS, 1968 (22)). INABA *et al.* (1968 (23)) reported the replicating form of mitochondrial DNA of AH-130 cells; the fact that replicating form was found in the same fraction of open circular form of mitochondrial DNA suggested the possibility that this form of DNA changed to open circle and replication began. These changes of molecular forms were thought to have something to do with a kind of replicating mechanism at replicon level. Marmur's method of DNA extraction adopted here did not affect such a denaturing effect, so these two stranded molecules could be said to be due to replication of DNA. Defective SV 40 genomes were found to replicate (BUTEL *et al.* 1968 (8)). The state of replication of defective SV 40 genome was observed electronmicroscopically in the present study.

SUMMARY

The correlation of infectivity to the length of SV 40 DNA by dilute and undiluted passage was described. DNA was extracted from purified virions by Marmur's method, and propagation of virus was done using VERO cells. The infectivity of SV 40 (simian virus 40) ran parallel with the length of its DNA. Undiluted passage caused shortening of DNA and decrease in infectivity, but when these undiluted group was passaged dilutely, length of DNA approached the original length and the infectivity recovered. In undiluted passage group small circular DNAs under 1.0 μ, so far not reported in the SV 40 DNA, were found in low frequency. Replicating form and dimers were also noted from virions and the nuclei of SV 40 infected VERO cells.

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