PERSISTENCE OF RNA ATTACHED TO NASCENT SHORT DNA PIECES IN BACILLUS SUBTILIS CELLS DEFECTIVE IN DNA POLYMERASE I

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SUMMARY: When cells of a polA^{ts} strain of Bacillus subtilis in logarithmic growth phase are raised to the restrictive temperature, a large amount of newly synthesized short DNA pieces accumulates. Analysis with spleen exonuclease has indicated that RNA-linked nascent DNA pieces accumulate in the polA^{ts} cells about 4.5 times as much as in the polA^{+} cells. These observations suggest that B. subtilis DNA polymerase I is involved in the removal of the RNA attached to the nascent short DNA pieces as well as in the filling in of the gaps between these pieces.

Our recent analyses of nascent DNA pieces by spleen exonuclease and modified polynucleotide kinase methods have shown that RNA is attached to the 5'-end of nascent short DNA pieces in Escherichia coli (1-4). Such a result is expected if the synthesis of these pieces, in discontinuous replication, is primed by RNA that is removed before the joining of these pieces. The requirement of the concerted action of both 5'→ 3' exonuclease and polymerase activities of E. coli DNA polymerase I for the removal of the attached RNA was suggested by the accumulation of the RNA-linked nascent DNA pieces upon inhibition of either one of these activities (2-4).

Also in Bacillus subtilis, RNA is attached to the 5'-end of nascent short DNA pieces as indicated in the present study. B. subtilis DNA polymerase I was originally purified by Okazaki and Kornberg (5). The purified enzyme, in contrast to E. coli DNA polymerase I, was found to have little if any nuclease activity. Therefore, we were interested in knowing whether B. subtilis DNA polymerase I might play a role in the removal of the attached RNA. The present study indicates that inhibition of DNA polymerase I in B. subtilis results in

*Deceased.
Abbreviations: 5'-OH DNA, 5'-hydroxyl terminated DNA; 5'-P DNA, 5'-phosphoryl terminated DNA.

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the accumulation of RNA-linked nascent DNA pieces. Thus B. subtilis DNA polymerase I seems to play a similar role as E. coli DNA polymerase I in discontinuous DNA replication.

MATERIALS AND METHODS

Bacteria: B. subtilis JH406 (trpC2, citC) and JH406 1-6 (trpC2, polA10), constructed by transformation of JH406 with DNA from BC26(10) (polA10), were provided by Dr. N. R. Cozzarelli (6). Cells were grown in modified Spizizen medium (7) containing 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.1% sodium citrate·$2H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 1% glucose, 1% Casamino Acids, 50 μg/ml L-tryptophan.

Enzymes and radioactive compound: Hog spleen exonuclease, T4 polynucleotide kinase and E. coli alkaline phosphatase have been described previously (2, 8). [Methyl-^{3}H]thymidine (20 or 43.1 Ci/mmol) was purchased from the New England Nuclear Corp.

Analyses of nascent DNA pieces: Pulse-labeling, DNA extraction and alkaline sucrose gradient sedimentation were carried out as described by Okazaki (9). Termination of pulse-labeling was achieved by pouring the culture into an equal volume of ethanol-phenol mixture (9).

To prepare nascent DNA pieces for the assay of RNA-linked DNA molecules, cells were suspended in SSC-27% sucrose-20 mM EDTA and nucleic acid was extracted by a modified Thomas procedure (9) except that lysozyme treatment was carried out for 10 min at 37°C with 100 μg/ml of lysozyme. Further purification was carried out essentially in the same way as described by Kurosawa et al. (2). Size fractionation by Sepharose 4B was omitted.

RESULTS

Accumulation of nascent short DNA pieces on inhibition of DNA polymerase I: DNA polymerase I of B. subtilis JH406 1-6 (polA^{ts}) is active at 37°C but loses its activity at temperatures above 49.5°C. Assay of DNA polymerase activity according to Okazaki and Kornberg (5) in crude extracts showed that the specific activity of mutant enzyme was 5.6% and 3.6% of that of wild type enzyme at 49.5°C and 51.5°C, respectively, while at 37°C the mutant enzyme showed 86% activity of the wild type enzyme.

Decrease in DNA polymerase activity in vivo resulted in the marked accumulation of nascent short DNA pieces. B. subtilis JH406 1-6 (polA^{ts}) and JH406 (polA^{+}) cells were pulse-labeled with [^{3}H]thymidine at 2 min after temperature shift from 37°C to 49.5°C or 51.5°C. The elevation of temperature caused an increase of DNA synthesis rate that reached a maximum at 2 min after the temperature shift and decreased thereafter in the same fashion in both cells. Sedimentation analysis of the labeled DNA showed that the joining

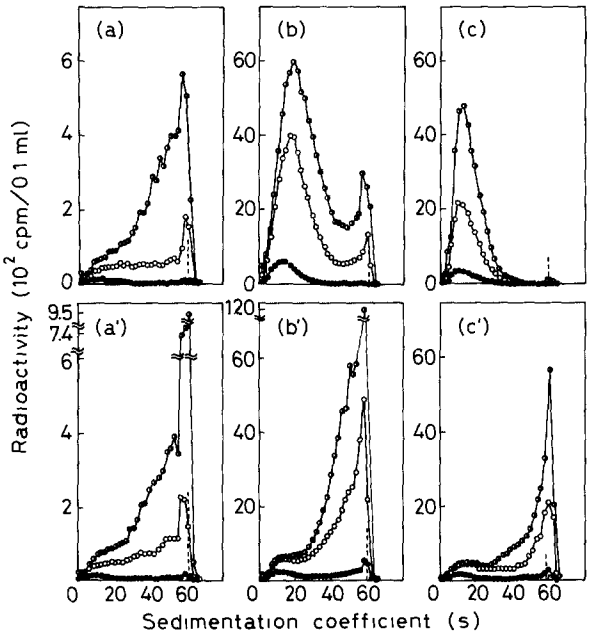

Fig. 1. Alkaline sucrose gradient sedimentation of DNA from B. subtilis $polA^{ts}$ and $polA^{+}$ cells pulse-labeled at various temperatures. B. subtilis JH406 1-6 ($polA^{ts}$) and JH406 ($polA^{+}$) were grown in modified Spizizen medium to a titer of 5 x 10^7 cells/ml at 37°C. 6 ml portions of each culture were pulse-labeled with 0.1 μM-[^{3}H]thymidine (20 Ci/mmol) for the indicated times at 37°C ((a) $polA^{ts}$; (a') $polA^{+}$), at 49.5°C beginning at 2 min after the temperature shift from 37°C ((b) $polA^{ts}$; (b') $polA^{+}$), or at 51.5°C beginning at 2 min after the temperature shift from 37°C ((c) $polA^{ts}$; (c') $polA^{+}$). The pulse was terminated by the addition of ethanol-phenol mixture. DNA was extracted in the denatured state and sedimented through an alkaline sucrose gradient in a SW41 rotor for 11.5 hr at 25,000 revs/min. The vertical broken lines indicate the boundary of the 82% sucrose cushion. -●--●-, 20 sec; -o--o-, 60 sec, -◑--◑-, 120 sec.

of short DNA pieces in the $polA^{ts}$ cells was retarded greatly at 49.5°C (Fig. 1b) and almost completely inhibited at 51.5°C (Fig. 1c). Such accumulation of the radioactive short DNA pieces was not found when the $polA^{ts}$ cells were pulse-labeled at 37°C (Fig. 1a).

To test the reversibility of the accumulation, $polA^{ts}$ cells were pulse-labeled for 1 min at 51.5°C and then transferred to 37°C. After 5 min-incubation at 37°C, a large portion of the radioactivity appeared in long DNA regions, whereas the radioactivity in the short DNA regions decreased markedly (Fig. 2a). In the control culture kept at 51.5°C, the radioactivity in the

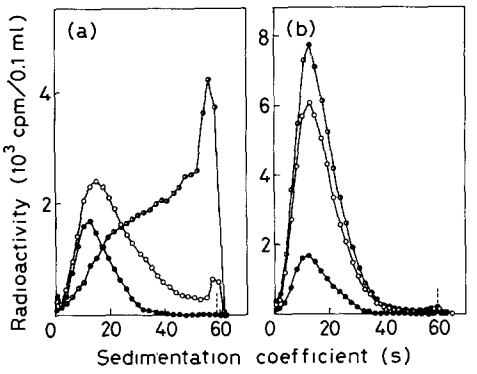

Fig. 2. Effect of incubation at low temperature subsequent to pulse-labeling at high temperature on the sedimentation pattern of radioactive DNA from polA^{ts} cells. (a) Each 6 ml culture of JH406 1-6 (5 x 10^7 cells/ml) was pulse-labeled with 0.1 μM-[^{3}H]thymidine (20 Ci/mmol) for 1 min at 51.5°C beginning at 2 min after the temperature shift from 37°C and then incubated at 37°C for the indicated times. (b) Each 6 ml culture (5 x 10^7 cells/ml) was pulse-labeled for the indicated times at 51.5°C as in Fig. 1. DNA extraction and alkaline sucrose gradient sedimentation were carried out as in Fig. 1. (a) -●--●-, 0 min; -o--o-, 2 min, -◐--◐-, 5 min. (b) -●--●-, 1 min; -o--o-, 3 min; -◐--◐-, 6 min.

short DNA regions continued to increase and little radioactivity could be observed in the long DNA regions (Fig. 2b). Thus the effect of high temperature was reversed by subsequent incubation at 37°C. On the other hand, when polA^{ts} cells were labeled for 5 min with [^{3}H]thymidine and chased with unlabeled thymidine for 3 min at 37°C and then transferred to 51.5°C, no degradation of the prelabeled DNA to short DNA pieces was observed during the incubation for up to 8 min (data not shown). These results indicate that the accumulated short DNA pieces observed at 51.5°C are not likely to be degradation products formed by the incubation at high temperature.

Accumulation of RNA-linked nascent DNA pieces on inhibition of DNA polymerase I: To explore RNA attachment to the accumulated short DNA pieces at 51.5°C, polA^{ts} and polA^{+} cells were pulse-labeled with [^{3}H]thymidine at 51.5°C for 20, 40 and 60 sec. The nascent pieces were isolated by centrifugation in neutral sucrose and Cs_2SO_4 gradients, treated with polynucleotide kinase and unlabeled ATP to mask any pre-existing 5'-OH ends of DNA. Then radioactive DNA bearing 5'-OH ends, created upon alkaline hydrolysis of the RNA moiety of

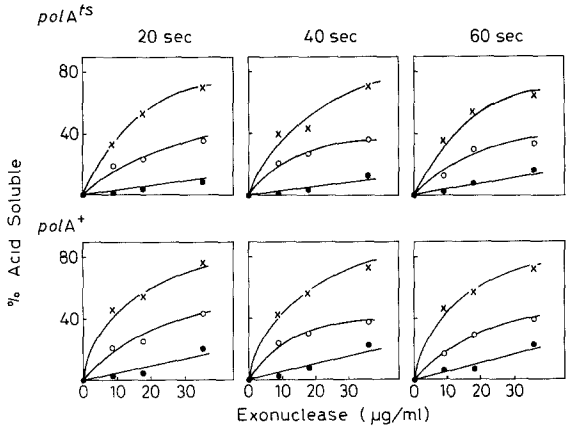

Fig. 3. Assay for the 5'-OH ends in alkali-treated nascent pieces from JH406 1-6 (polA^{ts}) and JH406 (polA^{+}). Cultures (40 ml) were grown at 37°C to 6 x 10^7 cells/ml, transferred to 41.5°C and 2.75 min later pulse-labeled with 0.1 μM-[^{3}H]thymidine (43.1 Ci/mmol) for 20, 40 or 60 sec. The labeling was stopped with an ethanol-phenol mixture and nucleic acid was extracted as described in Materials and Methods. After ethanol precipitation the nucleic acid was heat denatured and sedimented through neutral sucrose gradients. The nascent pieces (100~1800 nucleotides) were concentrated by ethanol precipitation, further purified by Cs_2SO_4 density equilibrium centrifugation and then incubated with unlabeled ATP and polynucleotide kinase. After hydrolysis with 0.15 M NaOH at 37°C for 20 hr, nascent DNA pieces were digested with spleen exonuclease directly (o), after polynucleotide kinase treatment (•) or after alkaline phosphatase treatment (x). The amount of radioactive sample used in each digestion (corresponding to the 100% value) was: polA^{ts} (20 sec), 700 cpm; polA^{ts} (40 sec), 900 cpm; polA^{ts} (60 sec), 1000 cpm; polA^{+} (20 sec), 500 cpm; polA^{+} (40 sec), 450 cpm; polA^{+} (60 sec), 570 cpm.

RNA-DNA molecules, were assayed with spleen exonuclease. From the data shown in Fig. 3 the proportion of the 5'-OH terminated molecules was estimated to be approximately 47%, 50% and 38% of the total radioactive short DNA pieces from polA^{ts} cells labeled by the 20, 40 and 60 sec-pulses, respectively. With polA^{+} cells, the proportion was estimated to be 41%, 43% and 33% of the short pieces labeled by the 20, 40 and 60 sec-pulses, respectively. The labeled 5'-OH DNA was in fact derived from the RNA-linked DNA pieces, since the similar results were obtained using RNase IA plus RNase T1 instead of alkali (Fig. 4c) and hardly any 5'-OH DNA was detected without alkali or RNase treatment (Fig. 4a). Fig. 5 shows the relative amount of the RNA-linked DNA pieces in polA^{ts} and polA^{+} cells, calculated from the radioactivity of short

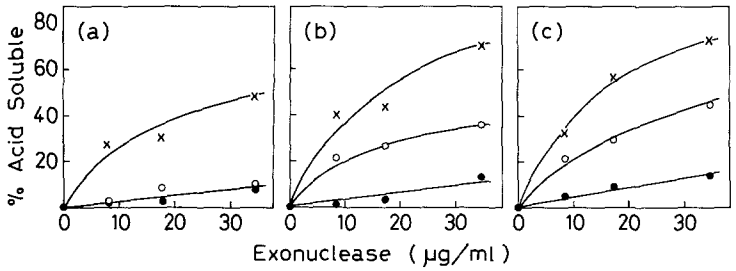

Fig. 4. Assay for the 5'-OH ends in alkali- or RNase-treated nascent pieces from JH406 1-6. A portion of the sample of polA^{ts} (40 sec) in Fig. 3 was withdrawn before alkali treatment and divided into three (a, b and c); (a) was left untreated; (b) was treated with 0.15 M NaOH at 37°C for 20 hr; and (c) was treated with 100 μg RNase IA/ml and 10 μg RNase T1/ml in 10 mM Tris·HCl (pH 8.0)-0.1 mM EDTA at 37°C for 90 min. Each portion (700 to 1200 cpm) was digested with spleen exonuclease directly (o), after polynucleotide kinase treatment (•) or after alkaline phosphatase treatment (x).

DNA pieces and the ratio of 5'-OH and 5'-P DNA in the alkali-treated short DNA pieces. Virtually all the short DNA pieces were labeled by the 60 sec-pulse, as judged from sedimentation analysis. From the data with this pulse time, the polA10 mutant was estimated to contain 4.5 times as many RNA-linked DNA pieces as the wild type.

DISCUSSION

B. subtilis wild type cells have at least three distinct DNA polymerases (6, 10). It has been suggested that DNA polymerase I functions primarily in DNA repair (11, 12), while DNA polymerase III is required for replication process (13-16). However, the present results indicate that DNA polymerase I plays a key role in the process of discontinuous DNA replication.

Analysis with spleen exonuclease has indicated that RNA is attached to the 5'-end of nascent short DNA pieces. These RNA-linked nascent short DNA pieces accumulate on inhibition of DNA polymerase I activity. It is also possible to assay RNA-linked DNA pieces by using T4 polynucleotide kinase. After isolation of RNA-linked DNA pieces, 5'-end of the RNA portion of these pieces can be labeled with [γ-^{32}P]ATP by the successive treatment with bacterial alkaline phosphatase and T4 polynucleotide kinase (4). ^{32}P incor-

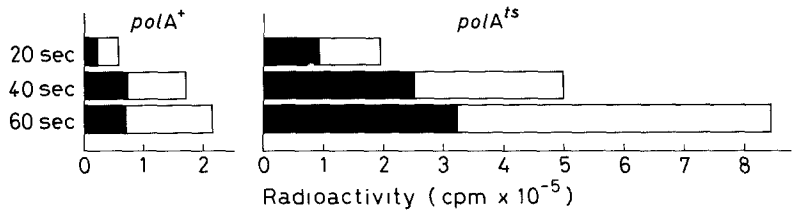

Fig. 5. Radioactivity incorporated into RNA-linked and RNA-free nascent DNA pieces in polA^{ts} and polA^{+} strains. The radioactivity incorporated into RNA-linked nascent DNA pieces in 40 ml culture during each pulse time was calculated from the radioactivity of the nascent short DNA pieces (pooled radioactivity in neutral sucrose gradient), and the ratio of 5'-OH and 5'-P DNA obtained from Fig. 3. Total incorporations into DNA were: polA^{ts} (20 sec), 4.42 x 10^5 cpm; polA^{ts} (40 sec), 1.30 x 10^6 cpm; polA^{ts} (60 sec), 2.19 x 10^6 cpm; polA^{+} (20 sec), 3.47 x 10^5 cpm; polA^{+} (40 sec), 1.85 x 10^6 cpm; polA^{+} (60 sec), 2.76 x 10^6 cpm. ■ , RNA-linked; □ , RNA-free.

porated into RNA-linked DNA pieces, measured by alkali-labile radioactivity, indicated that the amount of the RNA-linked DNA pieces in polA10 mutant at 51.5°C was 5 to 6 times that in the wild type (data not shown). These results suggest that B. subtilis DNA polymerase I is involved in the removal of the RNA attached to the nascent short DNA pieces.

Purified B. subtilis DNA polymerase I has little if any nuclease activity as reported by Okazaki and Kornberg (5). However, the present work has indicated that RNA-linked DNA pieces accumulate upon inhibition of this enzyme. This observation raises the possibility that B. subtilis DNA polymerase I has intrinsic 5'→ 3' exonuclease activity responsible for the removal of primer RNA. Since the extracts of B. subtilis have high proteolytic activity capable of cleaving E. coli DNA polymerase I to the large (the polymerase fragment) and the small (the 5'→ 3' exonuclease fragment) fragments (17), the purified B. subtilis DNA polymerase I might have lost its nuclease component during its purification. Reinvestigation of B. subtilis DNA polymerase I is in progress. Another possibility is that B. subtilis has a separate nuclease which functions in cooperation with DNA polymerase I in the RNA removal.

Three enzymes are now known to be involved in the removal of the RNA attached to the nascent short DNA pieces; E. coli DNA polymerase I (1-4), T7

gene 6 exonuclease (18) and B. subtilis DNA polymerase I. Studies with these enzymes will give us more precise picture of the excision mechanism of primer RNA.

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