THE ROLE OF DNA POLYMERASE I-ASSOCIATED 5'-EXONUCLEASE IN REPLICATION OF COLIPHAGE M13 REPLICATIVE-FORM DNA

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Summary — The conversion of both parental- and progeny-nascent open circular M13 RF DNA into covalently closed RF I is drastically reduced in an E. coli mutant deficient in the 5' → 3' exonuclease associated with DNA polymerase I. The nascent progeny RF DNA also contains a significant proportion of fragments of smaller than unit length.

Contrary to earlier conclusions as to the nonessentiality of Escherichia coli DNA polymerase I, the recent isolation of a temperature-sensitive conditional lethal mutant E. coli polA480ex (originally called polAex1) containing deficient and temperature-sensitive 5'→3' exonuclease associated with DNA polymerase I indicates the essential nature of the enzyme (1, 2). Filamentous coliphage M13 contains circular ssDNA of 2 X 10^6 daltons. The DNA, after infection, is converted into double-stranded pRF DNA. The next stage of phage DNA involves replication of RF DNA; finally progeny ssDNA is synthesized on RF DNA template [see Ray (3) for a review]. In both parental and progeny RF DNA synthesis, the nascent RF DNA is present as RF II DNA and is subsequently sealed to produce RF I DNA (3).

Kornberg and co-workers established the role of RNA priming in M13 parental RF synthesis in an in vitro system (4). The synthesis of complementary-strand DNA utilizes an RNA primer at the 5' end at a fixed site on the viral-strand template to generate RF II DNA. Sub-

Abbreviations: ssDNA, single-stranded DNA; RF DNA, replicative form DNA; pRF, parental RF; RF I DNA, covalently closed RF DNA; RF II DNA, RF DNA with one or more discontinuities in either or both strands.

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sequently the RNA is replaced by DNA during gap-filling and sealing to produce RF I DNA. Schekman et al. (4) proposed that the removal of RNA primer and gap-filling could be carried out by DNA polymerase I containing both the polymerase and 5' exonuclease activity.

In vivo studies (5) suggested that progeny RF DNA replication requires RNA priming as well, involving host RNA polymerase. Later in vivo experiments (6, 7) showed that M13 RF replication requires the dnaG function, which has RNA polymerase activity for initiation of phage DNA synthesis (8). Thus it appears reasonable to expect that nascent progeny RF DNA, like the pRF DNA, also contains RNA primers.

The purpose of this preliminary report is to present evidence that the 5'→3' exonuclease associated with DNA polymerase I is indeed involved in the final sealing of nascent RF DNA during both parental and progeny RF DNA synthesis, in the absence of progeny ssDNA synthesis, presumably by removing RNA primers at the 5' ends of nascent DNA chains. Chen and Ray (9) recently showed that the 5'→3' exonuclease activity is required in M13 progeny ssDNA synthesis

MATERIALS AND METHODS

Bacterial strains and phages — E. coli RS5052 (E. coli polA480ex 5'→3' $exo^{ts}thy^{-}su^{-}F^{+}$), originally isolated by Konrad and Lehman (1), was a gift of W. Masker. A spontaneous revertant, RS5052rev, was isolated which, unlike the mutant, is viable at 43°C and resistant to methyl methanesulfonate. It behaves exactly like wild-type E. coli X1090 (K12 5274, $thy^{-}su^{-}$). M13 gene 5 amber-mutant phage, am5H3 (M13 am5), and wild-type M13 phage were gifts of D. Pratt.

Media and buffers — L-broth contained 10 g of Difco bactotryptone, 5 g of Difco yeast extract, 10 g NaCl, and 1 g glucose/liter (supplemented with 20 mg/liter of thymidine). Supplemented M9 medium (7) contained 4 mg/liter of thymidine.

Isolation of intracellular phage DNA — Log-phase cultures of E. coli, grown under aeration and infected with phage (100–200 phage/cell), were mixed with an equal volume of an ethanol–phenol mixture (10) at -20°C to stop bacterial synthesis. The cells were then washed twice with 0.1 M NaCl, 50 mM TrisCl (pH 8.0), and 1 mM EDTA buffer containing 10^{-2} M KCN at 0°C and lysed as described before (11). The isolation of purified phage has been described (11).

Ultracentrifugation procedures — The cellular lysates were centrifuged in 5–20% sucrose gradient in 1 M NaCl, 20 mM TrisCl (pH 8.0), and 2 mM EDTA (11). Fractions of 0.7 ml were collected from the top, and 0.1–0.2 ml aliquots were assayed for radioactivity. Two types of band sedimentation in alkaline sucrose (5–20% sucrose in 0.8 M NaCl, 0.2 M NaOH, 2 mM EDTA) were carried out. For short centrifugations, phage DNA (0.1 ml) was layered on 4.9-ml gradients in Beckman SW50.1 swinging-bucket rotor tubes and spun at 45,000 rpm at 20°C for 80 min. Fractions were collected from the bottom and assayed for radioactivity. For extended centrifugations, DNA samples (0.2–0.4 ml) were spun in 10.5-ml gradients containing 1 ml 60% CsCl, and 60% sucrose (w/w) cushion in a Beckman SW41 swinging-bucket rotor at 38,000 rpm for 12 h at 5°C. The method of radioactivity measurement and the chemicals used have been described (11).

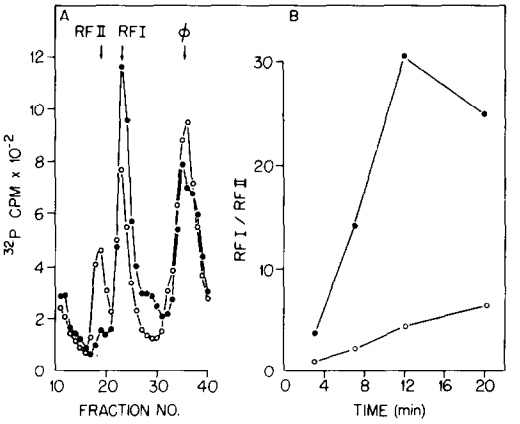
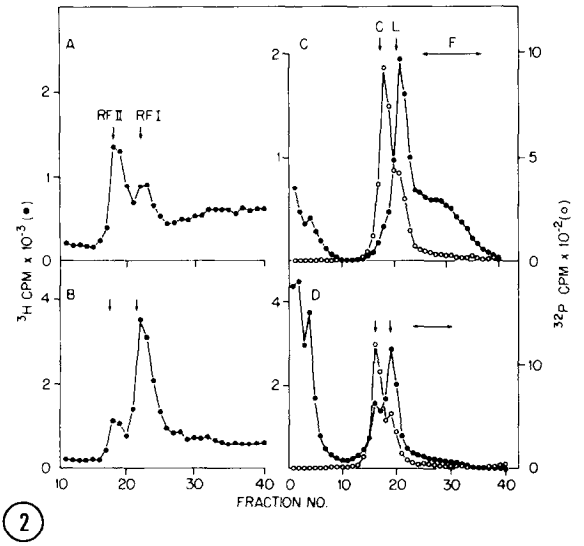


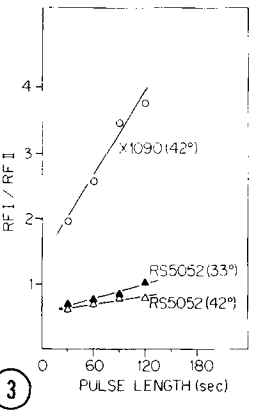
Figure 1. Parental RF synthesis in RS5052 and RS5052rev. Log-phase cultures of RS5052 and RS5052rev, grown at 33°C in supplemented L-broth, were shifted to 43°C 5 min before the addition of chloramphenicol (100 μg/ml). Ten min later, they were infected with ^{32}P-labeled wild-type M13 (3 X 10^5 PFU/cpm) at a multiplicity of ca. 200 and maintained at 43°C. At different times, 10-ml aliquots were taken and mixed with ethanol-phenol, washed, lysed, and subjected to band sedimentation in sucrose gradient as described in Materials and Methods. (A) Sedimentation profile of intracellular DNA 7 min after infection. Sedimentation was from left to right. The positions of RF II, RF I DNAs, and unaltered phage (φ) are indicated. (B) The ratio of RF I to RF II in the cultures as a function of time after infection is measured from the total radioactivity under the peaks. Symbols: O—O, RS5052; ●—●, RS5052rev.

RESULTS AND DISCUSSION

M13 parental RF synthesis in E. coli polA480ex — M13 pRF synthesis is carried out entirely by the host functions both in vivo and in vitro (3). We investigated the role of the 5'→3' exonuclease associated with DNA polymerase I in M13 pRF synthesis at 43°C by infecting RS5052 and RS5052rev pretreated with chloramphenicol (to prevent phage-specific protein synthesis) with [^{32}P]M13 phage and followed the fate of parental DNA by sucrose gradient sedimentation (Fig. 1). The total label in RF DNA (1.4 to 1.9 X 10^4 cpm) did not increase after 7 min postinfection, and was comparable in the mutant and the revertant. However, a remarkable difference between the mutant and the revertant was seen in their ability to convert newly synthesized RF II into RF I. Evidently the 5'→3' exonuclease is involved in the sealing of RF II DNA molecules. Since the in vitro studies of Schekman et al. (4) indicated that the complementary-strand DNA in M13 pRF has an RNA primer at the 5' end, our in vivo results support the idea (1, 2, 4) that the exonuclease has a vital role in the removal of RNA primers at the 5' ends of DNA chains. However, we should point out that even though, under our experimental conditions, the 5'→3' exonuclease associated with DNA polymerase I is undetectable in RS5052 (2), the conversion of RF II to RF I does occur, albeit at a slow rate



(2)



(3)

Figure 2. Progeny RF synthesis in RS5052 and RS5052rev. Log-phase cultures in supplemented M9 medium grown at 33°C were shifted to 43°C 10 min before infection with M13 am5 at a multiplicity of 50–100. Ten min later, the cultures were labeled with [^{3}H]thymidine (20 μCi/ml) for 1 min before addition of the poison, and then processed as described in Fig. 1. (A) and (B): Band sedimentation of cell lysate in neutral sucrose gradient of RS5052 and RS5052rev respectively. Other details are as in Fig. 1. (C) and (D): Band sedimentation in alkaline sucrose of pooled RF DNA from gradients in (A) and (B) respectively, as described in the text. Sedimentation was from right to left. The positions of RF I DNA and ssDNA: namely, C, circular; L, linear; and F, fragments arising from RF II DNA are indicated. [^{32}P]M13 ssDNA is used as the internal marker.

Figure 3. Conversion of RF II to RF I DNA. Log-phase cultures in supplemented M9 medium of wild-type E. coli X1090, and RS5052 were infected with M13 am5 at 33°C. Aliquots of cultures were shifted to 42°C 10 min after infection and then pulse labeled for different times with 20 μCi/ml [^{3}H]thymidine. Total RF DNA was isolated as described in Fig. 2. The RF I/RF II ratio was determined for each sample by a short ultracentrifugation in alkaline sucrose as described in Materials and Methods. Symbols: O—O, X1090 at 42°C; ▲—▲, RS5052 at 33°C; △—△, RS5052 at 42°C.

(Fig. 1B). This could be the result either of a trace of this enzyme activity in the mutant or of another 5' exonuclease activity. The 5'→3' exonuclease associated with DNA polymerase III in E. coli (12) may be such an enzyme.

Progeny RF DNA synthesis in E. coli polA480ex — The synthesis of M13 progeny RF DNA can be studied exclusively in a host infected with M13 am5 (3). We followed the progeny RF DNA synthesis at 43°C in RS5052 and RS5052rev by pulse labeling M13 am5-infected cultures with [^{3}H]thymidine (Fig. 2A, B). It is evident that here again there is a significant difference in the RF I/RF II ratio between RS5052 and RS5052rev.

The structure of these RF DNAs was further analyzed by extended band sedimentation of pooled total RF DNA (fractions 16 through 27 of gradients in Fig. 2A, B) in alkaline sucrose, where RF I sediments into the cushion while single strands from denatured RF II sediment as circular, unit-length and shorter than unit-length DNA (Fig. 2C, D). It is obvious again that the proportion of RF I in RS5052rev is much higher than that in RS5052. Also in RS5052, in contrast to the situation in RS5052rev, a large fraction of the pulse label in RF II is present as shorter than unit-length fragments (presumably replication intermediates). Thus, the deficiency of 5'→3' exonuclease apparently causes retarded sealing of the fragments into unit-length DNA strands. However, experiments involving chase with unlabeled thymidine showed that a slow synthesis of RF I does occur in RS5052 (data not shown).

Similar results were also obtained when M13 RF DNA was pulse labeled at the restrictive temperature after RS5052 and RS5052rev cultures were infected with M13 am5 and maintained at the permissive temperature for 10 min before the temperature was shifted (data not shown). These results strongly suggest that, as with the parental RF DNA, the 5'→3' exonuclease activity of DNA polymerase I plays an important role in the conversion of nascent RF II into RF I DNA. This is more clearly seen in Fig. 3, where the ratio of RF I to RF II DNA is measured as a function of time of pulse labeling of M13 am5-infected E. coli χ1090 and RS5052 The RF I/RF II ratio was quantitated in this experiment by pooling RF DNA from neutral sucrose gradients similar to those described in Fig. 2A, B and subsequent short centrifugation in alkaline sucrose to separate RF I from RF II as described in Materials and Methods. It is evident that while the ratio of RF I to RF II reflects the combined effect of RF synthesis and subsequent sealing, the sealing was extremely efficient in wild-type E. coli where, even after a 30-sec pulse label, two-thirds of the RF DNA was present as RF I. In contrast, in RS5052, the conversion of RF II to RF I DNA was remarkably depressed at both 33 and 42°C, although the total [^{3}H]thymidine incorporation during pulse label in RF DNA at both 33 and 42°C was only slightly lower than that in χ1090 (data not shown). Uyemura et al. (2) showed an acute deficiency of 5'→3' exonuclease associated with DNA polymerase I even at 30°C in polA480ex compared with that in the wild type.

These results are consistent with the proposed vital role of DNA polymerase I, along with its 5'→3' exonuclease, in removing RNA primers and gap-filling in both parental and progeny M13 RF DNA. This role includes the sealing of discontinuous fragments in nascent RF II DNA. We have recently found that the nascent RF II DNA contains multiple gaps and a stretch of ribonucleotides. In addition, the fragments contain sequences of both virus and complementary strands (unpublished results).

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