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Shinichiro Yamamoto* Takuzo Oda†

*Okayama University,

†Okayama University,

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Shinichiro Yamamoto and Takuzo Oda

Abstract

Electron microscopic observation of replicating SV 40 DNA has revealed the existence of two types of RF, e form and (1 form. The frequency of RF at 54 hours after infection was 8.9% for the e form and 4.3% for the (1 form. Morphological evidence exhibits that in (1 form RF the tails are, predominantly, shorter than the viral genome and double length SV-40 genomes are also capable of replication in SV-40 infected VERO cells.

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ELECTRON MICROSCOPIC STUDIES OF REPLICATING SV 40 DNA

Shinichiro YAMAMOTO and Takuzo ODA

Department of Biochemistry, Cancer Institute, Okayama University Medical School, Okayama, Japan (Director: Prof. T. ODA)

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The existence of replicating form (RF) of circular DNA molecules with one linear single tail (σ form) was reported in ϕx 174 (1), *E. coli* 15 (2), colicin factor E 1 (3) and lamda phage (4). Previous studies of the biophysical properties of SV 40 DNA revealed the existence of RF of circular DNA molecules with two branch points and no free end (θ form) (5). In the present report we have isolated σ form RF of SV 40 DNA from virus-infected VERO cells, applying the method of HIRT (6). In addition to θ form RF single tailed circular molecules were observed not only in monomers but also in double length SV 40 DNA.

MATERIALS AND METHODS

Tissue culture

VERO cells, an established cell line from African green monkey kidney cells, were used for the propagation of virus. The cells were grown in a dilution bottle (5.5×11.0 cm) using medium YLE containing 0.1% PVP, 10% heat-inactivated bovine serum. Two bottles of monolayer culture of VERO cells at the 450th passage were used at the same time, one is for noninfected control and the other for SV 40 infection.

Labeling of SV 40 DNA with ^{3}H-thymidine

Monolayer culture of VERO cells were infected with 1 ml SV 40 virus having the infectivity of 10^6 PFU/ml, absorbed for two hours at 37°C. After infection SV 40 virus was removed and supplemented with 10 ml YLE medium containing 10% bovine serum. At 16 hours after infection 5μc/ml ^{3}H-thymidine (20.6 c/mmole) was added to the bottle. At 53 hours after infection radioactive medium was removed and replaced by fresh medium containing 10% bovine serum and kept for one hour to exhaust thymidine pool. Infected cells were washed with phosphate buffered saline (PBS(−), pH 7.6) three times and lysed according to the method of HIRT (6). Uninfected controls were mock infected with PBS (−) instead of SV 40 virus and other treatments were the same as in SV 40-infected cells.

Selective extraction of SV 40 DNA

SV 40-infected cells were lysed by adding 5 ml of 0.6% sodium lauryl sulfate

containing 0.01 M EDTA, pH 7.5 and the lysate was treated with 2 mg/ml pronase (Nagarse) at 37°C for one hour. Five M NaCl was added to make the final concentration of 1 M. Most of the cellular DNA was precipitated by centrifugation of 17,000×g for 30 minutes and the supernatant was treated with 0.3 mg/ml RNase at 37°C for one hour.

Equilibrium density gradient centrifugation

Solid CsCl (3.60 g) was added to 4 ml above supernatant (mean density 1.55 g/cm^3) and centrifuged at 45000 rpm for 20 hours in an RPS 65 TA rotor of a Hitachi ultracentrifuge. Eight drop fractions (0.15 ml) were collected from the bottom successively. 0.5 ml SSC (0.15 M NaCl + 0.015 M Na-citrate, PH 7.6) was added to each fraction. 0.05 ml of each fraction was assayed for radioactivity by a Packard liquid scintillation counter.

Electron microscopy

DNA was prepared for electron microscopy essentially as described by KLEINSCHMIDT *et al.* (7). A solution containing 15 μg/ml DNA, 1 M ammonium acetate, 0.01% cytochrome c and 0.5% formalin were spread over a 0.3 M ammonium acetate solution containing 0.5% formalin. The film was picked up on a carbon coated copper grid and the grid was fixed by submersion in 95% ethanol and air-dried. The grid was rotary shadowed with platinum palladium and observed in a Hitachi 11 D electron microscope. Magnification calibration was done with carbon grating replicas. Contour lengths of DNA were measured with a map ruler.

RESULTS

CsCl density gradient centrifugation

The sedimentation behaviour of replicating SV 40 DNA was analyzed (Fig. 1). Both infected and noninfected cells had one major peak at fraction 6 or 7, but in the noninfected cells no middle peak was noted and infected cells had two more minor peaks at fractions 11 and 17. These three peaks (fractions 7, 11 and 17) of the infected cells were examined by electron microscopy. Fractions 7 and 11 consisted of cellular DNA, namely, long linear fiber and no circular DNA but a heterogeneous population of molecules was observed from fraction 17 of the infected cells consisting of three types; twisted or open circular molecules, circular molecules with two branch points (θ form) and circular molecules with a single tail (σ form). The latter two were thought to be RF of SV 40 DNA. Fractions 16-20 of the infected cells were collected and examined. Out of 370 molecules of SV 40 DNA, θ form and σ form existed by 33 and 16 molecules, the frequency of which corresponded to 8.9% and 4.3% respectively.

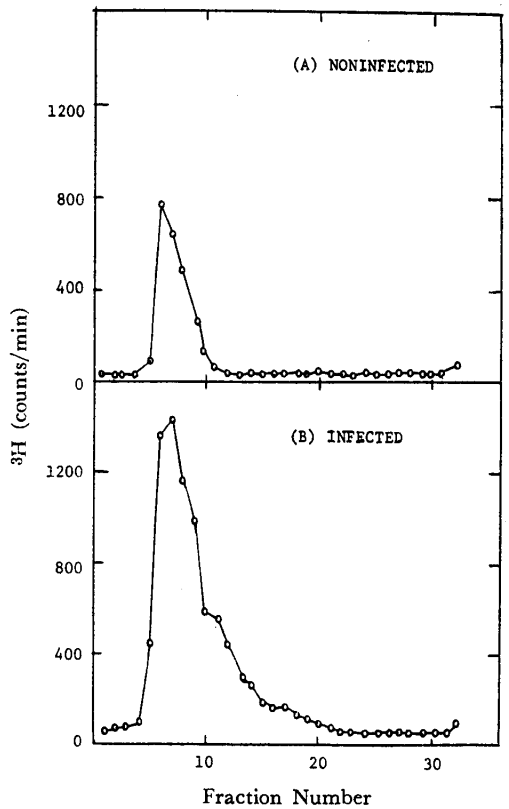

Fig. 1 CsCl density gradient centrifugation of DNA from (A) noninfected VERO cells and (B) SV 40 infected VERO cells.

Centrifugation was done at 5,400 rpm for 20 hours in an RPS 65 TA rotor of Hitachi ultracentrifuge. Eight drops were collected from the tube bottom and each fraction was added with 0.5 ml SSC. ^{3}H count is shown. Note one major peak at fraction 7 and minor peaks at fractions 11 and 17 of the infected cells.

RF SV 40 DNA

Of the circular molecules with tails, sixteen were spread well enough to be measured. Of these sixteen molecules, eleven possessed rings of viral DNA length and five possessed rings twice this length (Fig. 2). All of the molecules with tails (except one) had tails of less than viral length. One molecule had a tail 19% longer than the ring to which it was attached (Fig. 2, No. 11). The rate of circle length to tail length had considerably uniform distribution. The replication rate ranged from 10% to 95% almost uniformly. Representative molecules are shown in Fig. 3. A double length ring with a tail is shown in Fig. 3-d. We have found from this

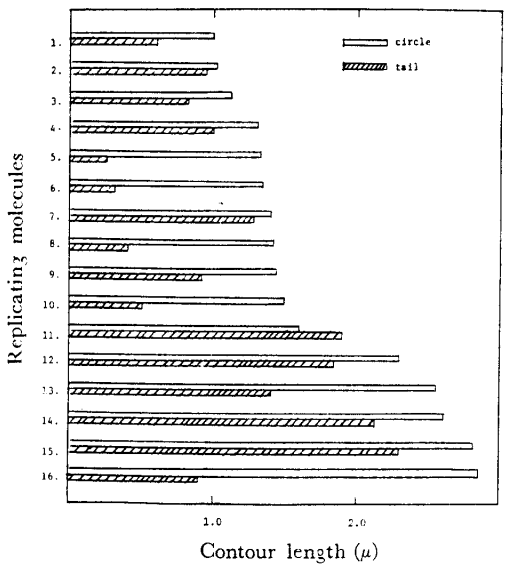

Fig. 2 Distribution of tail lengths

Tail length is compared with the circle length. One molecule (No. 11) has a longer tail than the circle. The replication rate (ratio of tail to circle) is uniformly distributed.

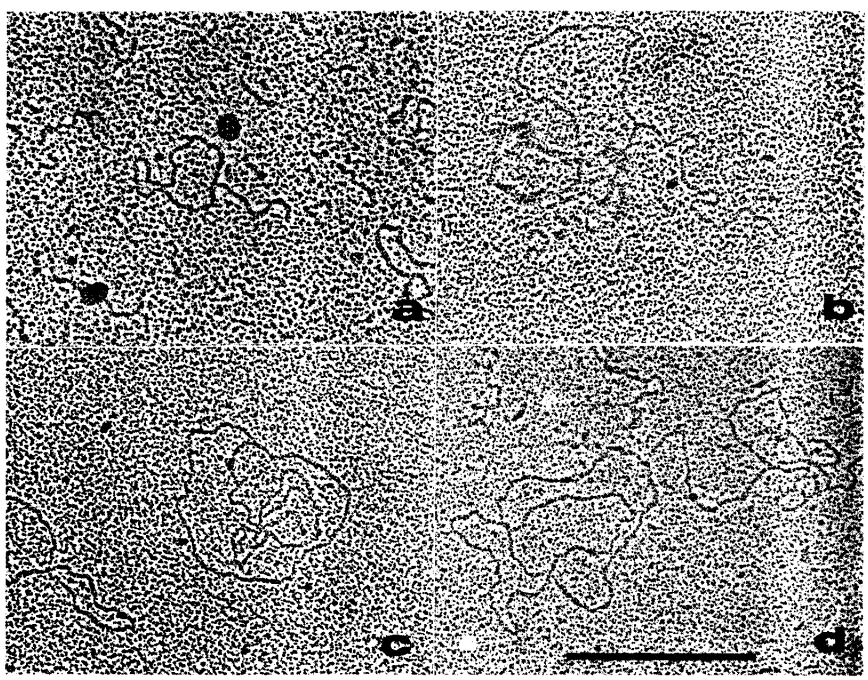

Fig. 3 Tailed molecules in DNA from fractions 16 to 20 of SV 40 infected VERO cells (Fig. 1). d. shows a double length DNA with a tail. The bar indicates 0.5μ.

experiment and former experiment (8) the existence of much deleted molecules which undergo the replication. As shown in Fig. 2, the smallest molecule of RF was 1.0μ and from the former experiment we have found θ form RF of the contour length of 0.71 to 1.0μ.

DISCUSSION

RF of SV 40 DNA were isolated from virus-infected VERO cells. Two kinds of RF, *e. g.* θ form and σ form RF were noted at 8.9% and 4.3% of all the viral DNA examined, respectively. Concerning σ form RF, the replication rate, namely, the ratio of tail to circle seems to be in uniform distribution, which was also noted in θ form RF of SV 40 DNA (8). In lambda phage RF in an early stage was found more than that in late stage (4) and they pointed out the possibility that the rates of DNA replication vary, dependent upon the extent of replication; namely, the first portion of the molecules might be replicated more slowly than the remainder, giving rise to a pool of incomplete intermediates possessing a short tail. On the contrary LEVINE *et al.* (9) reported that about 75% of the replicating molecules had completed 90 to 65% of the replication and ascribed the skew toward the late stage of replication to a rate-limiting step in the replication process. In our experiment the skew of RF to neither early nor late stage of replication was observed. The speed of replication of SV 40 DNA in VERO cells seems to be uniform from beginning to end of replicating process.

The tail length in σ form RF, is generally shorter than the circle, which were verified in ϕx 174 (1) and colicin factor E 1 (3). One molecule possessing a tail longer than the circle was noted in our experiment. KIGER and SINSHEIMER (4) observed the same phenomena and supposed the cause that if maturation of phage DNA and DNA replication are not coordinated, conditions may exist under which DNA replication could occur at a greater rate than maturation. Replication might then continue past the point of initiation on the ring and generate a tail longer than one phage DNA. This assumption might be applied to SV 40 DNA replication.

As pointed out in Fig. 2, the existence of double length rings with tails is an intermediate of replication. In lambda phage (4) morphological evidence was observed showing double length rings to which tails are attached. The fact that double length rings of viral DNA could replicate was demonstrated not only in lambda phage but in SV 40. Another findings are the existence of small circular RF of SV 40 DNA. The

contour length of SV 40 DNA was reported to be 1.4 to 1.7 μ (5, 10, 11), while molecules smaller than 1.0 μ were noted to replicate in our experiment. YOSHIIKE (11) reported that successive undiluted passage caused the shortening of DNA approximately by 15% from plaque formers and the shorter DNA was thought to be defective virus DNA. We have demonstrated RF of SV 40 DNA smaller than 1.0 μ (5, 8) and ascribed the cause to the deletion of DNA. Small RF not only in θ form (5, 8) but in σ form observed in our experiment suggests that greatly deleted defective SV 40 DNA are capable of replication as in case of plaque formers.

SUMMARY

Electron microscopic observation of replicating SV 40 DNA has revealed the existence of two types of RF, θ form and σ form. The frequency of RF at 54 hours after infection was 8.9% for the θ form and 4.3% for the σ form. Morphological evidence exhibits that in σ form RF the tails are, predominantly, shorter than the viral genome and double length SV-40 genomes are also capable of replication in SV-40 infected VERO cells.

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