Inhibition of Thymidine Transport in dnaA Mutants of Escherichia coli*

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DnaA protein is the initiation factor of chromosomal DNA replication in Escherichia coli. We report here our evidence that thymidine transport across cytoplasmic membranes in temperature-sensitive dnaA mutants is greatly decreased at a permissive temperature for growth of the mutants. Complementation analysis with a plasmid containing the wild type dnaA gene and P1 phage-mediated transduction confirmed that mutations in the dnaA gene were responsible for the phenotype. A low level of nucleoside transport in the dnaA mutant was specific for thymidine; transport activities for other nucleosides were much the same as those in wild type cells. Membrane vesicles prepared from the dnaA mutant showed much the same activity of thymidine transport as did those from the wild type cells. No significant difference in the activity of thymidine kinase, which is known to facilitate thymidine transport, was seen between the mutant and the wild type cells. An increase in the pool of dTTP, a negative regulator for thymidine kinase, was observed in the dnaA mutant. Based on these observations, we suggest that inhibition of thymidine transport in dnaA mutants is caused by increases in the dTTP pool.

Thymidine transport systems are ubiquitously present in prokaryotic and eukaryotic cells. Pulse-labeling experimentation with radiolabeled thymidine has been accepted widely as a method of determining the level of DNA synthesis in cells. Little attention, however, has been directed to whether the transport activity of thymidine is influenced experimentally. Genetic and biochemical studies using E. coli identified two different transport systems for nucleosides, termed the NupC and the NupG systems (16–19), and these can be distinguished by the lack of potential of the NupC system to transport guanosine (16–19). Transcriptional regulation of the nupC and nupG genes has been reported (19). Nucleoside transport activity plus DNA synthesis seem to be needed for related activity to occur.

We report here the influence of the dnaA mutation in thymidine transport in E. coli; thymidine transport across the cytoplasmic membranes in the dnaA mutants was inhibited drastically, which in turn suggests a role for DnaA protein in the regulation of nucleoside transport. A putative mechanism for the inhibition of thymidine transport in dnaA mutants is discussed.

EXPERIMENTAL PROCEDURES

Bacterial Strains and a Plasmid—All strains of E. coli used in the present experiments are listed in Table I. E. coli strains ME8923 (nuPC146) and ME8935 (nuPC157) were kindly provided by Dr. A. Nishimura (National Institute of Genetics, Japan) (20). P1 phage-mediated transduction was performed as described (21, 22). For complementation analysis, we used the pHB10S plasmid, which contains the wild type dnaA gene (21, 22). pAST1 plasmid, a thyA overexpression plasmid (23), was kindly provided by Dr. M. Iwakura (National Institute of Bioscience and Human Technology, Japan).

Measurement of Growth of Cells—Exponentially growing E. coli cells in LB medium were diluted appropriately. The suspension was spread on LB agar plates. The plates were incubated at 30 °C for 12 h. Colonies were counted, and viable cell numbers were determined.

Determination of DNA in Cells—Exponentially growing E. coli cells in LB medium were harvested periodically by centrifugation and were suspended in 300 μl of 9.9% NaCl. Toluene (10 μl) was added to the suspension, which was then mixed vigorously. 4,6-Diamidino-2-phenylindole dihydrochloride (24) solution was then added to the suspension to a final concentration of 0.1 μg/ml. Fluorescence by λ-excitation (346 nm) and λ-emission (452 nm) was measured.

Measurement of Transport Activity of Nucleosides into Cells—Exponentially growing E. coli cells in LB medium were treated with nalidixic acid (200 μg/ml, final concentration) at 37 °C for 10 min. Radiolabeled nucleosides were added to the culture, and incubation was run at 37 °C for 20 s. Transport reaction of radiolabeled nucleosides into cells was terminated by adding unlabeled nucleosides at a final concentration of 50 μM. Samples were passed through nitrocellulose membranes (Millipore), radioactivity remaining on the filters was counted in a liquid scintillation counter, and the amount of nucleoside transported into cells was calculated (19).

Preparation of Membrane Vesicles and Transport Experiments—Preparation of membrane vesicles from the dnaA46 mutant and wild type cells and examination of their activities for nucleosides transport were done as described by Munch-Petersen et al. (19). In brief, expo-

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experimentally growing cells in LB medium were harvested when the optical density at 660 nm reached 0.8. Vesicles were prepared according to Kaback (25), omitting the final sucrose gradient centrifugation. Protein determination of the membrane vesicles was done as described (26).

The standard reaction mixture contained 0.1 m potassium phosphate (pH 6.0), 0.1 m MgSO₄, 0.15 mm phenazine methosulfate, 30 mm ascorbate, and 4 μCi radiolabeled nucleoside. The transport reaction was initiated by addition of membrane vesicles to the mixtures; the reaction was performed at 20 °C for 4 min and was terminated by adding 50 μl unlabeled nucleoside. Samples were passed through nitrocellulose membranes (Millipore), and the radioactivity remaining on the filters was counted in a liquid scintillation counter. The amount of nucleosides transported into membrane vesicles was calculated (19).

**Measurement of Activity of Thymidine Kinase in Cell Extracts**—The activity of thymidine kinase in crude extracts from the dnaA46 mutant and wild type cells was measured, as described elsewhere (27). Experimentally growing cells were collected by centrifugation when the optical density at 660 nm reached 0.8. The precipitates were washed with buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, and 1 mM EDTA, then were suspended in the same buffer. After sonication, the suspension was centrifuged at 20,000 × g for 30 min, and the supernatant was used for the assay. Protein in the extracts was determined according to Lowry et al. (28).

The standard reaction mixture contained 62.5 mM Tris-HCl (pH 8.0), 0.1 M MgSO₄, 0.15 mM phenazine methosulfate, 30 mM ascorbate, and 4 μCi radiolabeled nucleoside. The transport reaction was initiated by addition of membrane vesicles to the mixtures; the reaction was performed at 20 °C for 4 min and was terminated by adding 50 μl unlabeled nucleoside. Samples were passed through nitrocellulose membranes (Millipore), and the radioactivity remaining on the filters was counted in a liquid scintillation counter. The amount of nucleosides transported into membrane vesicles was calculated (19).

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in the dnaA mutant cannot be explained by a decrease in the rate of synthesis of DNA; rather the inhibition of thymidine transport in the mutant may occur.

Incorporation of \(^{3}H\)thymidine into trichloroacetic acid-insoluble fractions in the wild type cells was inhibited (less than 1% of control) by pretreatment of the cells with 200 µg/ml nalidixic acid for 10 min (data not shown). Thus, when examining the activity of thymidine transport in cells, we preincubated the cells with nalidixic acid so that the effect of the DNA synthesis reaction on the transport reaction would be eliminated. The activity of thymidine transport in KS1001 growing at 37 °C was most apparent at a late stage of log phase of cell growth (Fig. 2). On the contrary, low activity of thymidine transport was observed in KS1003 growing at 37 °C, at the corresponding stage (Fig. 2). The \(K_m\) value for the thymidine transport in wild type cells was 1 µM (data not shown), and the transport experiments in Fig. 2 were done in the presence of 15 µM thymidine. When cells were grown at 28 °C, no significant difference in the activity of thymidine transport was observed between KS1001 and KS1003 (data not shown). These results suggest that the inhibition of incorporation of \(^{3}H\)thymidine into trichloroacetic acid-insoluble fractions of the dnaA mutants growing at 37 °C is caused by a decrease in the activity of thymidine transport in the mutant.

To confirm that the dnaA mutation is responsible for the inhibition of thymidine transport in KS1003, we transformed the strain with pHB10S, a plasmid carrying the wild type dnaA gene. P1 phages proliferated in mutant cells, transduced into W3110 cells, and the transductants were selected on tetracycline-containing plates (21, 22). The activity of thymidine transport in the transformants recovered to much the same level as that in wild type cells (Fig. 2), whereas the recovery was nil when the cells were transformed with the vector pBR322 (data not shown). Transformation of the wild type strain with pHB10S did not affect the transport activity (data not shown).

We then asked whether the phenotype of the low level of thymidine transport in KS1003 would be cotransducible with the temperature-sensitive growth of this mutant in P1 phage-mediated transduction experiments. In the dnaA46 mutant, Tn10 with a tetracycline-resistant marker is located at a position close to the dnaA gene. P1 phages proliferated in mutant cells, transduced into W3110 cells, and the transductants were selected on tetracycline-containing plates (21, 22). Temperature sensitivities of cell growth and activities of thymidine transport were examined. Of the 53 independent transductants, 7 showed a high temperature-resistant growth and a high activity of thymidine transport, whereas all of the remaining 46 showed temperature-sensitive growth and a low activity of thymidine transport. No transductant showed either a high temperature-resistant growth and a low activity of thymidine transport or a temperature-sensitive growth and a high activity of thymidine transport. Thus, both phenotypes (a temperature-sensitive growth and a low activity of thymidine transport) were cotransducible. From the results of plasmid complementation and P1 phage-mediated transduction, it seems clear that the dnaA46 mutation is responsible for the low activity of thymidine transport in the KS1003 strain.

Temperature-sensitive dnaA mutants have been isolated and the mutations identified (12). We examined the activity of thymidine transport in several other dnaA mutants. All dnaA mutants tested showed lower activity of thymidine transport than did the wild type strain (Fig. 3). The activity of thymidine transport in the dnaA204 mutant was more than half that in the wild type cells, whereas the activity in the dnaA64 mutant was less than one-tenth that in the wild type cells (Fig. 3). Thus, the extent of inhibition of the transport showed an allele specificity. Plasmid complementation with pHB10S confirmed that mutations in the dnaA gene are responsible for the phenotype of all of these mutants (data not shown).

Effect of nupC or nupG Mutation on the Inhibition of Thymidine Transport in the dnaA46 Mutant—Genetic and biochemical studies revealed that two transport proteins, NupC and NupG, play a major role in the nucleoside transport in E. coli (16–19). Transcription factors that regulate the expression of genes encoding the proteins, the nupC and nupG genes, have been identified (19). DnaA protein regulates the expression of a number of genes through specific binding to DnaA boxes (12) or through other mechanisms (13–15). Therefore, the possibility that inhibition of thymidine transport in dnaA mutants may relate to contribution of DnaA protein to expression of the nupC or the nupG gene would need to be ruled out. If such is indeed the case, in the presence of nupC or nupG mutation, the dnaA46 mutation should not affect thymidine transport. The dnaA46 mutation was then transduced into a nupC or nupG mutant (ME8923 and ME8935, respectively) by P1 phage-mediated transduction, and the activity of thymidine transport in the transductants (KY1001 and KY1002, respectively) was examined. Even in the presence of the nupC or nupG mutation, the dnaA46 mutation caused inhibition of thymidine transport (Fig. 4), indicating that the inhibition of thymidine transport in the dnaA46 mutant cannot be explained by the effect of the dnaA46 mutation on expression of either the nupC or the nupG gene.
in the dnaA46 mutant suggests that alteration in the NupC and NupG systems is not involved in the inhibition. Because unknown transport systems other than the NupC and NupG systems may contribute to the inhibition of thymidine transport in the dnaA46 mutant, we prepared membrane vesicles from the dnaA46 mutant and wild type cells and examined their transport activities for nucleosides in vitro. As shown in Fig. 6, the activity for thymidine transport of membrane vesicles prepared from the dnaA46 mutant grown at 37 °C was slightly higher than from the wild type cells. Inhibition of thymidine transport in the dnaA46 mutant apparently cannot be reproduced with membrane vesicles in vitro. We also observed significant transport activities for other nucleosides in membrane vesicles from the dnaA46 mutant, comparable to that from the wild type cells (data not shown). Thus, a mechanism other than alteration in transport machinery in membranes is involved in inhibition of thymidine transport in the dnaA46 mutant. Components in the cytosol, which had been removed during preparation of membrane vesicles, seem to be responsible for the inhibition of thymidine transport in the dnaA mutant. We assumed that these unidentified components would specifically affect thymidine transport because inhibition of nucleoside transport in the dnaA46 mutant was observed only in case of thymidine.

Thymidine Kinase Activity and dTTP Pool in the dnaA46 Mutant—It was reported that thymidine kinase is necessary for thymidine transport but not so for other nucleosides (32). This enzyme catalyzes the phosphorylation of thymidine and facilitates thymidine transport into cells (32). Therefore, we considered the possibility that the dnaA mutation decreases the activity of thymidine kinase. We then compared the activity of thymidine kinase in a crude extract from the dnaA46 mutant with that from the wild type cells. As shown in Fig. 7, a significant amount of thymidine kinase activity, comparable to that from the wild type cells, was observed in the crude extract from the dnaA46 mutant.

The activity of thymidine kinase is negatively regulated by dTTP (33). When we examined thymidine kinase activity in the crude extracts, the samples were diluted to more than 1,000 times before being subjected to assay (Fig. 7). Therefore, the effect on the enzyme activity of dTTP in cells would not reflect the result shown in Fig. 7. To rule out the possibility that the activity of thymidine kinase was inhibited by a higher concen-
The activity for thymidine transport in membrane vesicles prepared from KS1001 (wild type) and KS1003 (dnaA46) cells growing at 37 °C was measured, as described under "Experimental Procedures." The amounts of thymidine transported into membrane vesicles (pmol/min) are shown.

To test this notion we examined the effect of an increase in thymidine transport across the cytoplasmic membrane (32), we propose that the inhibition of thymidine transport in the dnaA46 mutant is at least partly caused by an increase in the dTTP pool. The possibility that mechanisms other than an increase in the dTTP pool may be involved in inhibition of thymidine transport in the dnaA46 mutant would need to be ruled out.

We obtained evidence that thymidine transport in temperature-sensitive dnaA mutants was drastically inhibited. Experiments on nucleoside specificity for the inhibition of transport, genetic analysis using nupC and nupG mutants, and measurement of transport activity of thymidine in membrane vesicles all support the thesis that the inhibition of thymidine transport in the dnaA mutants is not caused by modulation of transport machinery in membranes. There was a 3- or 4-fold increase of the dTTP pool in the dnaA46 mutant. Based on previous observations that dTTP negatively regulates the activity of thymidine kinase (33) and that thymidine kinase is essential for thymidine transport across the cytoplasmic membrane (32), we propose that the inhibition of thymidine transport in the dnaA mutants is caused by an increase in the dTTP pool. Studies on the effect of overexpression of thymidylate synthase on the dTTP pool and the thymidine transport supported this proposal.

To monitor DNA synthesis in cells, one usually measures the incorporation of radiolabeled thymidine into trichloroacetic acid-insoluble fractions. Some growth conditions have been reported to affect the dTTP pool in cells (30). Therefore, our results show that attention should be directed to alteration in the dTTP pool when pulse labeling cells with radiolabeled thymidine for examination of DNA synthesis.

How the dnaA mutations increase the dTTP pool remains unknown. Because dTTP is one of the substrates of DNA synthesis, the inhibition of DNA synthesis caused by the dnaA mutations seems to increase the dTTP pool. However, our data seem to rule this out. The rates of growth and of DNA synthesis of the dnaA46 mutant growing at 37 °C were indistinguishable.
Thymidine Transport in dnaA Mutants.

Fig. 9. Effect of overexpression of thymidylate synthase on thymidine transport. W3110 cells transformed with pASTS1, thymidylate synthase overexpression plasmid (23), or the vector pBR322 were grown in LB medium containing 100 μg/ml ampicillin until the optical density at 660 nm reached 0.8. The thymidine transport activity was determined as described under “Experimental Procedures.”

from findings in the wild type cells. It was reported that the dTTP pool in cells was not altered when DNA synthesis was inhibited in the presence of nalidixic acid (30). Actually, in our assay for the transport of thymidine, cells were pretreated with nalidixic acid.

Filpula and Fuchs reported that the activity of RDP reductase in the temperature-sensitive dnaA mutants was increased when the incubation temperature was shifted to 42 °C, a nonpermissive temperature (34). We recently found that this enzyme was overproduced in the dnaA46 mutant growing at 37 °C.2 Because RDP reductase catalyzes the synthesis of dUDP and dCDP from UDP and CDP, respectively (35), and because dTTP is synthesized de novo from dUDP and dCDP (35), the possibility that the induction of RDP reductase in the dnaA46 mutant at 37 °C causes increases in the dTTP pool would need attention. The following evidence suggests that this notion can indeed be ruled out. First, the decrease in pool size in the dnaA mutants is specific for dTTP. The overproduction of RDP reductase should increase pool sizes of all four deoxyribonucleotides. Second, the dnaA46 mutant carrying the thyA mutation showed dnaA46 mutation-dependent inhibition of thymidine transport (data not shown). The thyA mutant cannot synthesize dTTP de novo (36, 37). Thus, if the inhibition of thymidine transport in the dnaA46 mutant is indeed caused by increases in the dTTP pool resulting from a high level of RDP reductase activity, the inhibition of thymidine transport by the dnaA mutation would not be observed in the thyA mutant. Therefore, the induction of RDP reductase may not explain the inhibition of thymidine transport in the dnaA46 mutant.

DNA protein, the initiator of chromosomal DNA replication in E. coli, plays a key role in regulating DNA replication. Our observations suggest that DNA protein is also involved in nucleoside transport. DNA protein may have an important role in the coupling of DNA replication with nucleotide metabolism, which provides substrates of DNA synthesis.

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1 The abbreviation used is: RDP reductase, ribonucleoside diphosphate reductase.
2 A. Ohba, T. Mizushima, and K. Sekimizu, unpublished results.