Maturation of Infectious Simian Virus 40 in the Presence of Ethidium Bromide

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SUMMARY
We have investigated the effect of ethidium bromide (EB) on synthesis of nuclear, virus and mitochondrial DNA in permissive cells infected with simian virus 40 (SV 40). In the presence of EB, no newly formed, closed-circular, mitochondrial DNA (M-DNA) could be detected by isopycnic EB-CsCl gradient centrifugation. Synthesis of nuclear and virus DNA was not inhibited by a concentration of 8 #g./ml., even though SV40-DNA and M-DNA are similar in their tertiary structure, in that both are supercoiled, closed-circular, double-stranded molecules. The yield of SV 40 progeny under these conditions was not reduced nor was their specific infectivity affected, as compared to control values.

INTRODUCTION
Ethidium bromide (EB) was found to inhibit differentially the incorporation of [3H]-thymidine into closed-circular mitochondrial DNA (M-DNA) (Nass, 1970; Radsak et al. 1971), but not into nuclear DNA. Since M-DNA and SV 40 DNA are similar in that both are supercoiled, closed-circular, double-stranded molecules, experiments were designed to determine whether the replication of SV 40 DNA is also sensitive to EB. During the course of this work, it was reported (Eason & Vinograd, 1971) that EB present in infected cultures influenced the superhelix density of intracellular SV 40 virus DNA, while the superhelix density of the virus DNA from the virus particles was unaffected by the drug. EB added shortly after infection was reported not to reduce significantly the virus yield or the total SV 40 DNA in BSC-1 cells. The results of the present study demonstrate quantitatively the effect of EB during the lytic cycle on the yield of virus DNA and virus particles, and on their infectivity. While EB has a selective inhibitory effect on the replication of closed-circular M-DNA after infection with SV 40, virus DNA synthesis is not inhibited. Recently, it was reported that infection with SV 40 results in an enhanced synthesis of both nuclear and M-DNA in lytically infected African green monkey kidney (AGMK) cells (Levine, 1971). Moreover, it has been shown that cultures which have incorporated 5-iodo-2'-deoxyuridine (IUDr) into their nuclear DNA regain the ability to synthesize cellular DNA after infection with polyoma virus (Kasamaki, Ben-Porat & Kaplan, 1968). Therefore we wished to know whether the selective inhibitory effect of EB on M-DNA synthesis might be overcome by SV 40 infection.

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METHODS

*Virus.* SV 40, strain RH911 (Swetly *et al.* 1969), was grown in CV-1 cells; the crude virus suspension had a titre of \(5 \times 10^8\) p.f.u./ml.

*Chemicals.* EB (2,7-diamino-10-ethyl-9-phenylphenantridinium-bromide) was purchased from CalBiochem, Los Angeles, Calif.; [H]-methyl-thymidine (specific activity, 20 c/m mole) from New England Nuclear, Boston, Mass.; caesium chloride, optical grade, from the Harshaw Chemical Co., Solon, Ohio; and DNase I, from the Sigma Chemical Co., St Louis, Mo.

*Cells.* CV-1 cells, a continuous line of African green-monkey cells (Jensen *et al.* 1964) (35th to 50th passage), were used for all experiments.

*Infection of cell cultures.* For the measurement of total and virus DNA synthesis, CV-1 cells were grown by plating \(1 \times 10^6\) cells in 50-mm. Petri dishes in Eagle's basal medium (BME) supplemented with \(10^5\) foetal calf serum, 100 units of penicillin and 100 \(\mu\)g. of streptomycin. After 4-6 days, when the monolayers were completely confluent, the cultures were infected with SV 40 at a m.o.i. of 10. After an adsorption period of 90 min., medium, containing EB in different concentrations, was added.

*Measurement of DNA synthesis.* The total DNA synthesis in infected CV-1 cells was measured by the incorporation of [H]-thymidine into acid-insoluble material of CV-1 cells. Labelling was carried out for 1 to 24 hr with various amounts of isotope, according to the purpose of the experiment (see Results). At the end of the labelling period, the coverslip cultures were washed twice with phosphate-buffered saline (PBS), fixed for 15 min. in Carnoy's solution, treated for 30 min. with 5% trichloracetic acid (TCA) and dried. The coverslips were transferred into scintillation vials and the radioactivity was measured in a Beckman scintillation spectrophotometer.

For measurement of the M-DNA synthesis, cells were grown in roller bottles, and when confluent, medium containing different concentrations of EB was added. The M-DNA was labelled with 2 \(\mu\)c/ml. of [H]-thymidine (50 ml. total vol./roller bottle) over a period of 24 to 48 hr after the change of medium.

*Isolation and purification of mitochondria.* At the end of the labelling period the mitochondria were isolated and purified according to the method of Nass & Buck (1969) from approximately \(5 \times 10^6\) cells. The purified mitochondria were treated with 50 to 100 \(\mu\)g. of DNase I for 30 min. at room temperature (Buck & Nass, 1968; Clayton & Vinograd, 1969; Nass & Buck, 1969).

*Extraction of virus and M-DNA.* Virus and M-DNA were extracted from the SV 40-infected cultures and the purified mitochondria, respectively, by the method described by Hirt (1967), using 1 ml. of 0.6% sodium dodecyl sulphate (SDS) in 0.01 M-tris-HCl, pH 7.5, and 0.001 M-EDTA. The high-mol. wt nuclear DNAs were precipitated by adding 0.17 ml. of 7 M-CsCl to obtain a final concentration of 1 M. After storage overnight at 4\(^\circ\) the samples were centrifuged at 17,000g for 30 min. and the supernatant containing the low-mol. wt DNA (virus, mitochondrial, or both, as well as some cellular) fraction was dialysed against the same buffer for 48 hr. The samples were then submitted to isopycnic CsCl-EB gradient centrifugation to separate the closed-circular (component I) and the nicked-circular (component II) components in both the virus and M-DNA (Bauer & Vinograd, 1968). Fractions were collected on glass-fibre filters and the radio-activity was measured in a Beckman liquid scintillation spectrophotometer. Virus DNA synthesis was determined by measuring the incorporation of [H]-thymidine into virus DNA components isolated by band-centrifugation (Vinograd *et al.* 1963).
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The preparation of labelled SV 40 and the infectivity assay of SV 40 (plaque test) were carried out as described by Swetly et al. (1969). EB-CsCl isopycnic centrifugation was performed according to the procedure described by Radloff, Bauer & Vinograd (1967).

RESULTS

Effect of EB on DNA synthesis in uninfected CV-1 cells

Total DNA synthesis. Total DNA synthesis was measured by the incorporation of [3H]-thymidine into acid-insoluble material on coverslip cultures. As shown in Fig. 1, in uninfected confluent cultures of CV-1 cells, which were not treated with EB, the total DNA synthesis was typical for contact-inhibited cells. However, in the presence of EB (4 and 8 μg/ml, respectively), DNA synthesis was increased up to 50 % above the level of the untreated control culture and this level was maintained throughout the observed period. These results are also representative for the nuclear DNA synthesis, since the amount of M-DNA comprises less than 1 % [0.2 % in logarithmically growing mouse cells (Nass, 1966); 0.1 to 0.2 % in AGMK cells (Levine, 1971)] of the total DNA content of the cells. This ratio of approximately 1 % is not exceeded under stationary tissue culture conditions.

M-DNA synthesis. For a comparison with the data obtained for nuclear DNA synthesis in the presence or absence of EB we studied M-DNA synthesis as well. After the labelling period, the mitochondria were isolated and purified (Methods). M-DNA was extracted from the purified mitochondria by the method of Hirt (1967) and the samples were submitted to isopycnic CsCl-EB gradient centrifugation to separate closed-circular and nicked-circular M-DNA, under the conditions described by Bauer & Vinograd (1968). Fractions were collected on glass-fibre filters and the radioactivity was measured in a Beckman liquid scintillation spectrophotometer.

As shown in Fig. 2(a), in the uninfected control (0°C) in the absence of EB, M-DNA showed two separate peaks in the CsCl-EB gradient, component I (closed-circular) and component II (nicked form of component I). Uninfected controls (4°C) treated with 4 μg/ml of EB revealed only one peak, component II M-DNA, in the CsCl-EB gradient analysis, with component I being completely absent. This result was confirmed by band-centrifugation of this sample (4°C). From Fig. 2(a) and Fig. 4 (0°C) it is evident that component II of the M-DNA sample was not contaminating linear cellular DNA, which would sediment on the top of the gradient.
Fig. 2. Characterization of M-DNA from purified mitochondria from SV 40-infected cells treated with EB in an isopycnic CsCl-EB gradient.

(a) Uninfected cultures. 0C = uninfected control, no EB treatment. Peak I represents closed-circular M-DNA, Peak II the nicked form of circular M-DNA. Peaks I and II correspond to components I and II of SV 40 DNA, respectively. 4 C = uninfected control, treated with 4 mg./ml. of EB after mock infection. No component I of M-DNA can be demonstrated.

(b) Infected cultures. 0V = SV 40-infected culture without EB. M-DNA exhibits same pattern as in uninfected control. 4 V = SV 40-infected culture treated with 4 µg. of EB. In contrast to 4 C, where component I of M-DNA is absent, we find a small, but significant peak in the position of Peak I.

Effect of EB on DNA synthesis in CV-1 cells infected with SV 40

Tissue-culture conditions for these experiments were identical to those described above for uninfected cultures. Confluent coverslip cultures were infected with SV 40 at an m.o.i. of 10. After an adsorption period of 90 min., medium containing EB in different concentrations was added.

Total DNA synthesis. The total DNA synthesis was measured by 1 hr pulse labelling with 1 µc/ml. of [3H]-thymidine in 24-hr intervals as described above. Virus-induced nuclear DNA synthesis can be clearly shown 24 hr after infection and reaches its maximum at 48 hr after infection (Fig. 3). The increase of the total DNA synthesis is mainly due to the induction of nuclear DNA synthesis after infection with SV 40 (Kit et al. 1967). Under these conditions the presence of 4 and 8 µg., respectively, of EB/ml. in the medium after the adsorption period does not exert a significant influence on the nuclear DNA synthesis, as compared to the untreated control cells.

M-DNA synthesis. To study the effect of EB on the M-DNA of SV 40-infected cultures we used essentially the same conditions as described for the uninfected controls (see Methods).
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As shown in Fig. 2(b), in the SV 40-infected cultures in the absence of EB (0 μg), the M-DNA again exhibited the pattern of two peaks, as in the uninfected controls. When we analysed the M-DNA of infected cultures treated with 4 μg. of EB, however, we found two peaks—a large peak of component II, and a small, but significant, peak of component I. When we submitted this sample to a sedimentation analysis by band centrifugation (Vinograd et al. 1963), however, we could show that the highest peak corresponded to the position of component I SV 40 DNA (Fig. 4). The reason for the decrease of component I in the 4 μg sample, as compared to the other three samples presented in Fig. 2, is not known. A possible explanation might be a synergistic action of SV 40 and EB on the mitochondria, their DNA, or both.

Effect of EB on virus DNA synthesis in SV 40-infected CV-1 cells

Confluent cultures of CV-1 cells in Petri dishes were infected with SV 40 (see Methods). After adsorption, EB, at concentrations of 4, 8, 16 and 32 μg./ml. respectively, was added to the medium used for refeeding the cultures. The cultures were labelled with [3H]-thymidine at 48 to 72 hr post infection and the virus DNA of the cultures was harvested from the cells according to the method of Hirt (1967). Samples were subjected to sedimentation analysis by band centrifugation and the radioactivity of the SV 40 peak position was calculated (Fig. 5 and Table 1).

EB in concentrations up to 8 μg./ml. had no inhibitory effect on the synthesis of SV 40 virus DNA, and, in fact, even stimulated the virus DNA replication (Table 1).

Effect of EB on the production and infectivity of SV 40 virus particles

SV 40 yield. To study the effect of EB on the production of virus particles, we infected confluent CV-1 cells grown in roller bottles with SV 40 at an m.o.i. of 10. After adsorption,
Fig. 4. Band sedimentation of M-DNA in a neutral CsCl gradient. • — •, 0 C = same sample as in Fig. 2(a). Uninfected control, no EB treatment. The two peaks represent components I and II of M-DNA. ○ — ○, SV 40 marker. 4 V, same sample as in Fig. 2(b). SV 40-infected culture treated with 4 μg. of EB. In the position of component I of M-DNA, only a very small peak is found, whereas in the position of the SV 40 marker (indicated by arrow) a much greater amount of radioactivity appears. SV 40 DNA extracted from purified SV 40 virus was used as a marker.

Fig. 5. Band sedimentation of virus DNA in a neutral CsCl gradient. 0 V, SV 40-infected control without EB treatment. 8 V, SV 40-infected culture in the presence of 8 μg. EB/ml. Note increase of virus DNA in 8 V sample in comparison to untreated control. Calculation of net radioactivity of virus DNA was done by measuring the area as indicated by shading.

medium containing EB (8 μg./ml.) or without EB (control), was added to the cultures. On the third day after infection both cultures were labelled with [3H]-thymidine (0.2 μCi/ml.) for 2 days. After the appearance of a significant c.p.e. on the fifth day after infection, we harvested the cultures. The virus was quantitatively purified according to the procedure of Yoshiike (1968). The yield of full particles of the two samples was determined by measuring the extinction at 258 nm. The correlation between the particle number of SV 40 and the $E_{258}$ was studied by Yoshiike (1968), who showed that 1 $E_{258}$ corresponds to $6.4 \times 10^{12}$ virus
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Table 1. Effect of EB on virus DNA synthesis in SV 40-infected CV-1 cells

<table>
<thead>
<tr>
<th>EB concentration (µg./ml.)</th>
<th>% of control*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100†</td>
</tr>
<tr>
<td>4</td>
<td>132</td>
</tr>
<tr>
<td>8</td>
<td>139</td>
</tr>
<tr>
<td>16</td>
<td>90</td>
</tr>
<tr>
<td>32</td>
<td>58</td>
</tr>
</tbody>
</table>

* Results are mean values of three experiments.
† Mean value for 100 % equals 6670 counts/min.

Table 2. Effect of EB on the production and infectivity of SV 40 virus

<table>
<thead>
<tr>
<th>Sample</th>
<th>$E_{268}$</th>
<th>Counts/ min./ml.</th>
<th>p.f.u./ml.</th>
<th>Specific infectivity (p.f.u./$E_{268}$)</th>
<th>Specific radioactivity (counts/ min./$E_{268}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV 40 (control)</td>
<td>0.620</td>
<td>12,180</td>
<td>2 × 10⁹</td>
<td>3.2 × 10⁹</td>
<td>19,650</td>
</tr>
<tr>
<td>SV 40 (8 µg. EB/ml.)</td>
<td>0.660</td>
<td>13,125</td>
<td>1.1 × 10⁹</td>
<td>1.7 × 10⁹</td>
<td>19,890</td>
</tr>
</tbody>
</table>

particles. The two $E_{268}$ values (Table 2) do not differ significantly from each other. Therefore, the full particle yield from the culture in the presence of 8 µg. EB/ml. equals the one of the control.

Specific infectivity of SV 40 virus grown in the presence of EB. In a plaque assay we tested the infectivity of the virus grown in the presence of EB (8 µg./ml.) and of the virus grown in normal medium. For this comparison we introduced the term of specific infectivity, defined by the quotient (p.f.u./ml.$/E_{268}$).

The results (Table 2) show that virus grown in the presence of 8 µg./ml. had a specific infectivity of $1.7 \times 10⁹$ as compared with $3.2 \times 10⁹$ for the control. Considering the accuracy of the plaque assay in general, we cannot conclude from these data that 8 µg. EB/ml. significantly reduced the specific infectivity of SV 40.

Specific radioactivity. The incorporation of $[^3H]$-thymidine-labelled SV 40 DNA into virus did not differ when the medium contained 8 µg./ml. of EB from that in the absence of EB. This value was expressed as

$$\text{specific radioactivity} = \frac{\text{counts per min.}/\text{ml. (of virus)}}{E}$$

From these data we could conclude that the encapsidated virus DNA molecule contained the same amount of tritiated thymidine molecules, whether EB was present or not. Thus, EB did not affect the intracellular thymidine pool.

DISCUSSION

It has been reported (Nass, 1970; Radsak et al. 1971; Smith, Jordan & Vinograd, 1971) that in mammalian cells, EB in appropriate concentrations inhibits specifically the incorporation of $[^3H]$-thymidine into closed-circular M-DNA templates. Similar results have been obtained in yeast by Goldring et al. (1970), and Perlman & Mahler (1971). Treating uninfected CV-1 cells with 8 µg. of EB/ml. we could confirm that no $[^3H]$-thymidine is incorporated into component I of M-DNA. Under these experimental conditions EB has no inhibitory effect on the replication of nuclear DNA. On the contrary, we consistently found
a higher incorporation of radioactivity, after labelling with [\textsuperscript{3}H]-thymidine, into the nuclear DNA in the treated cultures than in the untreated controls. This finding is in good accord with the results reported by Nass (1970) and Radsak \textit{et al.} (1971).

When we studied the effect of EB on nuclear, mitochondrial, and virus DNA syntheses in SV\textsubscript{40}-infected CV-1 cells, we were especially interested in whether SV\textsubscript{40} DNA would react in the same way as M-DNA to EB, since these two types of DNA have some common characteristics: both are supercoiled, closed-circular, double-stranded molecules. The assumption that they would react in the same way was supported by a recent paper of Eason \& Vinograd (1971), who reported that the intercalating dye EB influenced the density of intracellular SV\textsubscript{40} DNA in productively infected cells. The induction of nuclear DNA synthesis in AGMK cells after SV\textsubscript{40} infection has been demonstrated (Hatanaka \& Dulbecco, 1966; Kit \textit{et al.} 1967). Our data show that EB present in concentrations up to 8 \(\mu\text{g.}/\text{ml.}\) does not suppress this stimulation of nuclear DNA synthesis. As in uninfected cells, EB in a concentration of 8 \(\mu\text{g.}/\text{ml.}\) again showed its inhibitory effect on the closed-circular M-DNA, as determined by [\textsuperscript{3}H]-thymidine incorporation. This phenomenon was interesting in relation to the recently reported result of Levine (1971), who showed the induction of M-DNA synthesis in AGMK cells lytically infected with SV\textsubscript{40}. The induction of nuclear DNA synthesis in the host cell seems to be a special feature of the oncogenic DNA viruses, SV\textsubscript{40} and polyoma, and seems to apply to M-DNA synthesis too, since the latter has now been demonstrated after infection with both kinds of oncogenic DNA viruses. Vesco \& Basilico (1971) found the induction of M-DNA synthesis after infection with polyoma in permissive cells. In the cell system used in our experiment, we could not demonstrate the induction of M-DNA synthesis described by Levine. But even under the condition of virus-induced stimulation of nuclear DNA synthesis (Hatanaka \& Dulbecco, 1966; Kit \textit{et al.} 1967), EB exhibited its specific inhibitory effect on M-DNA synthesis. Dulbecco, Hartwell \& Vogt (1965) suggested a correlation between the induction of nuclear DNA synthesis and the ability to transform, i.e. the presumable integration of the virus genome into the DNA of the host cell. If this correlation would apply for M-DNA synthesis as well, the low rate of induction after virus infection minimizes the chance for virus genome integration into the M-DNA genome of the infected host cell. Benjamin (1968) has shown the absence of virus genomes in M-DNA of SV\textsubscript{40}-transformed cells.

In experiments designed to study the effect of different concentrations of EB in the culture medium on the synthesis of intracellular virus DNA, a concentration of EB up to 8 \(\mu\text{g.}/\text{ml.}\) of medium, present from the time of infection until harvest at 72 hr post infection, did not reduce virus DNA synthesis, as measured by [\textsuperscript{3}H]-thymidine incorporation during the last 24 hr period. There was even a significant enhancement of the intracellular SV\textsubscript{40} DNA replication with increasing concentrations of EB, reaching a maximum production at a concentration of 8 \(\mu\text{g.}/\text{ml.}\) when synthesis was increased up to 13 \% of that of the control without EB. In the presence of the extremely high concentration of 32 \(\mu\text{g.}\) of EB/ml., the virus DNA synthesis decreased to 58 \% of the control.

From this result we assume that the mode of replication of M-DNA and SV\textsubscript{40} DNA are different. The data obtained do not allow us to determine whether this difference is due to a different binding capacity of the two DNA molecules to the intercalating dye EB, or to a difference in the sensitivity to EB of the enzymes involved in the replication of M-DNA or virus DNA. Meyer \& Simpson (1969) demonstrated the specific inhibitory effect of EB on M-DNA polymerase and Fan \& Penman (1970) demonstrated the inhibition of DNA-dependent mitochondrial RNA polymerase in mammalian cell systems.

Recently Eason \& Vinograd (1971) reported that intracellular SV\textsubscript{40} DNA is hetero-
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geneous in superhelix density, whereas, by the same criteria, virus DNA extracted from purified virus is homogeneous. When the cells were grown in the presence of EB (10 µg./ml.), however, the intracellular closed virus DNA was found to be homogeneous in superhelix density and to have the same superhelix density as the virus DNA. We wanted to determine whether the distinctive effect of EB on the tertiary structure of newly formed intracellular SV 40 DNA influenced the yield of virus particles and their infectivity. As the data (Table 2) show, the production of SV 40 virus particles and their specific infectivity, as well as the encapsidation of virus DNA into the virus particle in the presence of 8 µg./ml., did not differ significantly from the control. Whether the virus particles formed in the presence of EB are photosensitized needs to be investigated.

These data suggest that the insensitivity of SV 40 DNA replication to the inhibitory effect of EB, as compared to the M-DNA synthesis, might be due to the fact that SV 40 is replicated using the nuclear DNA-synthesizing system. The increase of nuclear DNA synthesis in the presence of EB in concentrations of 4 and 8 µg./ml. correlates with the increase of virus DNA synthesis under the same conditions. Furthermore, the function of M-DNA replication does not seem to be required for SV 40 replication. Experiments to study the specific infectivity of the virus progeny produced in the presence of 8 µg. EB/ml. also showed no difference in the incorporation of [3H]-thymidine into the SV 40 virus particle. Our results show that in SV 40-infected cells EB inhibits selectively the synthesis of closed-circular M-DNA. Moreover, appropriate concentrations of EB can even enhance the synthesis of SV 40 DNA. This strongly selective inhibition by EB may be useful for the investigation of nuclear, cytoplasmic and virus interaction in virus oncogenesis.

Note added in proof. After this paper was completed, it was reported (Kasamatsu, H., Robberson, D. L. & Vinograd, J., 1971. A novel closed-circular mitochondrial DNA with properties of a replicating intermediate. Proceedings of the National Academy of Sciences of the United States of America 68, 2252) that M-DNA contains a short replicated heavy segment of DNA that is hydrogen-bonded to the circular light strand. The inserted single strand is dissociable from the circular duplex molecule and is therefore called ‘displacement loop’ or ‘D-loop’. This phenomenon, observed specifically in M-DNA, might be responsible for the specific action of EB on these closed-circular molecules.

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