REPLICATION OF BACTERIOPHAGE M13 DNA IN PLASMOLYSED ESCHERICHIA COLI CELLS

Walter L. Staudenbauer and Peter Hans Hofschneider

Max-Planck-Institut für Biochemie, 8033 Martinsried b. München, Germany

Received July 30, 1973

Summary: Plasmolysed M13 infected E. coli cells utilize deoxynucleoside triphosphates to synthesize phage-specific DNA in an ATP-dependent, nalidixic acid sensitive, semi-conservative replication process. Whereas the major fraction of the reaction product consists of replicative form I molecules (RF) labeled asymmetrically in the viral strand, a minor fraction of the label is found in mature viral single strands. We therefore conclude that the system is capable of initiating second rounds of replication, for which ring closure seems to be a precondition.

INTRODUCTION

For a better understanding of bacteriophage M13 replication it is necessary to develop an *in vitro* system capable of performing the various processes involved in M13 DNA replication. Detailed information on the replication of ØX 174 has been obtained by studying phage DNA replication in infected cells made permeable to nucleotides by treatment with ether (1). However, it appears that this system stops after one round of replication. Thus we decided to test another system for M13 DNA replication *in vitro*, i.e. the plasmolysed cell system by Gros et al. (2,3), which allows semi-conservative DNA replication (4).

The data presented in this paper show that plasmolysed cells prepared from M13 infected mitomycin-treated cells late in the infection perform an asymmetric semi-conservative synthesis of single stranded phage DNA for more than one round of replication.

MATERIALS AND METHODS

E. coli 159 (F^+ thy^- hcr^-) obtained from D. Pratt (5) was grown in M 9 medium (6) supplemented with 10 µg/ml thymidine at 37°C. Preparation of sedimentation markers and assay of radioactivity have been described previously (7).

TEN buffer contains 0.05 M Tris-HCl pH 8.0, 0.005 M EDTA and 0.1 M NaCl.

Preparation of plasmolysed cells. A 2 l culture of E. coli 159 was harvested at a density of 2 x 10^8 cells/ml by low speed centrifugation at room temperature and resuspended in 200 ml prewarmed medium. Mitomycin C (Boehringer) was added to give a concentration of 50 µg/ml and the culture was incubated at 37°C without aeration in the dark for 10 min. 1.8 l medium were added and the culture divided into two parts. Each part was pelleted by low speed centrifugation at room temperature and the cell pellets resuspended in 400 ml medium and aerated at 37°C. 5 min later one culture was infected with M13 at a m.o.i. of 25 and aeration continued for 45 min. Each culture was then poured on an equal volume of ice and harvested by low speed centrifugation at 4°C. The pellets were washed once with 450 ml of cold 0.05 M Tris-HCl pH 8.0 - 0.01 M $MgCl_2$ - 0.1 mM EDTA. The cells were finally plasmolysed by resuspending in 0.05 M Tris-HCl pH 8.0 - 5 mM EGTA - 2 M sucrose at a concentration of about 5 x 10^{10} cells/ml. Plasmolysed cells were stored at -20°C for several months without loss of activity.

Standard incubation mixtures (0.2 ml) contained 20 mM morpholino propane sulphonic acid pH 7.8, 100 mM KCl, 10 mM NH_4Cl, 7.5 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM ATP, 20 mM phosphoenol pyruvate, 50 µg/ml pyruvate kinase (Boehringer), 0.25 mM NAD, 0.5 mM each of CTP, GTP and UTP, 0.05 mM each of dATP, dCTP and dGTP, and 0.01 mM ^{3}H-TTP (specific activity 200 cpm/pmole). 20 µl of the plasmolysed cell suspension (corresponding to 1 x 10^9 cells) were added to the incubation mixture and the tubes incubated for 30 min at 33°C. The incubation was stopped by adding 0.5 ml 10% TCA and placing the tubes in an ice bath.

RESULTS

Properties of DNA synthesis in M13 infected plasmolysed cells. Mitomycin C has been used previously for suppressing cellular DNA synthesis in infectious with M13 (5). As shown in Fig. 1 mitomycin treated plasmolysed cells are unable to incorporate a substantial amount of ^{3}H-TMP into an acid-insoluble product.

Table 1

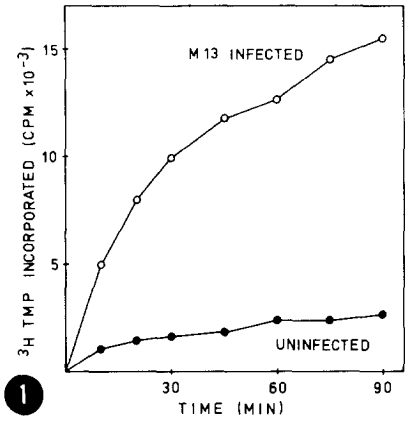
DNA synthesis in M13 infected plasmolysed cells

Additions or omissions	^{3}H-TMP incorporation
	%
Complete system	100
- $MgCl_2$	9
- $MgCl_2$ + EDTA (20 mM)	0
- Dithiothreitol	43
- Dithiothreitol + N-ethylmaleimide (5 mM)	7
- Phosphoenolpyruvate - pyruvate kinase	54
- ATP	24
- ATP + GTP (2 mM)	17
- 3 rNTP's	46
- 3 dNTP's	7
+ Chloramphenicol (100 µg/ml)	108
+ Nalidixic acid (100 µg/ml)	36
+ Rifampicin (50 µg/ml)	54
+ Actinomycin (50 µg/ml)	11
+ KCN (5 mM)	92

The complete system is the standard incubation mixture described in Materials and Methods. 100% activity corresponds to 40 pmoles of TMP incorporated by 10^9 cells per 30 min.

However after M13 infection a significant synthesis of DNA is observed. The rate of incorporation is approximately 50 pmoles TMP per 10^9 cells per 30 min at 33°C, corresponding to 15 molecules of phage DNA per cell. Synthesis is linear only during a short period of time but continues for more than 2 hours at a gradually decreasing rate.

As shown in Table 1, the activity requires Mg^{2+}, the four deoxynucleotides and ATP. The incorporation is inhibited by N-ethylmaleimide, nalidixic acid and actinomycin D, but not by KCN. Omission of ATP reduces the synthesis to background level observed in uninfected cells. None of the other ribonucleoside triphosphates can substitute for ATP. However, the omission of these leads to a 50% reduction as does the addition of rifampicin. This might point to a role of RNA polymerase in M13 ss DNA replication (8).



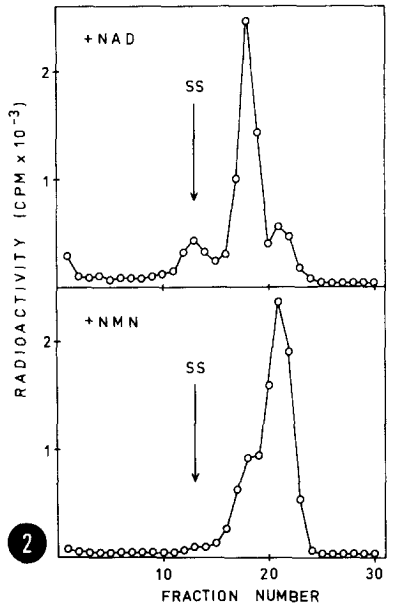
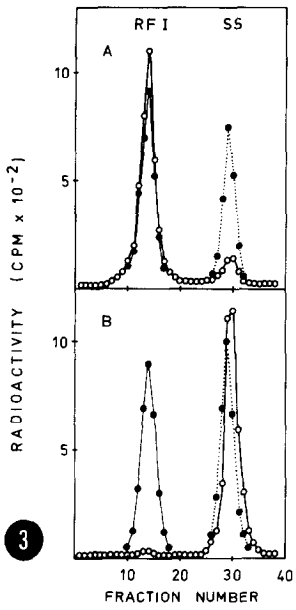


Fig. 1. Kinetics of ^{3}H-TMP incorporation by plasmolysed cells. Incubation mixtures (1 ml) were incubated at 33°C with 5 x 10^9 plasmolysed cells. At the times indicated 0.1 ml aliquots were removed and assayed for acid-insoluble radioactivity.

Fig. 2. Preparative sucrose gradient centrifugation of newly synthesized DNA. Incubation mixtures (2 ml) containing either 0.25 mM NAD or 2.5 mM NMN were incubated with 2 x 10^{10} M13 infected and then plasmolysed cells for 30 min at 33°C. Incubation was stopped by adding 0.2 ml of 0.5 M EDTA, followed by incubation with lysozyme (0.5 mg/ml) for 60 min in an ice bath. 0.1 volume of 5% sarcosyl was added and the lysate incubated with self-digested pronase (0.5 mg/ml) for 5 hours at 37°C. The highly viscous lysates were layered on 10-30% (w/w) sucrose gradients in 35 ml TEN (M NaCl) and centrifuged in a Spinco SW 27 rotor at 25 000 rpm for 17 hours at 4°C. 50 fractions were collected from the top of the gradient by pumping 50% sucrose into the bottom of the centrifuge tube. Aliquots were assayed for acid-insoluble radioactivity. Sedimentation is from right to left. The arrow indicates the position of a ^{32}P-labeled ss DNA sedimentation marker.

Characterization of the newly synthesized DNA. When the labeled DNA is extracted by treatment with sarcosylpronase and subjected to preparative sucrose gradient centrifugation about 70% of the label sediments in the region of replicative forms, mostly as covalently closed RF I (Fig. 2A). A small but significant amount of label sediments in the region of single stranded M13 DNA. If NAD is omitted and an excess of NMN added to inhibit the NAD-dependent DNA



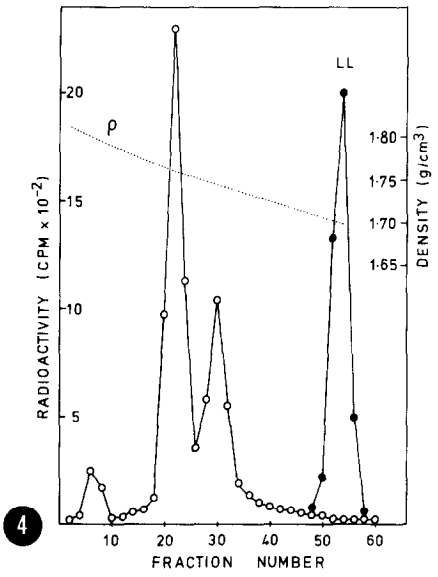


Fig. 3. Alkaline CsCl gradient analysis of labeled phage specific DNA. 0.2 ml aliquots of the pooled peak fractions from the gradients shown in Fig. 2 were denatured in 0.2 n NaOH and layered on 4.3 ml linear alkaline CsCl gradients (density 1.2 - 1.4 g/ml) as described previously (12). Centrifugation was performed in a Spinco SW 56 rotor at 45 000 rpm for 60 min at 4°C. 38 fractions were collected on filter paper discs and assayed for radioactivity. Sedimentation is from right to left.
(A) Fraction 17-19 from gradient 2A; (B) Fraction 20-22 from gradient 2B. o——o, ^{3}H-labeled DNA; ●——●, ^{32}P-labeled RF I; ●····●, ^{14}C-labeled ss DNA.

Fig. 4. Equilibrium sedimentation of Bromodeoxyuridine-labeled RF I in neutral CsCl. 2 x 10^{10} plasmolysed cells were incubated for 30 min at 33°C in 2 ml standard incubation mixture with ^{3}H-TTP replaced by 0.05 mM dBrUTP and dCTP replaced by 0.01 mM ^{3}H-dCTP (0.5 Ci/mmole). RF I was isolated as described in Fig. 2 and 4.0 ml sample mixed with 5.20 g CsCl (initial density 1.720 g/ml) and centrifuged at 40 000 rpm for 40 hours in a Ti 50 rotor. 80 fractions were collected and alternate fractions used for determination of refractive index and assay of radioactivity. The data of Szybalski (13) were used to calculate density from refractive index and the values obtained were corrected by referring to the position of ^{32}P-labeled RF I marker (density 1.701 g/ml). o——o, ^{3}H-labeled DNA; ●——●, ^{32}P-labeled RF I.

ligase most of the label is found in open circular RF II molecules (Fig. 2B). Under these conditions practically no labeled single strands are found.

The peak fractions (fraction 17-19 from gradient 2A, fraction 20-22 from gradient 2B) were pooled separately, dialysed against TEN buffer and further analysed by alkaline CsCl band sedimentation. The main peak from gradient 2A

(+NAD) cosedimented with denatured RF I (Fig. 3A), whereas the material from gradient 2B (+NMN) sediments slightly slower than circular single stranded phage DNA (Fig. 3B) as expected for linear single strands. The label from the single strand region in Fig. 2A cosediments in alkaline CsCl with circular M13 ss DNA (not shown).

Evidence for asymmetric semi-conservative replication. In order to show that phage DNA synthesis in plasmolysed cells results from semi-conservative replication and is not due to repair synthesis, newly synthesized DNA was labeled with a density marker. TTP was replaced in the incubation mixture by its analogue dBUTP and ^{3}H-dCTP was used for radioactive label. The labeled RF I was isolated by sucrose gradient centrifugation and further analysed by neutral CsCl equilibrium centrifugation as described in Fig. 4. Three peaks of radioactivity were observed banding at the densities of 1.745, 1.760, and 1.800 respectively. On the basis of the higher thymine content of the viral strand of the RF (9) we consider the main peak as heavy-light RF containing an heavy viral and a light complementary strand. The lighter peak consists of light-heavy RF with the density label in the complementary strand. The heavy peak is tentatively identified as heavy-heavy RF.

DISCUSSION

Plasmolysed M13 infected cells have been shown to utilize deoxynucleoside triphosphates to synthesize phage specific DNA in an ATP-dependent, nalidixic acid sensitive, semi-conservative replication process. The distinct density shifts observed after incorporation of 5-bromouracil clearly exclude any repair-like synthesis. Under standard conditions the label is mostly found in RF I molecules. Blocking the DNA ligase by NMN leads to the accumulation of RF II molecules containing the label in linear strands of unit length. The observed asymmetry in the distribution of label between viral and complementary strands agrees with *in vivo* data demonstrating that late in M13 infection asymmetrically labeled RF molecules serve as precursors for single stranded phage

DNA (10). The rate as well as the extent of M13 replication in plasmolysed cells is similar to ØX 174 replication in ether-treated cells (1,11). However as shown by the shift of label into free single strands and the synthesis of heavy-heavy RF, the plasmolysed cell system is capable of initiating second rounds of replication. Ring closure is apparently required for reinitiation to occur since in the presence of NMN no labeled single strands are found.

Acknowledgement. This work was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

1. Vosberg, H.P., and Hoffmann-Berling, H., J. Mol. Biol. 58, 739 (1971)
2. Gros, F., Gallant, J., Weisberg, R., and Cashel, M., J. Mol. Biol. 25, 555 (1967)
3. Ben-Hamida, F., and Gros, F., Biochimie 53, 71 (1971)
4. Wickner, R.B., and Hurwitz, J., Biochem. Biophys. Res. Commun. 47, 202 (1972)
5. Pratt, D., and Erdahl, W.S., J. Mol. Biol. 37, 181 (1968)
6. Adams, M.H., Bacteriophages, Interscience Publishers, New York, 1959
7. Staudenbauer, W.L., and Hofschneider, P.H., Eur. J. Biochem. 30, 403 (1972)
8. Staudenbauer, W.L., and Hofschneider, P.H., Proc. Nat. Acad. Sci. 69, 1634 (1972)
9. Salivar, W.O., Tzagoloff, H., and Pratt, D., Virology 24, 359 (1964)
10. Ray, D.S., J. Mol. Biol. 43, 631 (1969)
11. Müller-Wecker, H., Geider, K., and Hoffmann-Berling, H., J. Mol. Biol. 69, 319 (1972)
12. Staudenbauer, W.L., and Hofschneider, P.H., Biochem. Biophys. Res. Commun. 42, 1035 (1971)
13. Szybalski, W., Methods in Enzymology 12, 346 (1968)