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Studies on the biological role of DNA methylation: III. Role in excision of one-genome long single-stranded ØX174 DNA

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ABSTRACT

Accumulation of replicative intermediates of the bacteriophage ØX174 was observed in E.coli C infected cells when phage DNA methylation has been inhibited by nicotinamide or when cells were infected with a temperature-sensitive mutant in gene A. Analysis of the accumulating replicative intermediates by electron microscopy revealed that these molecules are composed of double-stranded DNA rings with multiple-genome length single-stranded "tails". These results suggest that the single 5-methylcytosine residue present in the phage DNA serves as a recognition site for the gene A protein mediating the excision of one-genome long phage DNA. This excision process is obligatory for the final maturation of the phage.

INTRODUCTION

The final step in the bacteriophage ØX174 DNA replication is a still obscure circularization-excision process. The products of this event are: single-stranded phage DNA rings encapsidated in the coat proteins forming mature virus and RFII[1] molecules entering a new round of single-stranded DNA synthesis.

In earlier studies we have shown that a single cytosine residue in the phage DNA is methylated (1). The methylation process occurs at a late stage in phage DNA replication (2), catalyzed by a phage-induced transmethylation activity (3). The methylation is carried out at a specific region on gene H close to the termination site of phage DNA replication (4). We have recently shown that inhibition of phage DNA methylation by nicotinamide is accompanied by a corresponding decrease in phage production (5) and a concomitant accumulation of replicative intermediates (RI) composed of double-stranded DNA rings with multiple-genome long single-stranded "tails" (6). In the present report further evidence is provided for the nature of these abnormal RI molecules, based on electron microscopy.

The gene A product of ØX174 has been shown to be required for the specific cleavage of RFI molecules to produce RFII molecules that serve as

precursors for single-stranded DNA synthesis (7, 8, 9). It has also been shown to be an essential constituent of an in vitro system that synthesizes RF molecules (10, 11). The involvement of the gene A protein in a site-specific endonucleolytic cleavage during single-stranded DNA synthesis has been recently suggested by in vivo experiments with temperature sensitive mutants in gene A (12).

In the present study we show, by electron microscopy, that the same replicative intermediates with multiple-genome length "tails" which are found in infected cells in the presence of nicotinamide are also observed when cells are infected with a temperature sensitive mutant in gene A.

MATERIALS AND METHODS

All the experiments were performed with the E.coli H502 strain (endo-I$^-$, thy$^-$, hcr$^-$,su$^-$). The lysis defective mutant of øX174, am 3, served as wild type. The temperature sensitive, ts 56, a mutant in the C terminal end of Gene A was kindly provided by Dr.P.J. Weisbeek and ts 7, a temperature sensitive mutant in the N terminal end of gene A was generously obtained from Dr,M. Hayashi.

E.coli H502 cells were grown in the defined TPA medium described by Knippers et al. (13) supplemented with 5 µg/ml of thymidine to 5 x 10^8 cells/ml. To these logarithmically growing cells virus was added at m.o.i.= 5 and infection carried out as described in the text. With ts mutants m.o.i.= 1.

Preparative neutral velocity sedimentation. Lysates of the infected cells were prepared as described before (14) and layered on a linear 5 to 20% gradient of sucrose in 0.3M NaCl-0.05M Tris-0.003M EDTA pH 7.8. The gradients were centrifuged for 16 hrs at 18.000 rev/min in the SW 27 rotor at 10°C. Fractions of 1 ml were collected from the bottom of the tubes and analyzed for radioactivity. Peak fractions were combined and analyzed by electron microscopy.

Radioactivity measurements. Aliquots were withdrawn from the gradients fractions and DNA precipitated by cold 5% TCA. The precipitates were washed with cold 5% TCA on GF/C filters and counted in toluene PPO/POPOP scintillating fluid with a Tri-Carb liquid-scintillation spectrometer (Packard Instruments Co.).

Preparation of samples for electron microscopy. Labeled peak fractions were combined as indicated in the text and DNA precipitated by the addition of 0.1 vol. of 3M potassium acetate pH 5.5 and 2 vol. of ethanol. The precipitated DNA was dissolved in 0.05M Tris-0.003M EDTA pH 8.5.

The spreading and mounting of DNA for electron microscopy were according to Davis et al. (15) with the following modification: 10 µl of the DNA solution were gently mixed with 10 µl of 0.1M Tris pH 8.5, 0.01M EDTA, 20 µl of 100% formamide and 10 µl of 0.1% cytochrome C. The DNA mixture was then spread onto 50 ml hypophase containing 0.01M Tris pH 8.5, 0.001M EDTA and 17% (v/v) formamide. The spread DNA was picked up from the hypophase surface with freshly prepared collodion coated grids. The grids were stained for 30 sec with 5 x 10^{-5}M uranyl acetate in 90% ethanol and shadowed with a platinum-palladium (80:20) wire at an angle of 7°. The grids were analyzed in JEM 7A and Philips 300 electron microscopes. The magnification was calibrated several times during the work with carbon replica (58.400 lines per inch). Micrographs were taken at a magnification of 10,000 and 20,000. The negatives were enlarged and the DNA molecules traced and measured, øX174 RF was found to have an average circumference of 1.81 (= ±0.13).

RESULTS

Effect of nicotinamide: pulse experiments. E.coli H502 cultures, infected with the lysis defective mutant (am3) of øX174, were incubated at 37°C in the absence and presence of 200mM nicotinamide. Forty min after the onset of the infection, when active synthesis of single-stranded DNA is going on, the cultures were pulse labeled with (methyl-^{3}H) thymidine (2 µCi/ml; 26 Ci/mmole) for 25 sec. The pulse was terminated by rapid cooling of the cultures in liquid air. The cells were washed in the cold, lysed and DNA analyzed by velocity sedimentation on neutral 5-20% sucrose gradients (see Materials and Methods).

The sedimentation profile of the pulse-labeled DNA is represented in Fig.1. The pulse label of the DNA extracted from infected cultures in the absence of nicotinamide is distributed between RFII molecules sedimenting at 16S and RI molecules sedimenting as a broad leading shoulder at 18-25S (Fig. 1(a)). Pulse label in DNA extracted from infected cells in the presence of nicotinamide is distributed between RFII molecules (16S) and a fast sedimenting material (Fig. 1(b)). The labeled fractions of the two gradients were further analyzed by electron microscopy as described in Materials and Methods.

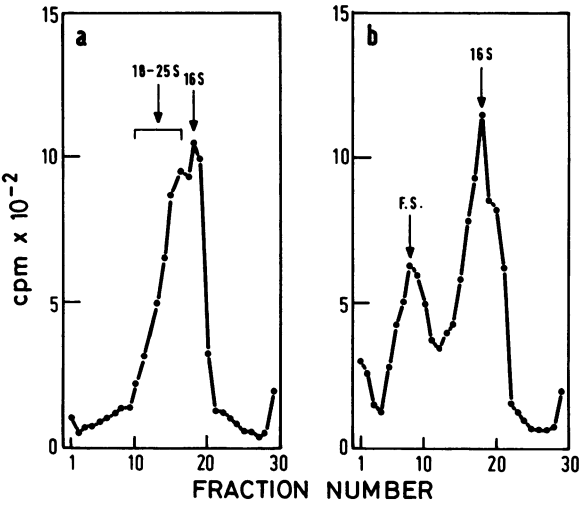

Fig.1. Velocity sedimentation through a neutral sucrose gradient of pulse labeled øX174 DNA. (a) without nicotinamide; (b) with 200mM nicotinamide. Direction of sedimentation is from right to left. The 16S arrow corresponds to øX174 RFII molecules. F.S. - designates fast sedimenting material (see text).

The DNA sedimenting at 18-25S in the lysates of infected cells without nicotinamide was found to be composed of RI molecules with various lengths of the single-stranded "tail". The length of the "tail" is equal to or shorter than the øX174 genome (Fig.2). The fast sedimenting material in the gradients of infected cells in the presence of nicotinamide is composed of RI molecules with multiple-genome long single-stranded DNA "tails" (Fig.3). In most instances the majority of these molecules are cleaved to multiple-genome long single-stranded linear DNA and double-stranded øXDNA rings, probably due to shearing inherent in the mounting procedure (Fig.4). All efforts to avoid this shearing were unsuccessful. However the fact that RFII molecules are observed in fast sedimenting material which separates properly from the 16S sedimenting material (RFII molecules), lends support to our interpretation that the very long single-stranded DNA derives from the abnormal RI molecules as illustrated in Figure 3.

Effect of nicotinamide: pulse and chase experiments. E.coli H502 cultures, infected in the absence and presence of 200mM nicotinamide were pulse-labeled as described in the preceding section and chased for 10 min, in the presence of a thousandfold amount of unlabeled thymidine. Lysates were pre-

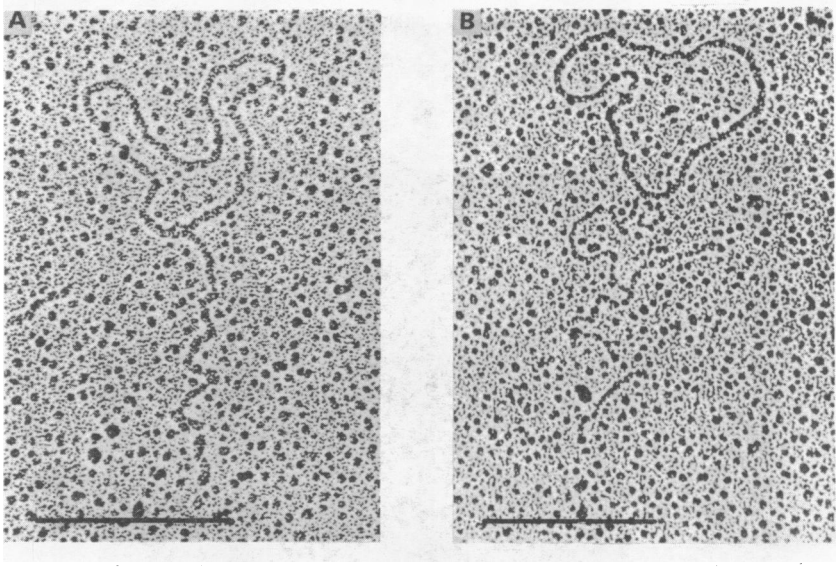

Fig. 2. Electron micrograph of ØX174 replicating intermediates. Fractions corresponding to material sedimenting in the region of 18-25S (see Fig. 1a) were combined, ethanol-precipitated and mounted for electron microscopy as described in Materials and Methods. (a) replicating intermediate with a single-stranded "tail" which corresponds to 0.41 øX genome length; (b) replicating intermediate with a single-stranded "tail" of 0.9 øX genome length. Bar represents 0.5 µm.

pared and analyzed by velocity sedimentation as described above. While all the label in DNA extracted from infected cells in the absence of nicotinamide was chased into single-stranded phage DNA (Fig.5a), the distribution of label in DNA extracted from cells infected in the presence of nicotinamide was between RFII molecules and fast sedimenting material (Fig.5b) as in the pulse experiments. The analysis of the DNA by electron microscopy revealed in the pulse and chase experiment without nicotinamide essentially only single-stranded rings (Fig.6), and in the experiment with nicotinamide RI molecules with multiple-genome long "tails" as represented in figures 3 and 4. These results indicate that the RI molecules are not chased into single-stranded rings when nicotinamide is present.

The fate of the abnormal RI molecules was studied by pulse and chase of cells infected in the presence of 200mM nicotinamide followed by washing the cells in fresh media without nicotinamide and incubation for another 10 min in a nicotinamideless medium. The lysates were analyzed by velocity sedimentation through a neutral sucrose gradient (Fig.7(a)). This analysis and electron microscopy of the peak fractions revealed that the abnormal

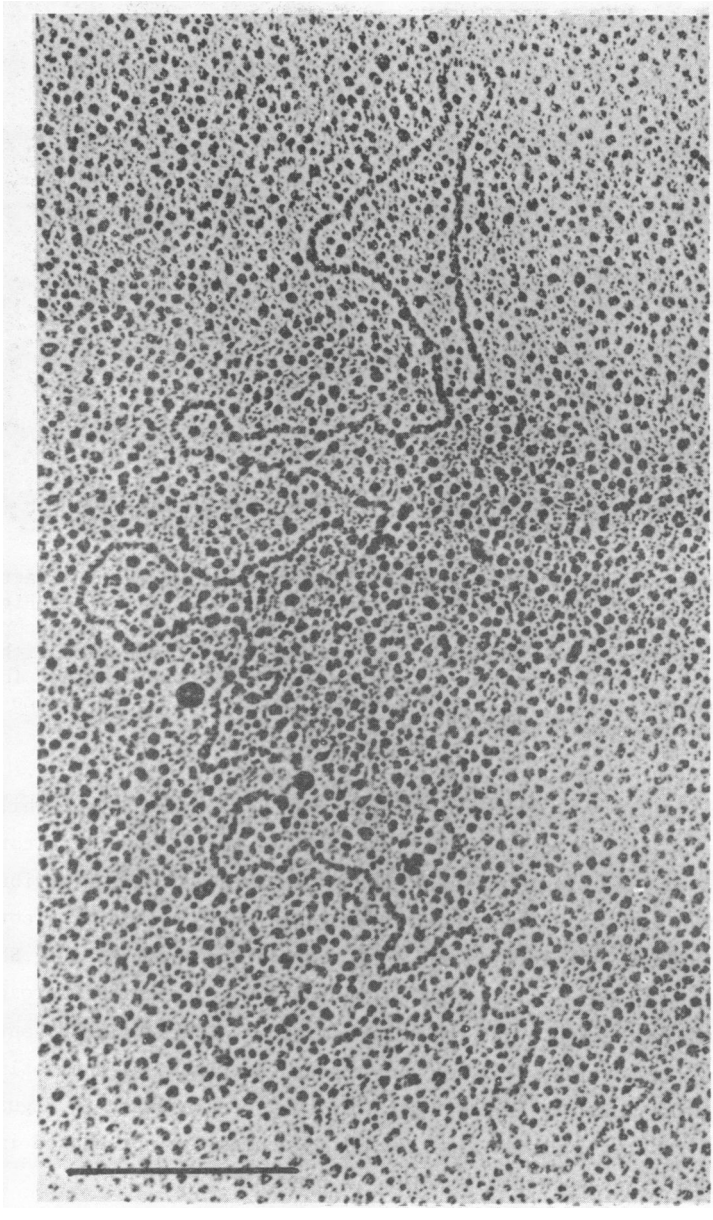

Fig. 3. Electron micrograph of øX174 replicating intermediates isolated from cells infected in the presence of 200mM nicotinamide. Fractions corresponding to the fast sedimenting material (see Fig.1b) were combined, ethanol-precipitated and mounted for electron microscopy as described in Materials and Methods. The RI molecule in the micrograph represents molecules with multiple-genome long single-stranded "tails". The single-stranded DNA "tail" is equal to 2.9 x øX genome length. Bar represents 0.5 µm.

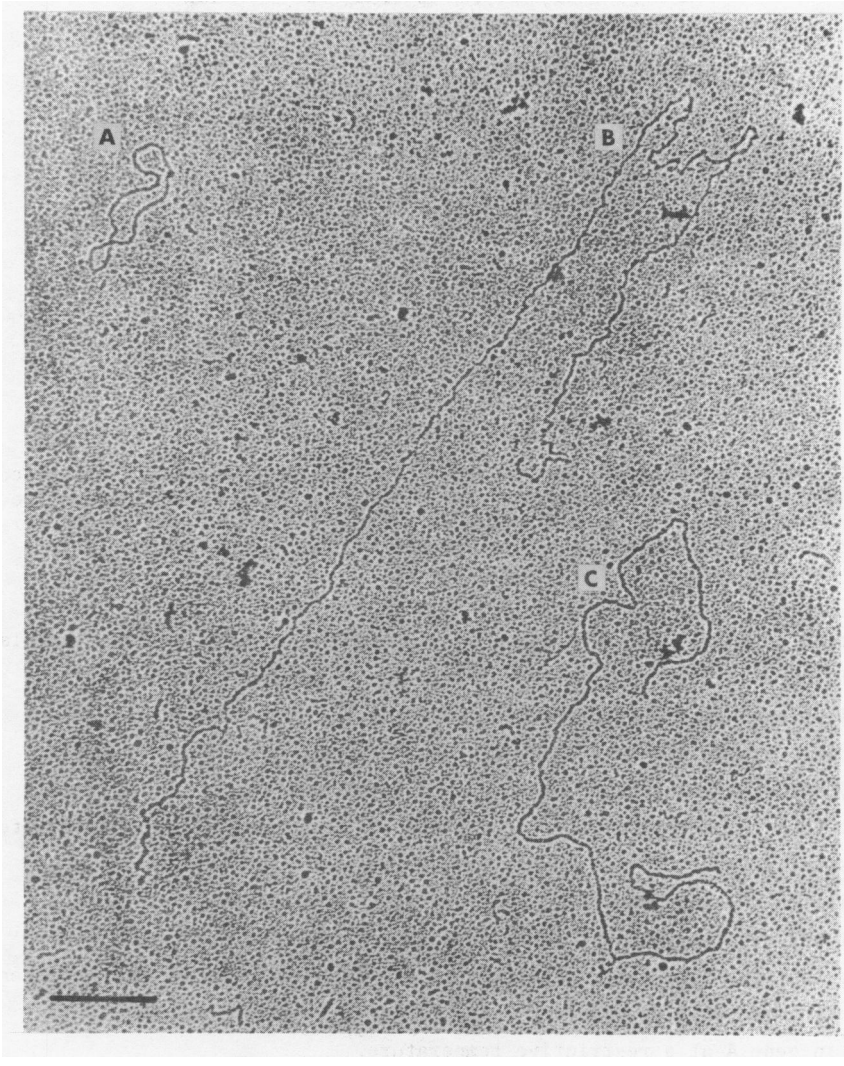

Fig. 4. Electron micrograph of fast sedimenting material (see Fig.1b). The majority of the multiple genome long single-stranded DNA was found to be detached from the double-stranded rings (for details see text). (a) double-stranded ring (1.8 μm circumference length); (b) linear single-stranded DNA (7.5 μm long, 4.3 times the length of the øX genome); (c) linear double-stranded DNA, probably host DNA (6.2 μm long). Bar represents 0.5 μm.

intermediates are completely chased into single-stranded DNA rings after the removal of the inhibitor (Fig.7(b)).

Infection with a temperature-sensitive mutant in gene A: pulse and chase experiments. The results presented in the previous section show that the

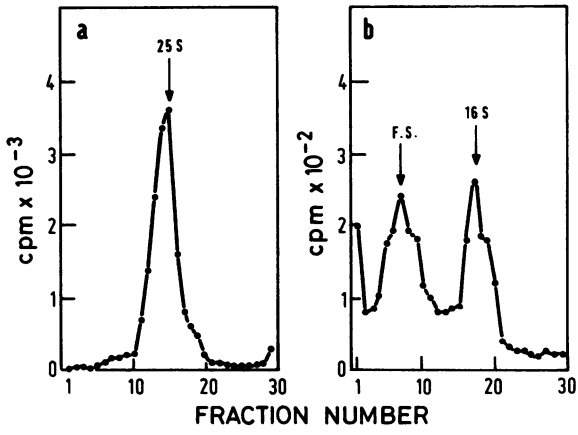

Fig. 5. Velocity sedimentation through neutral sucrose gradients of pulsed and chased øX DNA. (a) without nicotinamide; (b) with 200mM nicotinamide. Direction of sedimentation from right to left. The 25S and 16S arrows correspond to øX single-stranded DNA rings and RFII molecules respectively. F.S. - designates fast sedimenting material.

inhibition of phage DNA methylation by nicotinamide results in the accumulation of abnormal RI molecules composed of double-stranded DNA rings with multiple-genome long single-stranded DNA "tails". These molecules can be chased into single-stranded DNA rings only when nicotinamide is removed, thereby allowing methylation of the single stranded "tails". The results suggest that the methyl group in the phage DNA serves as a recognition site for a specific endonuclease that cleaves one-genome long single-stranded DNA prior to the final maturation of the virus. Since the phage gene A product has been suggested to function in this excision process (12), we assumed that the same abnormal RI molecules which accumulated in the presence of nicotinamide will be observed when cells will be infected with a ts mutant in gene A at a restrictive temperature.

E.coli H502 cells were infected with either ts 7 or ts 56, both mutants in gene A and incubated for 10 min at the permissive temperature (30°C), to allow RF replication, then shifted to the restrictive temperature (42°C) and pulsed and chased as described in the previous section. The cells were washed, lysed and the lysates were analyzed by velocity sedimentation on neutral sucrose gradients as described in Materials and Methods. In the case where the temperature shift-up was carried out 10 min after the infection the accumulation of fast sedimenting material was observed (Fig. 8(a)). On the other hand when the infection was allowed to go on at the permissive temperature for 20 min and then shifted to restrictive temperature, no fast

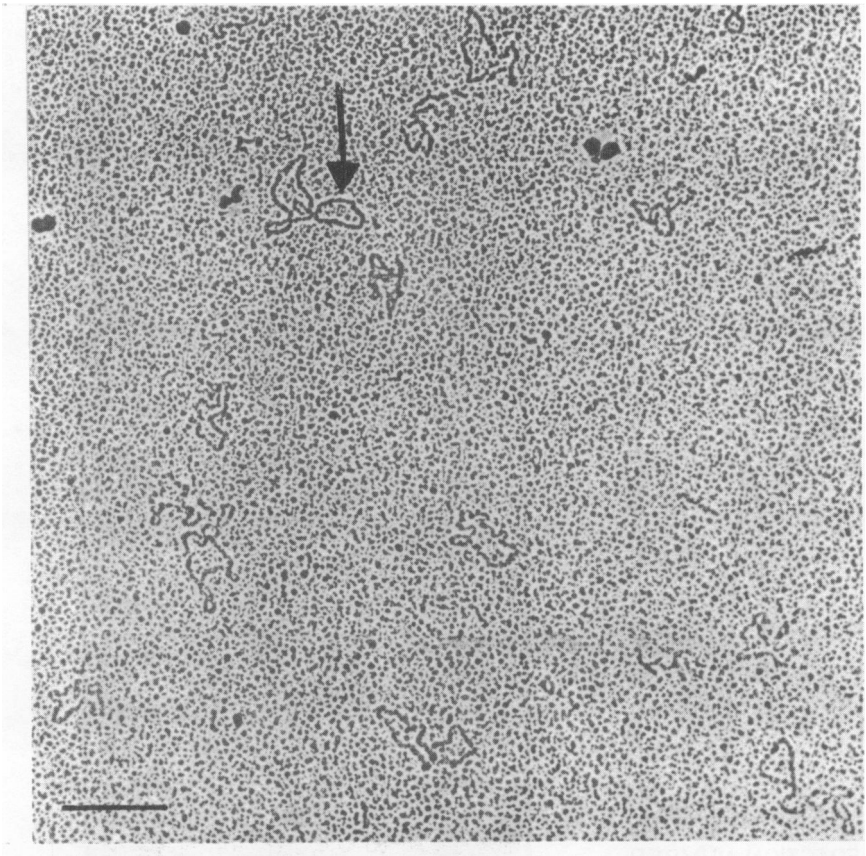

Fig. 6. Electron micrograph of material sedimenting at 25S (Fig.5a). Peak fractions were combined, ethanol-precipitated and mounted for electron microscopy as described in Materials and Methods. Almost the entire preparation contains single-stranded øX DNA rings. A single RFII molecule seen in the micrograph is indicated by an arrow. Bar represents 0.5 µm.

sedimenting material was observed and all the label was chased into material sedimenting as single-stranded DNA rings (Fig.8(b)). Similar results were obtained when the pulse and chase was performed 10 min after the shift. Electron microscopy of the fast-sedimenting material revealed that it was composed of RI molecules with multiple genome long single-stranded "tails" (Fig.9(a)). The material sedimenting at 25S (Fig.8(b)) proved to be solely single-stranded DNA rings (Fig.9(b)).

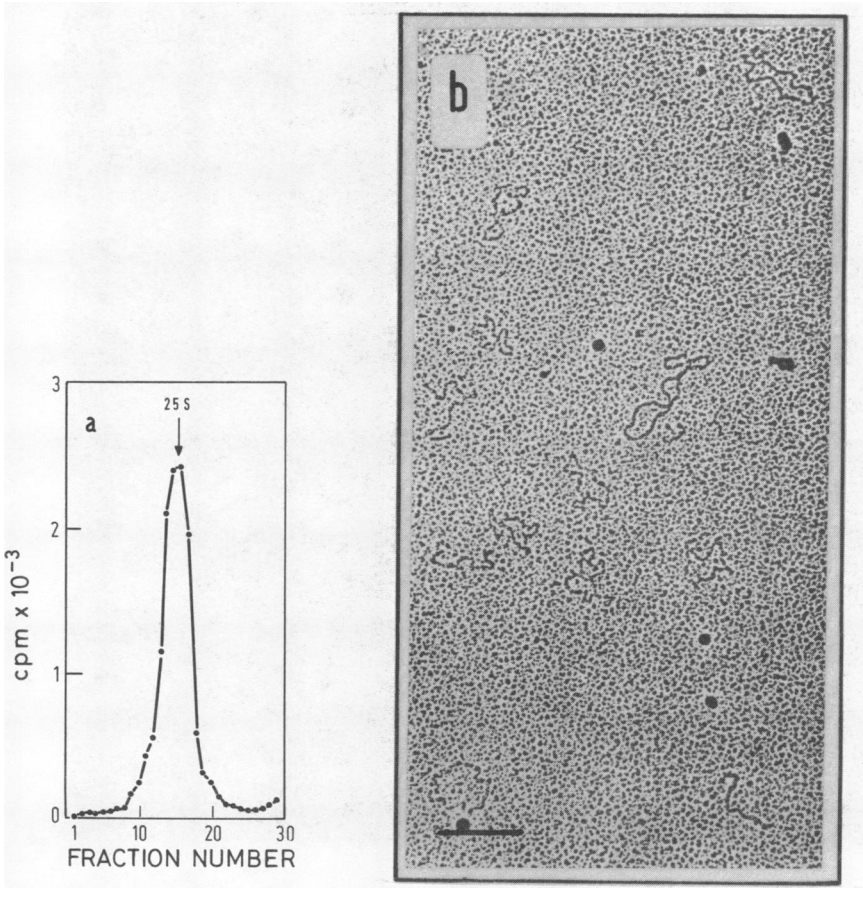

Fig. 7. (a) velocity sedimentation profile in a neutral sucrose gradient of DNA extracted from infected cells in the presence of 200 mM nicotinamide, pulse-chased and nicotinamide removed. Direction of sedimentation from right to left. The 25S arrow corresponds to single-stranded øX DNA rings.
(b) Electron micrograph of the 25S peak fractions. Peak fractions were combined, ethanol-precipitated and mounted for electron microscopy as described in Materials and Methods.

DISCUSSION

In the present report evidence is presented that abnormal replicative intermediates with multiple-genome long single-stranded "tails" are accumulating under two different conditions: (i) when methylation of the bacteriophage øX174 DNA is inhibited by nicotinamide (Fig.3); (ii) when E.coli cells are infected with a temperature-sensitive mutant in gene A of the bacteriophage øX174 (Fig.9). The accumulation of these RI molecules when

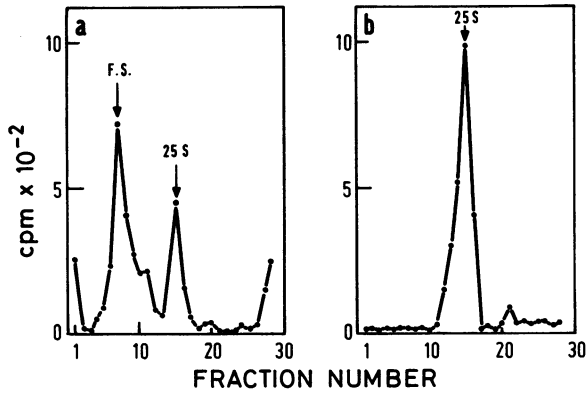

Fig. 8. Velocity sedimentation profile of øX DNA extracted from cells infected with a ts mutant in gene A. (a) after 10 min infection in the permissive temperature (30°C) the cells were transferred to 42°C, pulse labeled and chased; (b) same experiment as described in (a) except temperature shift was performed 20 min post infection. Direction of sedimentation from right to left. The 25S arrow corresponds to single-stranded øX DNA rings. F.S. - designates fast sedimenting material. Note absence of F.S. in gradient b.

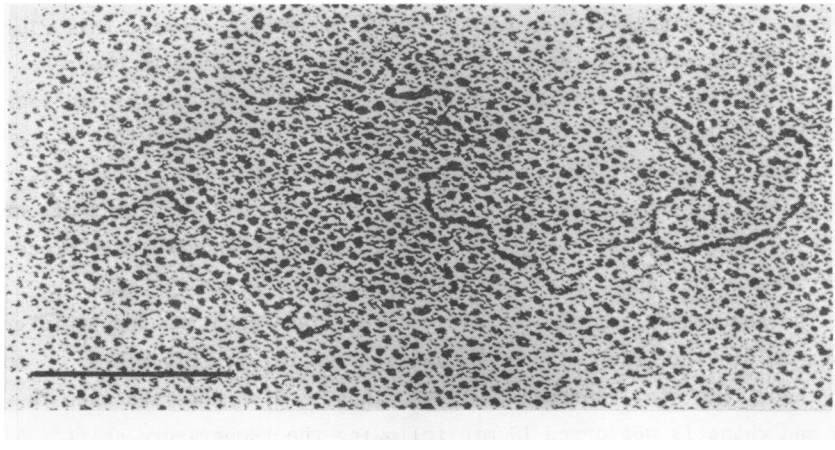

Fig. 9. Electron micrograph of fast sedimenting øX DNA extracted from cells infected with a ts mutant in gene A. Fast sedimenting DNA (Fig. 8a) was ethanol precipitated and mounted for electron microscopy, as described in Materials and Methods. RI molecules with multiple-genome long single-stranded "tails" were encountered. This electron micrograph shows such a RI molecule with a 3.8 µm long "tail" which corresponds to 2.1 x øX 174 genome. Bar represents 0.5 µm.

the DNA methylation is inhibited by nicotinamide suggests that the methyl group plays a role in the excision of one genome long single-stranded DNA, probably by serving as a recognition site for a specific endonuclease that cleaves the one genome long single-stranded DNA.

The accumulation of the same type of abnormal RI molecules when cells are infected with a temperature-sensitive mutant in gene A suggests that the gene A product, which has previously been shown to perform a specific endonucleolytic activity (8), is the protein that recognizes the methyl group and cleaves the DNA at the proper site (Fig. 10).

The function of the methyl group in the termination of one round of øX174 single-stranded DNA replication has been suggested by us before (6). The present results lend support to this conclusion. Since removal of the inhibitory nicotinamide results in the resumption of DNA methylation (5, 6), we were able to show that the abnormal RI molecules can still serve as precursors for øX single-stranded DNA rings, provided methylation is resumed (Fig.7).

The gene A product has been shown to be required for the specific cleavage of RFI to RFII molecules (7,8,9,10). It has been also suggested by Fujisawa & Hayashi (12) that gene A product plays a role in the cleavage of one genome long single-stranded DNA. Our data seem to support this claim although the results obtained by Fujisawa & Hayashi differ in the respect that they observed the accumulation of RI molecules with one genome long "tail" but failed to detect RI molecules with "tails" longer than one genome length. This difference in results is probably due to the different experimental conditions used by Fujisawa & Hayashi. In the light of our results we conclude that elongation of the single stranded "tail" proceeds in the absence of gene A product while termination is inhibited.

The abnormal RI molecules fail to accumulate when the temperature shift follows 20 min infection at the permissive temperature (Fig.8(b)). A complete chase into single-stranded øX DNA rings is also observed when the pulse and chase is performed 10 min following the temperature shift. A possible explanation for these results would be that the gene A product is required in the last step of phage DNA replication at a relatively high concentration and its synthesis rather than its activity is impaired at the elevated temperature.

Based on the results of the experiments with nicotinamide and the temperature-sensitive mutants in gene A we propose that the single 5-methylcytosine in the øX 174 DNA might serve as a recognition site for

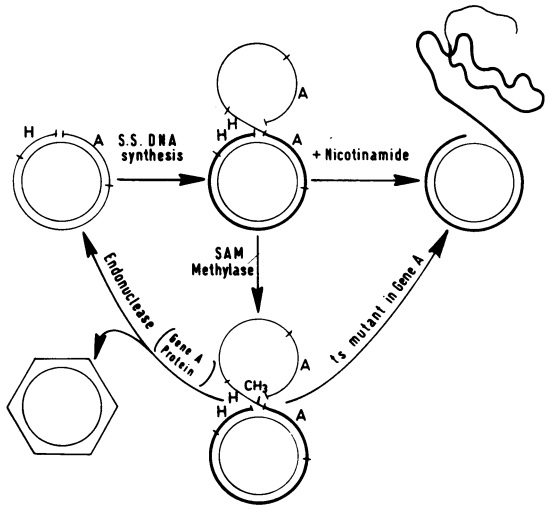

Fig. 10. Hypothetical scheme for the events in the single-stranded øX DNA synthesis in normal infections, infection in presence of nicotinamide and infection with a mutant in gene A. H and A correspond to genes H and A on the genetic map.

the gene A product. The position of the methyl group on gene H (4) is not identical with the presumed site of action of the gene A product at the origin of replication on gene A (17). We therefore assume that the methyl group serves as an "entry site" for the gene A product which then progresses along the DNA molecule and cleaves at the origin of replication. Such a mechanism has been shown to operate in the restriction process of phage f1 DNA by endo R.EcoB (18).

It should be pointed, however, that our data do not rule out the possibility that nicotinamide interferes directly with the action of the gene A product in addition to its inhibitory effect on methylation. *In vitro* experiments designed to clarify this point are currently underway.

While restriction endonucleases act only on under-methylated DNA, the gene A product appears to have the opposite specificity since its activity is dependent on methylation of a specific site of the phage DNA. A specificity similar to that of the gene A product was reported for an endonuclease isolated from *Diplococcus pneumoniae* which cleaved methylated DNA only (16).

While this work was in preparation, Scott *et al.* (19) reported their observation of *in vitro* synthesis of øX single stranded DNA from RFI in the presence of øX *cis* A, *E.coli* rep, DNA pol III and DNA binding protein.

None of these proteins has a known methylase activity. How this in vitro synthesis relates to the mechanism of in vivo single-stranded DNA synthesis suggested by our experiments remains to be clarified.

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1 Abbreviations used: RF, the double-stranded replicative form DNA of øX. In RFI both strands are circular and covalently closed. In RFII the complementary strand is closed and the viral strand is nicked in one specific site.

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