DNA Synthesis in Resting African Green Monkey Kidney Cells Infected with Defective SV 40

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SUMMARY

SV40 (small-plaque type) generates heterogeneous defective virus particles during serial undiluted passages. DNA synthesis in resting African green monkey kidney cells infected with such defective particles was studied by autoradiography and band-sedimentation. The defective particles were found to have capacities to induce synthesis of cell DNA and to direct synthesis of virus DNA. The progeny virus DNA was more heterogeneous than the standard DNA.

INTRODUCTION

Small-plaque type SV40 yields various defective particles during serial undiluted passages in African green monkey kidney (GMK) cells (Uchida, Watanabe & Kato, 1966; Uchida et al. 1968). The defective DNA molecules are heterogeneous in size and somewhat shorter than the complete molecules (Yoshiike, 1968). They contain deletions, insertions and substitutions (Tai et al. 1972; Yoshiike, Furuno & Uchida, 1974). Furthermore, host DNA sequence in supercoiled SV40 DNA has been shown to increase during serial undiluted passages (Lavi & Winocour, 1972; Martin et al. 1973).

Infection of GMK cells with nondefective SV40 results in the induction of cell DNA synthesis and the sequential appearance of virus specific antigens, newly replicated virus DNA, virus particle protein, and progeny virus particles. Some defective particles have been shown to direct synthesis of T antigen but not of virus protein and some, to produce V antigen but not infectious progeny (Uchida et al. 1968). This paper describes the induction of cell DNA synthesis and the replication of virus DNA in resting GMK cells infected with the small-plaque type SV40 defective particles.

METHODS

Virus. SV40 strain 777 (small-plaque) was used. Assay for infectivity, and T and V antigen-forming activities, purification, and fractionation of the virus were as previously described (Uchida et al. 1968). The standard virus sample was purified from a dilute-passage stock. We combined the leading heavier fractions in a CsCl gradient, excluding the tailing lighter fractions. The virus particles had a buoyant density of 1.34 g/ml in CsCl solution. The standard sample had a sp. infectivity of 1 p.f.u./100 virus particles and was assumed to be free of defective viruses. Its antigen-forming activities were normalized so that it had sp. act. of 1 V antigen-forming unit (v.f.u.)/100 virus particles and 1 T antigen-forming unit (t.f.u.)/100 virus particles. The defective virus particle sample was obtained after three serial undiluted passages. The leading heavier fractions in a CsCl gradient were excluded.
and the appropriate lighter fractions were combined. The virus particles had a mean buoyant density of 1.33 g/ml in CsCl solution. The defective virus particle sample had normalized sp. act. of 1 p.f.u./12000 virus particles, 1 v.f.u./1200 virus particles, and 1 t.f.u./140 virus particles, indicating that it was enriched with T antigen-forming defective SV40 (T particle) by a factor of about 100.

**Cell culture.** The primary GMK cell cultures were stored at −80 °C and the secondary cultures were used for the experiments. The coverslip cultures (1.2 x 3.2 cm) were grown in medium LE (Earle’s balanced salt solution containing 0.5% lactalbumin hydrolysate supplemented with 5% bovine serum). The culture medium was replaced with LE without serum on days 2 and 6 after cell seeding. On day 8 when the synthesis of DNA decreased to the lowest level (K. Kato, unpublished observations), the cultures were infected with the virus (0.1 ml). After an adsorption period of 2 h the cultures were incubated at 36 °C with the spent medium LE (that had been used for cell cultivation before infection) containing 0.1% anti-SV40 rabbit serum.

**Determination of DNA synthesis.** At various times after infection the coverslip cultures were labelled with [3H]-thymidine (2 μCi/ml) for 1 h, fixed in Carnoy’s solution for 15 min, washed with 2% perchloric acid solution, and then dipped in Sakura NR-M2 emulsion for autoradiography. The proportion of DNA synthesizing cells was calculated on the basis of over 1000 cells. For characterization of DNA synthesized the infected cultures were labelled with [3H]-thymidine (100 μCi/ml) for indicated periods. DNA, extracted by the pronase-SDS-phenol method (Berns & Thomas, 1965), was mixed with [32P]-labelled standard SV40 DNA and analysed by band-sedimentation in CsCl solution (Vinograd et al. 1963). For characterization of virus DNA the cultures infected with the standard and the defective samples were labelled with [14C]-thymidine (3.6 μCi/ml) and with [3H]-thymidine (140 μCi/ml), respectively, during the late stage of infection. Virus DNA was selectively extracted by the method of Hirt (1967). The Hirt’s supernatant fraction was deproteinized by extraction with phenol followed by chloroform extraction. Supercoiled circular DNA was isolated by sedimentation at 37000 rev/min for 44 h in CsCl solution (ρ = 1.58 g/ml) containing 500 μg ethidium bromide per ml (Radloff, Bauer & Vinograd, 1967). After ethidium bromide was removed by extraction with isoamyl alcohol followed by ether extraction (Hudson et al. 1969) and CsCl, by dialysis against SSC/100 (0.0015 M-NaCl, 0.00015 M-sodium citrate), the two virus DNAs were mixed and analysed by band sedimentation in neutral and alkaline CsCl solutions (Vinograd et al. 1963).

**Radiochemicals.** [6-3H]-thymidine (TRA. 61: sp. act. 5.0 Ci/mmol) and [2-14C]-thymidine (CFA. 219: sp. act. 58 mCi/mmol) were purchased from The Radiochemical Centre, England. [32P]-orthophosphate (P-32-I, carrier free; radioactivity, 1.5 x 102 mCi/ml) in 1 N-HCl was obtained from Japan Radioisotope Association, Japan.

**RESULTS**

*Induction of DNA synthesis in resting GMK cultures*

After infection with SV40, DNA synthesis is induced in GMK cells whose DNA synthesis has been suppressed by contact inhibition (Hatanaka & Dulbecco, 1966; Kit et al. 1967), by X-irradiation (Gershon, Sachs & Winocour, 1966), or by treatment with actinomycin D (Kato, 1968) or FudR (Ossowski & Reich, 1972). We determined by autoradiography the proportion of DNA synthesizing cells in cultures infected with the standard or defective virus. In preliminary studies we determined DNA synthesis at 24 h after infection with the standard virus, using various input multiplicities (0.1 to 100 p.f.u./cell). Since an input
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Fig. 1. Patterns of DNA synthesis induced after infection of resting GMK cells with standard and defective SV40. The resting GMK cultures (8 days old) were infected with SV40, washed with phosphate-buffered saline after the adsorption for 2 h, and incubated at 36 °C in the medium LE containing anti-SV40 rabbit serum (0.1%). The infected cultures were labelled with [3H]-thymidine for 1 h at 24, 48 and 72 h, fixed, and processed for autoradiography. ×—×, uninfected; ○—○, infected with the standard sample at 1 p.f.u./cell; ●—●, at 10 p.f.u./cell; ○—○, infected with the defective sample at 0.01 p.f.u. (1 t.f.u.)/cell; ○—○, at 0.1 p.f.u. (10 t.f.u.)/cell.

multiplicity of one or more p.f.u./cell was required for detection of induced DNA synthesis, we used two multiplicities, 1 and 10 p.f.u. (or 1 and 10 t.f.u.) per cell, for the standard virus in the present study. The patterns of induced DNA synthesis after infection with the standard SV40 (m.o.i. = 1, 10) are shown in Fig. 1. Comparable results were obtained with the defective virus particle sample, as shown in Fig. 1, at two multiplicities, 0.01 and 0.1 p.f.u. or 1 and 10 t.f.u. per cell. These results clearly indicate that at least some of the defective mutants produced during serial undiluted passages have capacities to induce DNA synthesis in resting GMK cells. Similar results were obtained when GMK cultures were infected with either the standard or defective virus in the presence of a low dose (0.1 or 0.2 μg/ml) of actinomycin D (the data are not shown).

Characterization of DNA synthesized in resting GMK cells after infection with defective SV40

Infection of resting monkey cells with SV40 results in the induction of cell DNA synthesis followed by replication of progeny virus DNA (Hatanaka & Dulbecco, 1966; Kit et al. 1967). To characterize DNA synthesized after infection with defective SV40, [3H]-labelled DNA extracted from infected cultures was mixed with [32P]-labelled standard SV40 DNA and sedimented through neutral CsCl density gradients. For comparison DNA from the cultures infected with standard SV40 was analysed similarly. Fig. 2 shows the sedimentation patterns of DNA labelled with [3H]-thymidine between 22 and 28 h after infection. Most of the DNA synthesized during this period (the early stage of infection) in the cells infected with the standard virus as well as the defective virus sedimented faster than virus DNA.
Fig. 2. Sedimentation patterns of DNA synthesized during the early (22 to 28 h) stage of infection with standard and defective SV40. [\(^{3}H\)]-labelled DNA (O---O) from infected cultures was mixed with [\(^{32}P\)]-labelled (●—●) standard SV40 DNA containing 21S (supercoiled) and 17S (nicked) components, and analysed on CsCl gradients. (a) DNA extracted from cultures infected with the standard sample at 1 p.f.u./cell. (b) As in (a) but 10 p.f.u./cell. (c) DNA from cultures infected with the defective sample at 0.01 p.f.u./cell. (d) As in (c) but at 0.1 p.f.u. (1 t.f.u.)/cell.

Fig. 3. Sedimentation patterns of DNA synthesized during the late (46 to 56 h) stage of infection with standard and defective SV40, analysed as in Fig. 2. (a) DNA from the cultures infected with the standard sample at 1 p.f.u./cell. (b) As in (a) but at 10 p.f.u./cell. (c) DNA from cultures infected with the defective sample at 0.01 p.f.u. (1 t.f.u.)/cell. (d) As in (c) but at 0.1 p.f.u./cell. O—O, [\(^{3}H\)]-DNA; ●—●, [\(^{32}P\)]-DNA.
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Fig. 4. Sedimentation patterns of virus DNA synthesized in standard ([14C]-labelled) and defective ([3H]-labelled) SV40 infection. The selectively extracted two virus DNAs were centrifuged on neutral (pH 7.6) and alkaline (pH 12.0) CsCl gradients. (a) Sedimentation in neutral CsCl solution. ●, [14C]-SV40 DNA in standard SV40 infection; ○, [3H]-DNA in defective SV40 infection. (b) Sedimentation in alkaline CsCl solution. ●—●, [14C]-DNA in standard SV40 infection; ○—○, [3H]-DNA in defective SV40 infection. (c) Comparison of the band width between the two virus DNAs synthesized in standard and defective SV40 infection. The radioactivity counts of fraction 15 to 40 in (b) were combined and the proportion of each fraction to the total counts (%) was calculated. ●—●, [14C]-DNA in standard SV40 infection; ○—○, [3H]-DNA in defective SV40 infection.

Since this fast-sedimenting DNA has been established to be mostly cellular in origin (Kit et al. 1967), we conclude from Fig. 2 that at least some of the defectives can induce synthesis of cell DNA in resting monkey cells.

Fig. 3 shows the sedimentation patterns of DNA labelled with [3H]-thymidine between 46 and 56 h. At this late stage, replication of cell and virus DNAs was evident. The sedimentation patterns of DNA synthesized in the GMK cells infected with the defective sample at multiplicities of 0.01 and 0.1 p.f.u. per cell (Fig. 3c and d) resembled those in the cells infected with the standard sample at 1 and 10 p.f.u. per cell, respectively (Fig. 3a and b). These results indicate that some of the defective mutants have capacities not only to induce cell DNA synthesis but to direct synthesis of virus DNA. It should be noted that the major peak of virus DNA that was synthesized in the cells infected with the defective SV40 did
not coincide completely with the standard DNA, whereas that of the cells infected with the standard SV40 did. To confirm the difference in sedimentation velocity between these two virus DNAs we extracted supercoiled circular DNA from the two cultures infected separately with the standard and the defective samples at an input multiplicity of 0.5 t.f.u. per cell. The virus DNAs labelled with [\(^{14}\)C]-thymidine (for the standard sample) and [\(^{3}\)H]-thymidine (for the defective sample) between 48 and 53 h were mixed and sedimented through neutral and alkaline CsCl density gradients. The sedimentation patterns in neutral CsCl solution shown in Fig. 4a are consistent with those in Fig. 2 and 3. Evidently, the virus DNA synthesized in cells infected with the standard sample sedimented faster than that in cells infected with the defective sample. Since the difference in sedimentation velocity between the two virus DNAs was also evident in the alkaline CsCl solution (Fig. 4b), this difference is ascribable to the difference in DNA mass or DNA length as discussed previously (Yoshiike, 1968). To compare homogeneity between the two virus DNAs we plotted the proportion of radioactivity of each fraction to the total counts of virus DNA, assuming that fractions from 25 to 40 in Fig. 4b contained virus DNA (Fig. 4c). As the band-width of the defective DNA is slightly broader than that of the standard DNA, the former is interpreted to be slightly more heterogeneous than the latter. We conclude from Fig. 4 that infection with the defective SV40 sample, which contains heterogeneous deletion mutants (Yoshiike, 1968), results in the production of defective DNA molecules that are somewhat shorter and slightly more heterogeneous than the complete SV40 DNA molecules.

**DISCUSSION**

The defective sample used in the present study had a specific infectivity of 1 p.f.u./12,000 virus particles and a specific T antigen-forming activity of 1 t.f.u./140 virus particles. Therefore, the two multiplicities employed for the defective sample were almost the same with those for the standard sample in terms of t.f.u./cell. The amount of virus DNA synthesized after infection with the defective sample was comparable to or more than that after infection with the standard sample at the corresponding multiplicities of infection expressed as t.f.u./cell (Fig. 3). A simple interpretation of these results would be that the T antigen-forming defective viruses (T particles) have the capacities to induce synthesis of cell DNA and to direct syntheses of T antigen and virus DNA. This interpretation is consistent with the previous results suggesting that the T particle genome can replicate itself (Uchida & Watanabe, 1969). Induction of DNA synthesis during the early stage of infection (at 24 h in Fig. 1) was more prominent in the cultures infected with the defective virus sample than in those infected with the standard sample at comparable input multiplicities (t.f.u./cell). We repeated similar experiments four times and obtained essentially the same results as shown in Fig. 1 in three cases. In one case the degree of induced DNA synthesis was the same between the cultures infected with the standard and defective samples. Since the DNA synthesized during this period was mostly cellular in origin (Fig. 2), the results may be interpreted to suggest that the T particles induce synthesis of cell DNA (and virus DNA) more efficiently than the standard viruses do, owing to the lack of the late functions. Alternatively, the defective sample may contain, besides T particles, some mutants that have the capacity to induce synthesis of cell DNA. Whether or not complementation between defective mutants plays a significant role in induction of cell DNA synthesis and in replication of virus DNA remains to be investigated.

The progeny virus DNA in defective SV40 infection was found to be somewhat shorter and slightly more heterogeneous than the standard SV40 DNA. This result is not surprising
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If the defective SV40 particles containing such DNA molecules (Yoshiike, 1968) can direct synthesis of virus DNA. From the present study it appears reasonable to assume that infection of monkey cells with a certain class of defectives (namely T particles) alone is sufficient for induction of cell DNA synthesis and production of defective virus DNA. In a preliminary study we examined formation of virus particles in defective SV40 infection. We infected GMK cultures separately with the standard and defective samples at an input multiplicity of 1 t.f.u./cell, labelled them with [3H]-thymidine, and harvested them at 72 h after infection. The radioactivity of the virus fraction (in a CsCl density gradient) obtained from the culture infected with the defective sample was less than one-fifth of that with the standard sample. This result suggests that coinfection with defective and complete viruses may be required for efficient formation of defective virus particles.

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REFERENCES


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