Functional aspects of *Escherichia coli* *rep* helicase in unwinding and replication of DNA

Irmtraud BÄUMEL, Thomas F. MEYER, and Klaus GEIDER

Max-Planck-Institut für medizinische Forschung, Abteilung Molekulare Biologie, Heidelberg

(Received August 16/October 28, 1983) — EJB 83 0898

The gene for *Escherichia coli* *rep* helicase (*rep* protein) was subcloned in a pBR plasmid and the protein overproduced in cells transformed with the hybrid DNA. The effect of purified enzyme on strand unwinding and DNA replication was investigated by electron microscopy. The templates used were partial duplexes of viral DNA from bacteriophage fd::Tn5 and reannealed DNA from bacteriophage Mu. The experiments with the two DNA species show DNA unwinding uncoupled from replication. The single-stranded phage fd::Tn5 DNA with the inverted repeat of transposon Tn5 could be completely replicated in the presence of the *E. coli* enzymes *rep* helicase, DNA binding protein I, RNA polymerase and DNA polymerase III holoenzyme. A block in the unwinding step increases secondary initiation events in single-stranded parts of the template, as DNA polymerase III holoenzyme cannot switch across the stem structure of the transposon.

The *Escherichia coli* gene *rep* has been implicated with replication of small bacteriophages [1]. The enzymatic involvement of *rep* helicase in strand unwinding has been shown *in vitro* for replication of double-stranded phage ϕ X174 [2] and phage fd DNA [3].

The action of *rep* helicase in the fork depends on ATP or dATP as energy source [4]. The polarity of unwinding by *rep* helicase is opposite to the direction of strand separation found for other *E. coli* DNA helicases [5]. The *rep* helicase separates the strands in the direction of DNA replication on the leading strand. A general method to assay the action of DNA helicases is the degradation of single-stranded DNA generated by S₁-endonuclease [6]. Partially unwound duplex DNA cannot be assayed by this method, as the strands will renature before completion of endonucleolytic degradation or endonucleolytic digest of partial duplexes cannot be distinguished from degradation of completely separated strands. However, electron microscopy is a convenient tool to study partial denaturation of double-stranded DNA [7].

The *E. coli* DNA helicases I, II and III require a DNA duplex to be partially single-stranded in order to unwind DNA [8]. *E. coli* *rep* helicase can initiate DNA unwinding at a nick if a helper protein like gene 2 protein for phage fd [3] or gene A protein for phage ϕ X174 DNA is present [9]. In the absence of these phage-specific proteins, unwinding by *rep* helicase also depends on single-stranded regions in the duplex DNA [10]. The enzyme might also be involved in replication of the host chromosome. Defects in the coding gene results in increased fork initiation [11]. The *rep* mutation does not seem compatible with the non-functional *uvrD* gene product (DNA helicase II) [12]. It has therefore been assumed that *E. coli* *rep* helicase and DNA helicase II simultaneously unwind the two strands in a replication fork.

Here we will describe electron microscopical investigations on enzymatic strand separation by *E. coli* *rep* helicase in the presence of *E. coli* DNA binding protein I. It will be shown that DNA replication by DNA polymerase III holoenzyme on a

single-stranded template can be blocked by stable, base-paired regions, if they are not enzymatically unwound. This eventually leads to unspecific initiation events on single-stranded areas of the molecule.

MATERIALS AND METHODS

Enzymes

The preparation of the proteins used in the assays have been described for fd gene 2 protein [13], *Escherichia coli* DNA polymerase III holoenzyme [3], and *E. coli* DNA binding protein I [14]. *rep* helicase was isolated from overproducing cells described below by using a published procedure [15]. One unit is defined as the amount catalyzing the synthesis of 1 pmol of deoxynucleotide into phage fd RF under conditions described for the standard replication assay [3]. RNA polymerase holoenzyme was prepared according to Burgess and Jendrisak [16]. The concentration of proteins was determined according to the dye absorption method of Glick [17] and verified on polyacrylamide gels by Coomassie blue staining.

DNA

Phage Mu and phage fd DNA were extracted from phage particles. Phage fd::Tn5 (fd-30) was obtained by R. Herrmann [18]. Phage Mu CIts, Δ 445-7, gin⁻, G(+) was kindly provided by D. Kamp. As substrate for strand unwinding, Mu DNA was alkali-denatured, neutralized with acid, heated to 50°C and slowly cooled. Reannealing was found to be complete as judged by screening in the electron microscope.

Cloning of the *rep* gene

A DNA segment carrying the *E. coli* *rep* gene was derived from plasmid pLC 44-7 [19]. This plasmid DNA was cleaved with *Hind*III endonuclease and the fragments inserted into the gene for tetracycline resistance of plasmid pBR322 [20]. After ligation, the hybrid plasmids were transferred into strain H517

Abbreviation. RF, replicative form DNA of small bacteriophages.

(F^+ , rep^-). Selection of transformants was done on plates with ampicillin. Tetracycline-sensitive colonies were inoculated in TC medium and the cells were plated on rgh agar. To screen for rep^+ cells, droplets with phage ϕ X174 or fd were applied to the surface. These phages can only be propagated on *E. coli* cells with a functional *rep* gene. Cells from positive clones were used for the isolation of *rep* helicase. A restriction map of plasmid pTM111 is presented in Fig.1. Further subcloning of the fragment with the *rep* gene did not produce stable plasmids expressing *rep* helicase.

DNA of phage fd::Tn5 was unwound by adding 54 ng fd::Tn5 single-stranded DNA, 550 ng DNA binding protein I and 5 units of *rep* helicase to 10 μ l of unwinding buffer (20 mM Hepes, pH 7.5; 100 μ g/ml bovine serum albumin, 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 70 μ M ATP). Incubation was done for 20 min at 30°C. 140 ng of reannealed Mu DNA were incubated for unwinding with 1500 ng of *E. coli* DNA binding protein I and 15 units of *rep* helicase in 10 μ l unwinding buffer for 20 min at 30°C. Unwinding was measured by electron microscopy.

Replication with DNA polymerase III holoenzyme was done in 10 μ l of replication buffer containing 40 mM Hepes, pH 7.5; 80 μ g/ml bovine serum albumin, 4 mM 2-mercaptoethanol, 5 mM $MgCl_2$ and 4% sorbitol for 20 min at 30°C. Nucleotide concentrations were 500 μ M ATP, 250 μ M each of CTP, GTP, and UTP, 50 μ M each of dATP, dGTP, and dTTP and 25 μ M [α - ^{32}P]dCTP (500 cpm/pmol). The assay mixture contained 8×10^9 molecules of single-stranded DNA, which is 54 ng DNA from phage fd : Tn5 or 29 ng DNA from phage fd wild type, in addition to 300 ng RNA polymerase holoenzyme, 15 units of DNA polymerase III holoenzyme, 550 ng DNA binding protein I and, if indicated, 5 units of *rep* helicase.

The reactions were stopped by addition of EDTA. Sorbitol-containing samples were centrifuged through tubes with Sepharose CL6B. The DNA was spread by modifying the cytochrome *c*/formamide technique of Davis and Davidson

For the unwinding studies we have chosen two distinct partially double-stranded DNA templates, i.e. reannealed bacteriophage Mu DNA (Fig. 2A) and single-stranded bacteriophage fd DNA which carries an insertion of transposon 5 in the intergenic region (Fig. 3A, B). Mu DNA is flanked at one end by a short heterogeneous host DNA sequence of about 100 nucleotides, at the other end by a longer host DNA sequence of about 3000 nucleotides [22]. Denaturation of the Mu DNA and subsequent reannealing results in non-pairing ends, because the population of molecules is only homologous in the viral part of the DNA sequence, whereas the host sequence attached is random. In phage fd::Tn5 DNA, the transposon consists of 2300 nucleotides with an inverted repeat of 1500 nucleotides, forming a stem or paired DNA, which has to be unwound before a DNA polymerase can replicate this region. Both DNA species were incubated with *rep* helicase and *Escherichia coli* DNA binding protein I and subsequently spread for electron microscopy. Fig. 2B shows a typical molecule of Mu DNA with partially unwound strands. Strand unwinding by *rep* helicase was observed for 4% of the molecules, the rest was found in the original conformation. In a quantitative estimation only partially unwound molecules were seen, which could be distinguished from the template molecules by longer single-stranded and shorter double-stranded regions. The enzyme can apparently not unwind the whole length of Mu DNA. As shown in Fig. 3C *rep* helicase is able to unwind the partially base-paired DNA of fd::Tn5. Similar to the result for Mu DNA, 4% of the molecules were unwound, but spontaneous reannealing could already have occurred during the unwinding reaction before spreading for the electron microscopic picture. Other ratios of *rep* helicase and DNA binding protein with respect to the DNA substrate did not improve the amount of separated DNA strands visualized. The addition of glyoxal to the reaction mixture immediately at the end of the incubation time also did not increase the percentage of unwound molecules. The low, but distinct, percentage of unwound molecules therefore may not necessarily reflect an optimum for the unwinding reaction rather than an equilibrium of strand separation and reannealing. If the unwinding reaction was performed in the absence of DNA binding protein I, strand separation was not observed in the electron microscopic assay.

It has been shown previously that single-strand to RF conversion of fd viral strands by a multi-protein system is initiated at position 5758 on the phage genome [23]. This system consisted of *E. coli* DNA binding protein I, RNA polymerase, and DNA polymerase III holoenzyme [24] and was also used for replication of phage fd::Tn5 DNA. Since in fd::Tn5 DNA the transposon is inserted at nucleotide 5559 (Fig. 3) [18], the

Fig. 2. *Phage Mu DNA with unpaired termini and its unwinding by rep helicase.* (A) Renatured Mu DNA as described in Materials and Methods; (B) Mu DNA partially unwound by *rep* helicase. Forks are indicated by arrows. Magnification was 6500-fold. 110 molecules were evaluated

Fig. 3. *Structure and enzymatic unwinding of phage fd DNA with an insertion of transposon 5 (fd-30) [18].* (A) Genetic and physical map of the DNA from phage fd-30. (B) Electron microscope picture of the single-stranded DNA from phage fd-30. (C) DNA from the phage unwound by *rep* helicase. The reaction mixture and the electron microscopic investigation are described in Materials and Methods. 150 molecules were evaluated. Magnification was 10800-fold

Fig. 4. *The effect of rep helicase on replication of fd::Tn5 DNA by DNA polymerase III holoenzyme.* (A) Replication in the absence of *rep* helicase (30-min incubation). (B and C). Replication in the presence of *rep* helicase; (B) incubation for 10 min; (C) incubation for 30 min. The transition of single-stranded to double-stranded DNA is indicated by arrows. Magnification was 10800-fold

RNA primer should only be extended for about 170 deoxynucleotides until the polymerase reaches the double-stranded stem of the insert. Our data for DNA synthesis with fd::Tn5 single strands are consistent with the assumption that DNA polymerase III holoenzyme cannot cross a fork, in contrast to DNA polymerase I [25] or to phage T7 DNA polymerase [26]. After a block for DNA synthesis of DNA polymerase III holoenzyme by the transposon insert, initiation of the conversion of single strands to RF DNA appeared to occur at secondary sites (Fig. 4A). We observed initiation of replication at various sites on the fd genome, as well as in the loop structure of the transposon DNA, resulting in multiple starts of DNA synthesis on the same template molecule (Fig. 4). The occurrence of single-stranded and double-stranded DNA can be judged by the shape of areas in the molecule. Kinky patches suggest single-stranded DNA. An independent measurement for unwinding can be derived from the length of

Table 1. *Single-strand to RF conversion of fd::Tn5 DNA in the presence and in the absence of rep helicase*

The replication was performed for 20 min with DNA binding protein I, RNA polymerase and DNA polymerase III holoenzyme with *rep* helicase, if indicated, and fd::Tn5 DNA as template, as described in Materials and Methods. The products were investigated by electron microscopy. Double-stranded DNA could be distinguished as solid bars in contrast to thinner kinky contours of single-stranded DNA. Replication from the normal fd origin to the transposon insert (170 base pairs) and subsequently halt was concluded by the appearance of secondary initiation events resulting in an extension of the double-stranded DNA from the stem into the loop structure (see Fig. 4A). The identified structures are given as a percentage of total molecules

Replication type	Proportion	
	with <i>rep</i> helicase	without <i>rep</i> helicase
	%	
No replication	9	11
Replication to stem of Tn5 and secondary initiation	53	88
Unwinding and replication of the stem structure	38	1
Total number of molecules evaluated	126	117

the stem structure relative to the whole fd::Tn5 molecule. Full length is observed for the molecule shown in Fig. 4A, residual pairing is seen in Fig. 4B and complete strand separation is demonstrated in Fig. 4C. As concomitant replication prevents strand reannealing, circles without loops are strongly increased (Table 1).

Addition of *rep* helicase to the replication proteins stimulated enzymatic replication with fd::Tn5 single strands for about 30% compared to DNA synthesis in the absence of *rep* helicase, but did not show any effect on the incorporation kinetics with wild-type fd single strands (data not shown). During the first minutes of incubation the action of *rep* helicase allowed DNA synthesis to intrude into the stem of the transposon (Fig. 4B). When the incubation time was extended to 30 min the base-paired structure was separated and the whole DNA formed a large double-stranded circle (Fig. 4C). At this stage gaps in the DNA were used for further unwinding which resulted in double-stranded molecules with single-stranded tails. Branches were not found for the conversion of wild-type fd single strands to RF DNA (data not shown). This suggests that the normal replication origin with its four hairpin structures may prevent unwinding events, whereas secondary initiation as seen in fd::Tn5 creates sites of single-stranded DNA which can be subsequently unwound by *rep* helicase in the presence of DNA binding protein I.

A quantitative evaluation of single-strand of RF conversion of fd::Tn5 DNA in the absence and in the presence of *rep* helicase is given in Table 1. Replication in the absence of *rep* helicase most often resulted in an early stop at the site of the transport insert, which was accompanied with a second initiation event visible as an extension of the double-stranded region as demonstrated in Fig. 4A. Replication possibly starts in the molecule shown about 3000 nucleotides from the insertion site of Tn5 and stops at the double-stranded stem. This was judged by the appearance of the complete stem and the thick contour of DNA in half of the large loop. The

addition of *rep* helicase resulted in unwinding of the stem structure seen by the conversion of large parts of the fd::Tn5 DNA into the double-stranded form (Fig. 4B and C). For shorter incubation times the stem structure was still partially intact (Fig. 4B), whereas longer incubation produced large double-stranded circles without pairing of the transposon repeats (Fig. 4C). We conclude that replication of the stem structure of the transposon was strictly dependent on concomitant unwinding by *rep* helicase.

DISCUSSION

Strand unwinding is a prerequisite of DNA synthesis in a replication fork. DNA helicases are possible candidates to participate in this process [27]. For DNA helicase II involvement in replication has been demonstrated [28]. Good evidence has been obtained for functions of *Escherichia coli rep* helicase in strand separation of duplex DNA of small bacteriophages [2, 3].

We have isolated the *rep* helicase from *E. coli* cells which contain the coding gene subcloned in a multicopy plasmid with a drug resistance marker. In order to study DNA unwinding uncoupled from replication we have chosen two DNA substrates with properties suitable for strand unwinding by *E. coli rep* helicase. The unpaired end of reannealed Mu DNA and the single-stranded loop structure of fd::Tn5 DNA provided a start for *rep* helicase to intrude into the double-stranded regions in order to separate the strands. The unwinding functions of *rep* helicase were investigated in the absence of accessory proteins required in the replication of duplex DNA of small bacteriophages like ϕ X174 gene A protein or fd gene 2 protein. Electron microscopic visualization of unwinding intermediates revealed *rep* helicase activity which is difficult to detect in biochemical assays for strand separation. In the enzymatic assay unwound strands or self-annealing DNA like the stem of a transposon rapidly reanneal before cleavage of the single-stranded DNA. We have demonstrated here that in long double-stranded molecules the *rep* helicase can unwind only parts of the duplex DNA. Shorter double-stranded sequences like the 1500-base-pair repeat of transposon-5 DNA can be completely separated by *rep* helicase. Without unwinding, the inverted repeat of the transposon near the initiation site of fd single-stranded to RF conversion seems to interfere with proceeding DNA synthesis. The block of replication apparently increases initiation of complementary strand synthesis at various sites on the genome. This is consistent with the result that the deletion of the replication origin for complementary strand synthesis of the filamentous phage M13 still allows phage propagation although at a reduced rate [29]. Also bacteriophage T7 exposes secondary replication origins if the normal initiation site is deleted [30]. On the other hand, RNA polymerase holoenzyme saturated with active σ -factor strongly diminishes initiation outside the primary origin of replication [31]. The use of secondary origins *in vitro* might therefore be a possible but rare event.

For replication of fd::Tn5 DNA *in vitro* in the presence of *rep* helicase the unwinding reaction is not fast enough to prevent a cessation of replication at the inverted repeat structure. As a consequence multiple initiation also occurs. Furthermore, *rep* helicase starts unwinding the double-stranded DNA segment whenever replication reaches another initiation site. This event is seen in the electron microscope as displaced single-stranded tails in the replicated fd::Tn5 DNA. The large hairpin region as used for the primary origin in wild-

type fd DNA [32] apparently prevents strand displacement after replication of the whole genome. This is concluded from the fact that replication of fd single strands did not produce double-stranded circles with tails.

REFERENCES

- Denhardt, D. T. (1977) in *Comprehensive Virology*, (Fraenkel-Conrat, H. & Wagner, R. R., eds) vol. 7, pp. 1–104, Plenum Press, New York.
- Kornberg, A., Scott, J. F. & Bertsch, L. L. (1978) *J. Biol. Chem.* **253**, 3298–3304.
- Geider, K., Bäuml, I. & Meyer, T. F. (1982) *J. Biol. Chem.* **257**, 6488–6493.
- Arai, N. & Kornberg, A. (1981) *J. Biol. Chem.* **256**, 5294–5298.
- Abdel-Monem, M. & Hoffmann-Berling, H. (1980) *Trends Biochem. Sci.* **5**, 128–130.
- Abdel-Monem, M., Dürwald, H. & Hoffmann-Berling, H. (1977) *Eur. J. Biochem.* **79**, 39–45.
- Schnös, M. & Inman, R. B. (1970) *J. Mol. Biol.* **51**, 61–73.
- Kuhn, B., Abdel-Monem, M., Krell, H. & Hoffmann-Berling, H. (1979) *J. Biol. Chem.* **254**, 11343–11450.
- Scott, J. F., Eisenberg, S., Bertsch, L. L. & Kornberg, A. (1977) *Proc. Natl Acad. Sci. USA*, **74**, 193–197.
- Yarranton, G. T. & Geftter, M. L. (1979) *Proc. Natl Acad. Sci. USA*, **76**, 1658–1662.
- Lane, H. E. D. & Denhardt, D. T. (1975) *J. Mol. Biol.* **97**, 99–112.
- Taucher-Scholz, G., Abdel-Monem, M. & Hoffmann-Berling, H. (1983) in *UCLA Symposia on Molecular and Cellular Biology*. (Cozzarelli, N. R., ed.) New Series, vol. X, A. R. Liss, New York, in the press.
- Meyer, T. F. & Geider, K. (1979) *J. Biol. Chem.* **254**, 12636–12641.
- Beyreuther, K., Berthold-Schmidt, V. & Geider, K. (1982) *Eur. J. Biochem.* **123**, 415–420.
- Eisenberg, S. J., Scott, J. F. & Kornberg, A. (1976) *Proc. Natl Acad. Sci. USA*, **73**, 1594–1597.
- Burgess, R. R. & Jendrisak, J. J. (1975) *Biochemistry*, **14**, 4634–4638.
- Diamant, B., v. Redlich, D. & Glick, D. (1967) *Anal. Biochem.* **21**, 135–146.
- Herrmann, R., Neugebauer, K., Zentgraf, H. & Schaller, H. (1978) *Mol. Gen. Genet.* **159**, 171–178.
- Scott, J. F. & Kornberg, A. (1978) *J. Biol. Chem.* **253**, 3292–3297.
- Bolivar, F. (1978) *Gene*, **4**, 121–136.
- Davis, R. W., Simon, M. & Davidson, N. (1971) *Methods Enzymol.* **21**, 413–428.
- Daniell, E., Abelson, J., Kim, J. S. & Davidson, N. (1973) *Virology*, **51**, 237–239.
- Geider, K., Beck, E. & Schaller, H. (1978) *Proc. Natl Acad. Sci. USA*, **75**, 645–649.
- Geider, K. & Kornberg, A. (1974) *J. Biol. Chem.* **249**, 3999–4005.
- Schildkraut, C. L., Richardson, C. C. & Kornberg, A. (1964) *J. Mol. Biol.* **9**, 24–45.
- Kolodner, R. & Richardson, C. C. (1978) *J. Biol. Chem.* **253**, 574–584.
- Geider, K. & Hoffmann-Berling, H. (1981) *Annu. Rev. Biochem.* **50**, 233–260.
- Klinkert, M.-Q., Klein, A. & Abdel-Monem, M. (1980) *J. Biol. Chem.* **255**, 9746–9752.
- Kim, M. H., Hines, J. C. & Ray, D. S. (1981) *Proc. Natl Acad. Sci. USA*, **78**, 6784–6788.
- Tamanoi, F., Saito, H. & Richardson, C. C. (1980) *Proc. Natl Acad. Sci. USA*, **77**, 2656–2660.
- Kaguni, J. M. & Kornberg, A. (1982) *J. Biol. Chem.* **257**, 5437–5443.
- Meyer, T. F., Geider, K., Kurz, C. & Schaller, H. (1979) *Nature*, **278**, 365–367.

I. Bäuml, T. F. Meyer, and K. Geider, Abteilung für Molekulare Biologie, Max-Planck-Institut für medizinische Forschung, Jahnstraße 29, D-6900 Heidelberg, Federal Republic of Germany