ASSAY OF RNA-LINKED NASCENT DNA PIECES WITH POLYNUCLEOTIDE KINASE*

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SUMMARY: The 5'-OH end of DNA created upon alkaline hydrolysis of the RNA-linked nascent DNA pieces can be labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. However, it is difficult to use this method for the assay of these molecules in the presence of RNA-free DNA pieces because of the exchange reaction between the γ-phosphate of ATP and the 5'-phosphate of DNA catalyzed by the kinase. This difficulty can be circumvented by performing the polynucleotide kinase reaction at 0°C, where little exchange reaction occurs. Using these conditions, E. coli polAexl, a mutant defective in the 5'→ 3' exonuclease activity of DNA polymerase I, is shown to contain several times as many RNA-linked DNA pieces as the wild type.

We showed (1-3) that RNA is attached covalently to the 5' end of the nascent DNA pieces in E. coli, suggesting involvement of RNA in the initiation of these pieces in discontinuous replication. One of the methods used for the detection and characterization of the RNA-linked DNA pieces relies on the ability of T4 polynucleotide kinase to label the 5'-OH end of DNA created upon alkaline hydrolysis of these molecules with [γ-^{32}P]ATP (3). A similar method was used for the analysis of RNA-linked nascent DNA pieces of polyoma (4).

However, during further studies we noted a difficulty in using this method for the detection and assay of the RNA-linked DNA pieces in preparations which also contain a large amount of RNA-free DNA pieces. With such preparations, an appreciable transfer of ^{32}P from [γ-^{32}P]ATP to DNA was found in the polynucleotide kinase reaction without prior alkaline hydrolysis, even though the sample had been incubated with unlabeled ATP and the kinase to mask any

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

pre-existing 5'-OH end of DNA. This high background, which obscures the labeling of the new 5'-OH end produced by alkaline hydrolysis of the RNA-linked pieces, is most probably due to the exchange reaction between the γ-phosphate of ATP and the 5'-phosphate of DNA catalyzed by polynucleotide kinase (5).

To facilitate the analysis of the RNA-linked DNA pieces with polynucleotide kinase, we have sought conditions under which 5'-OH DNA is labeled selectively with [γ-^{32}P]ATP by the kinase with little or no labeling of the 5'-P DNA. We have found that the exchange reaction can be minimized by performing the kinase reaction at 0°C. Using these conditions, we have demonstrated that *E. coli polA*exl, a mutant defective in the 5'→3' exonuclease activity of DNA polymerase I (6), contained several times as many RNA-linked DNA pieces as the wild-type strain.

MATERIALS AND METHODS

Bacteria: *E. coli* RS5064 (*polA*exl) was provided by Drs. I. R. Lehman and E. B. Konrad. *E. coli* W3110 (wild type) was from our laboratory stock. Cells were grown in Medium A (7) supplemented with 0.5% Casamino Acids and 20 μg/ml of L-tryptophan.

Enzymes: Polynucleotide kinase was prepared as previously (3) and further purified by hydroxyapatite chromatography (8). Bacterial alkaline phosphatase and pancreatic DNase have been described (3).

^{3}H-labeled 5'-P and 5'-OH DNA: DNA was extracted from *E. coli* B3 (*thy*$^{-}$) grown on [^{3}H]thymine (495 Ci/mole, New England Nuclear), heat denatured, digested with pancreatic DNase and fractionated by gel filtration on Sepharose 2B; the fractions with K_d values of 0.33 to 0.82 (200-2800 nucleotides) were pooled (5'-P DNA). 5'-OH DNA was prepared by treating the 5'-P DNA with alkaline phosphatase.

Polynucleotide kinase reaction: The reaction mixture contained 67 mM Tris-HCl (pH 8.0), 17 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 16 μM [γ-^{32}P]ATP (8,300-36,200 cpm/pmole, the Radiochemical Centre, Amersham), 1-33 nM DNA* and 3.3-60 units/ml of enzyme. With 5'-OH DNA, 1.5 mM KPO_4 (pH 7.5) was also included in the incubation mixture.

RESULTS

Conditions for polynucleotide kinase reaction: Because of the small quantity of the material available, the polynucleotide kinase reaction with the nascent DNA pieces is performed at DNA concentrations as low as 1 to 33 nM. Even at such low concentrations, 5'-OH DNA was completely phosphorylated

*Concentrations of DNA are expressed in terms of moles of polymer.

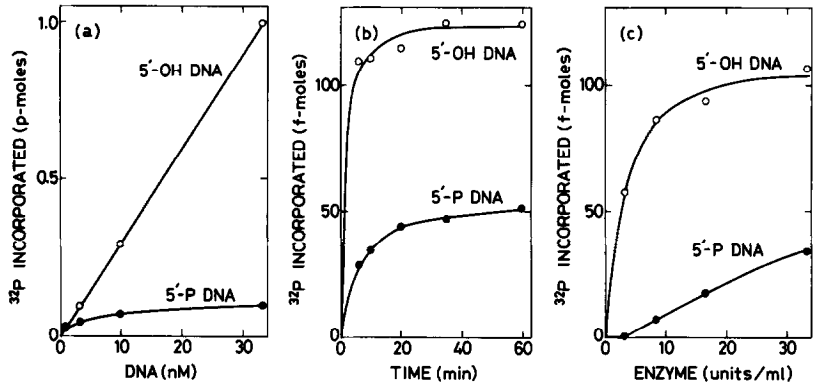

Fig. 1. Effect of DNA concentration (a), incubation time (b) and amount of enzyme (c) on the polynucleotide kinase reaction at 37°C. (a) 5'-OH or 5'-P DNA at the indicated concentrations was incubated for 30 min with 33 units/ml of enzyme in a 30-µl reaction mixture. (b) 5'-OH or 5'-P DNA (~3.3 nM) was incubated with 33 units/ml of enzyme for the times indicated in a 30-µl reaction mixture. (c) 5'-OH or 5'-P DNA (~3.3 nM) was incubated with the indicated enzyme concentrations for 30 min in a 30-µl reaction mixture.

during a 30-min incubation with 33 units/ml of enzyme at 37°C. The DNA concentration, however, had a striking effect on the labeling of 5'-P DNA by the exchange reaction. The relative extent of the labeling of 5'-P DNA decreased with increases of its concentration (Fig. 1a). Thus, 85, 40 and 10% of 5'-P DNA were labeled at DNA concentrations of 1, 3.3 and 33 nM, respectively. Reducing the incubation time or the amount of enzyme at a fixed DNA concentration decreased the extent of labeling of both 5'-OH and 5'-P DNA (Figs. 1b and c). At pH 9.0 the labeling of 5'-P DNA was supressed by 40%. The addition of neither d-pTp nor d-pTpT (13-300 nM), which may serve as substrates for the exchange reaction, affected the labeling of 5'-P DNA.

The rate of labeling of 5'-P DNA at the concentration of 3.3 nM was found to be strongly temperature dependent (Fig. 2). The temperature coefficient (Q_{10}) of the exchange reaction in various ranges of temperature between 0 and 37°C was 2.7 to 3.9, and the rate at 0°C was 1/86 of the rate at 37°C. The activation energy was calculated to be 20.5 kcal from the Arrhenius plot (Fig. 2b). Although the extreme rapidity of the labeling of 5'-OH DNA did not allow measurement of its rate under similar conditions, the rate measured at various

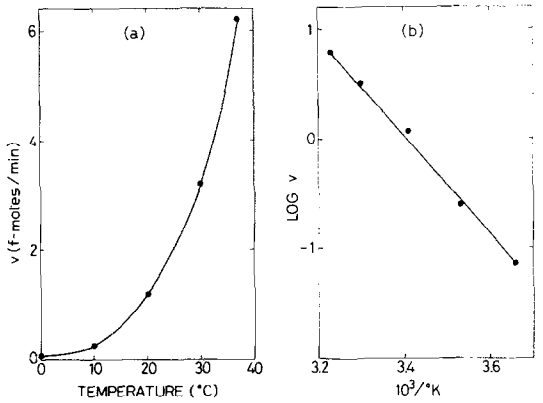

Fig. 2. Effect of temperature on labeling of 5'-P DNA by polynucleotide kinase. 5'-P DNA (~3.3 nM) was incubated with 33 units/ml of enzyme at the indicated temperatures for various times (5-120 min) to obtain the initial rate. (a) Reaction rates (v) at various temperatures. (b) Arrhenius plot of the data shown in (a).

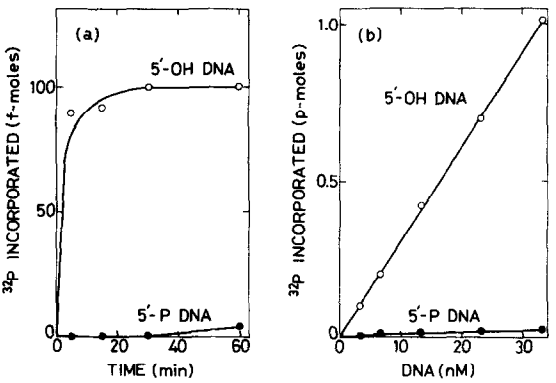

Fig. 3. Effect of incubation time (a) and DNA concentration (b) on the polynucleotide kinase reaction at 0°C. (a) 5'-OH or 5'-P DNA (~3.3 nM) was incubated with 33 units/ml of enzyme in a 30-µl reaction mixture. (b) 5'-OH or 5'-P DNA at the indicated concentrations was incubated for 60 min with 33 units/ml of enzyme in a 30-µl reaction mixture.

temperatures at a low enzyme concentration (2.4 units/ml) and a high DNA concentration (~16 mM) showed the temperature coefficient of phosphorylation to be 1.4 to 2.4, and its activation energy to be 12.3 kcal. The ratio of the rate at 0°C to the rate at 37°C was 1/13.6. At low DNA concentrations, the difference of the rates at the two temperatures would be even smaller, since the kinase activity is inactivated rapidly in the absence of a sufficient amount of DNA at 37°C, but not at 0°C.

At 0°C, 5'-OH DNA at the concentration of 3.3 nM was completely phosphorylated during a 30-min incubation with 33 units/ml of enzyme. Under these conditions, virtually no 5'-P DNA was labeled (Fig. 3a). After a 60-min incubation, about 3% of the 5'-P DNA was labeled. The effect of DNA concentration on the 60-min reaction at 0°C (Fig. 3b) indicated that over a wide range of DNA concentrations little exchange reaction occurs. Varying the enzyme concentration between 16 to 60 units/ml did not change the essential feature of the results.

RNA-linked DNA pieces in E. coli polAexl and wild type: The nascent DNA pieces were isolated from E. coli RS5064 (polAexl) and W3110 (wild type) which had been pulse labeled at 43°C. After incubation with unlabeled ATP and polynucleotide kinase (to mask any pre-existing 5'-OH end of DNA), the pieces were hydrolyzed with alkali, and the 5'-OH end of DNA was measured by using the polynucleotide kinase reaction with [γ-^{32}P]ATP at 0°C (Table 1). The preparations from RS5064 contained 5 to 6 times as many 5'-OH DNA pieces as the preparations from W3110. Although these preparations also contained 5'-P DNA, under the conditions used, the labeling of 5'-P DNA in the kinase reaction was less than 3% and virtually no ^{32}P transfer to DNA was found without prior alkaline hydrolysis. It is therefore evident that at the restrictive temperature the level of the RNA-linked DNA pieces is 5 to 6 times higher in the polAexl mutant than in the wild type. From the data, the average number of the RNA-linked DNA pieces per cell is calculated to be about 44 for the mutant and 8 for the wild type.

DISCUSSION

Because of the exchange reaction between the γ-phosphate of ATP and the 5'-phosphate of DNA (5), the assay of the RNA-linked DNA pieces with polynucleotide kinase requires either that these molecules be isolated quantitatively in a pure form or that the exchange reaction be minimized. While the former requirement seems difficult to be satisfied, our results show that the latter can be met simply by performing the kinase reaction at 0°C. The influence of

Table 1. RNA-linked DNA pieces in E. coli RS5064 (polAexl) and W3110 (wild type).

Experiment No.	Strain	5'-OH ends in alkali-treated pieces (f-moles/ml culture)
1	RS5064	64
2	RS5064	57
3	RS5064	66
4	W3110	11
5	W3110	11

Cultures (40-180 ml) were grown at 30°C to 8 x 10^8 cells/ml, transferred to 43°C and 3 min later pulse labeled with [^{3}H]thymidine (2.5-58 Ci/mmole, New England Nuclear) for 20 to 40 sec. The labeling was stopped with an ethanol-phenol mixture (7) and nucleic acid was extracted by the modified Thomas procedure (7). After ethanol precipitation the DNA was heat denatured and sedimented through neutral sucrose gradients; the material which sedimented slower than 16S was concentrated by ethanol precipitation. In Experiment 1, this material was incubated with unlabeled ATP and polynucleotide kinase (50 units/ml) in a 1.7-ml reaction mixture at 37°C for 30 min and hydrolyzed with 0.3 M NaOH at 37°C for 20 hr. In Experiments 2-5, the material recovered from the sucrose gradients was 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, the DNA was subjected to gel filtration on a Sepharose 4B column and the fractions with K_d values of 0.2 to 0.7 (100-2500 nucleotides) were pooled. In all experiments the alkali-treated sample was concentrated to less than 1/100 the culture volume and dialyzed against 10 mM Tris-HCl (pH 8.0)-0.1 mM EDTA. The sample was incubated with [γ-^{32}P]ATP and 40 units/ml of polynucleotide kinase in a 0.1- to 1.2-ml reaction mixture at 0°C for 60 min. After the addition of EDTA to 20 mM to stop the reaction, [γ-^{32}P]ATP was removed by alkali treatment (0.15 M NaOH at 37°C for 20 hr) and gel filtration on a Sephadex G75 column, and the acid-insoluble radioactivity was counted. The amount of 5'-OH DNA per ml of the culture was calculated from the amount of ^{32}P transferred per ^{3}H count and the ^{3}H radioactivity incorporated into the nascent DNA pieces per ml of the culture.

the exchange reaction may be sufficiently reduced by performing the reaction at high DNA concentrations (e.g. 0.3 μM). However, this is difficult in practice, as it necessitates the preparation of the nascent pieces from a large quantity of cells, by a procedure involving extensive concentration of the sample.

E. coli polAexl, a mutant defective in the 5'→3' exonuclease activity of DNA polymerase I, shows retarded joining of the nascent DNA pieces, as do the mutants defective in DNA ligase or the polymerase activity of DNA polymerase I

(6). The present study demonstrates an accumulation of the RNA-linked DNA pieces in the polAexl mutant, suggesting that the 5'→3' exonuclease plays a role in the removal of the RNA attached to the nascent DNA pieces.

A disadvantage of the polynucleotide kinase assay is that if there were covalently linked RNA-DNA molecules of similar size that were not replication intermediates, these irrelevant molecules would also be measured. We have recently developed a new method for the specific assay of the RNA-linked nascent DNA pieces, which relies on the ability of spleen exonuclease to degrade the radioactive 5'-OH DNA that is produced from the pulse-labeled nascent pieces upon alkaline hydrolysis (9). Assay with this new method has also showed (9) that the RNA-linked nascent DNA pieces are accumulated in E. coli polAexl as well as in E. coli polA12 (10) and polA1 (11), but not in E. coli ligts7 (12). The amount of the RNA-linked pieces in E. coli polAexl at 43°C measured by the spleen exonuclease assay was 5.2 times that in the wild type. The good agreement of the results obtained by the kinase and exonuclease methods strengthens the evidence for accumulation of RNA-linked nascent DNA pieces in the polAexl mutant and the reliability of both assays. It also makes the presence of the irrelevant RNA-DNA molecules less likely.

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