Search for Proteins Required for Accurate Gene Expression under Oxidative Stress

ROLES OF GUANYLATE KINASE AND RNA POLYMERASE

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Background: Oxygen radicals, formed in aerobically growing cells, oxidize guanine to 8-oxo-7,8-dihydroguanine, which causes base mispairing.

Results: Guanylate kinase and RNA polymerase are most responsible for preventing transcriptional errors caused by oxidative stress.

Conclusion: Mutations in the genes responsible for preventing phenotypic suppression were identified.

Significance: This study reveals a mechanism by which bacterial cells protect themselves against oxidative damage to RNA.

In aerobically growing cells, in which reactive oxygen species are produced, the guanine base is oxidized to 8-oxo-7,8-dihydroguanine, which can pair with adenine as well as cytosine. This mispairing causes alterations in gene expression, and cells possess mechanisms to prevent such outcomes. In Escherichia coli, 8-oxo-7,8-dihydroguanine-related phenotypic suppression of lacZ amber is enhanced by mutations in genes related to the prevention of abnormal protein synthesis under oxidative stress. A genome-wide search for the genes responsible, followed by DNA sequence determination, revealed that specific amino acid changes in guanylate kinase and in the β and β′ subunits of RNA polymerase cause elevated levels of phenotypic suppression, specifically under aerobic conditions. The involvement of the DnaB, DnaN, and MsbA proteins, which are involved in DNA replication and in preserving the membrane structure, was also noted. Interactions of these proteins with each other and also with other molecules may be important for preventing errors in gene expression.

The basal level of spontaneous errors in RNA synthesis is estimated to be 10⁻⁵ per residue, which is considerably higher than that for DNA synthesis (1, 2). The fidelity of transcription is predicted to be worse in aerobically growing cells, in which reactive oxygen species are generated as by-products of oxygen utilization. Although most of these radicals are eliminated by the actions of cellular antioxidation systems, some remain in the cell and damage its constituents, including proteins, lipids, and nucleic acids (3–5). Among the various types of oxidized purine and pyrimidine bases thus produced, 8-oxo-7,8-dihydroguanine (8-oxo-Gua)² is the most abundant and seems to affect the maintenance and transfer of genetic information. Unlike other types of oxidized bases, 8-oxo-Gua does not block nucleic acid syntheses but rather induces base mispairing. The 8-oxo-Gua can pair with both adenine and cytosine, thus causing alterations in the genetic information (6–8). When such mispairing occurs during DNA replication, base substitution mutations ensue. Likewise, the 8-oxo-Gua mispairing that occurs in RNA synthesis would cause errors in gene expression.

Organisms are equipped with elaborate mechanisms for counteracting such deleterious effects of 8-oxo-Gua. In Escherichia coli, three proteins with distinct enzyme activities are involved in the process (9, 10). The MutT protein, the product of the mutT gene, functions to prevent mutations caused by the oxidation of guanine in the nucleotide pool of the cell. An oxidized form of dGTP, 8-oxo-dGTP, is a potent mutagenic substrate for DNA synthesis, and the MutT protein hydrolyzes 8-oxo-dGTP and 8-oxo-dGDP to 8-oxo-dGMP, thereby preventing the misincorporation of 8-oxo-Gua into DNA. Because 8-oxo-Gua can pair with adenine and cytosine with almost the same efficiency, this misincorporation potentially causes both GC to TA and AT to CG transversions. However, in the mutT-deficient mutant, only AT to CG mutations were evident because the occurrence of the GC to TA mutation is still prevented by MutM and MutY, which act on the 8-oxo-Gua present in DNA (11–13). The MutM protein removes 8-oxo-Gua paired with cytosine, whereas the MutY protein excises adenine paired with 8-oxo-Gua. The occurrence of GC to TA transversions caused by 8-oxo-Gua-cytosine pairing in DNA is thus prevented by the concerted actions of these two enzymes. The frequencies of both GC to TA and AT to CG transversions in mutants lacking all three genes, mutT, mutM, and mutY, increase to a level several hundred times higher than that for wild-type cells.

The oxidation of guanine occurs also in the ribonucleotide pool of the cell, and the 8-oxo-GTP thus produced is misincorporated into RNA. The 8-oxo-Gua present in messenger RNA would cause errors during codon-anticodon pairing in the translation process, and thus, the persistence of 8-oxo-Gua in RNA may induce erroneous protein synthesis. The MutT protein also plays an important role in this process. In addition to
its action on the oxidized DNA precursors, the MutT protein has the ability to degrade 8-oxo-Gua-containing ribonucleoside tri- and diphosphates, 8-oxo-GTP and 8-oxo-GDP, to the monophosphate, thereby preventing the misincorporation of 8-oxo-Gua into RNA (14). Because the RNA and the DNA precursor pools are separated by a distinct nucleoside diphosphate reductase system (15), MutT appears to function independently for the ribonucleotide and deoxyribonucleotide pools.

This leads to the question of whether any other protein factors, beside MutT, are involved in the high fidelity of gene expression under oxidative stress. To identify such factors, we have applied a system related to partial phenotypic suppression using the lacZ amber mutation, which was previously used to establish the role of MutT in the prevention of transcriptional errors caused by oxidative damage (16). In this system, the action of an amber codon, UAG, was partially suppressed by the misincorporation of 8-oxo-Gua in place of uracil in the messenger RNA, which allows the formation of a small amount of active β-galactosidase protein. We hypothesized that if a larger amount of 8-oxo-Gua is formed and persists in the precursor pool and also in the RNA, larger degrees of phenotypic suppression would be observed. By screening mutants exhibiting such phenotypes from an E. coli gene library, we would be able to collect the genes involved in these processes. By then determining the sequences of the candidate genes, we would be able to identify the proteins responsible and further reveal specific amino acid residues necessary for executing these functions.

By performing these experiments, we found that certain base substitutions in the gmk and rpoC genes, encoding guanylate kinase and the β′ subunit of RNA polymerase, respectively, cause elevated levels of phenotypic suppression. We further noted that, among the mutations that arose in the rpoB gene, which codes for the β subunit of RNA polymerase, some exhibited a similar phenotype. In addition, we obtained evidence that proteins encoded by the dnaB, dnaN, and msbA genes may take part in this process. We herein describe the results of these studies in detail and discuss the potential applications of this method, our findings, and the products developed during these studies.

**EXPERIMENTAL PROCEDURES**

*Bacteria, Phages, and Plasmids*—All bacterial strains used are derivatives of E. coli K12. Strain CR63 was used as the wild-type bacterium in the present study. Strain BT369 is a derivative of 1000BT (17) and carries an amber (UAG) mutation in the lacZ gene. It was confirmed by DNA sequencing that BT369 carries a single base substitution in the glutamine codon (CAG) at the 1456th position of the lacZ gene. Strain BT370 was constructed by introducing the chloramphenicol resistance gene (Cm') into the mutT gene of BT369 by P1 transduction. As a result, BT370 carries a lacZ amber mutation, together with a deletion in the mutT gene. A set of Kohara's λ clones, which cover the entire E. coli K12 chromosome (18), was used for a complementation analysis. Plasmid pQE80L (Qiagen)-MutT, which carries the wild-type mutT gene (14), was also used.

*Media and Reagents*—Bacteria were grown in LB medium (10 g of Bacto-tryptone, 5 g Bacto-yeast extract, and 10 g of NaCl/liter). LB agar plates contained 15 g of agar/liter. For propagation of λ phages and the complementation test, tryptone broth plates (10 g of Bacto-tryptone, 5 g of NaCl, and 15 g of Bacto-agar/liter) and soft agar (10 g of Bacto-tryptone, 5 g of NaCl, and 6 g of Bacto-agar/liter) were used. For the isolation of mutant colonies exhibiting enhanced levels of phenotypic suppression, X-gal and isopropyl β-D-thiogalactoside (IPTG) were added to give concentrations of 250 μg/ml and 100 μM, respectively. For the selection of antibiotic-resistant cells, plates were supplemented with either 50 μg/ml ampicillin, 50 μg/ml chloramphenicol, or 150 μg/ml rifampicin. Chemicals and reagents were purchased from Sigma, BD Biosciences, and Wako Pure Chemicals (Osaka, Japan) unless otherwise specified.

*Cultivation under Anaerobic Conditions*—Cells were spread on LB plates and placed in a sealed polyethylene bag containing AnaeroPack/Anaero (Mitsubishi Gas Chemicals Co., Inc., Tokyo, Japan), which is a disposable oxygen-absorbing and carbon dioxide-generating reagent. This containment was placed in an air incubator at defined temperatures and kept for 4 days because cells grow slowly under these conditions.

*Complementation Test*—The complementation test of the temperature-sensitive characteristics of the isolated mutants was performed with the use of Kohara's λ phage clones (476 clones in total). A mixture of 10 clones (1 × 10¹⁰ phages/ml of the lysate) was mixed with a lysate of the wild-type λ phage (1 × 10¹⁰ phages/ml) to provide the CI protein because Kohara's λ phage clones lack the CI repressor. For the complementation test, temperature-sensitive mutant bacteria (~2 × 10⁶ bacteria/plate) were seeded on tryptone broth plates with soft agar, and then 2.5 µl of the mixture of phage clones was spotted onto each plate. After incubation at 42 °C overnight, the plate was examined. Next, the second round of the complementation test was performed with a single λ phage clone, chosen from the 10-clone batch. When mutations in the phages were complemented by the corresponding gene carried by the λ phage clone, a growth zone appeared.

*Penicillin Screening*—To enrich the temperature-sensitive mutants, 20 independent cultures of BT370 (lacZam mutT::Cm'), each derived from a single colony, were shaken at 30 °C overnight and refreshed at 30 °C until growth to a logarithmic phase. Then the cultures were transferred to the non-permissive temperature of 42 °C and cultivated for 20 min. For the penicillin screening, ampicillin was added to give a final concentration of 75 μg/ml, and cultivation was continued for 2 h. During the incubation at 42 °C, wild-type bacteria died due to lack of the cell wall synthesis, whereas temperature-sensitive mutants for growth survived. The cultures were diluted and spread onto LB plates containing 250 μg/ml X-gal and 100 μM IPTG. The plates were incubated for 2–3 days at the permissive temperature of 30 °C to allow for the formation of blue-colored mutant colonies (see Fig. 1).

*β-Galactosidase Assay*—An aliquot (0.55 ml) of overnight culture treated with tolune was mixed with the same volume of reaction buffer (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, pH 7.0, 0.01 M KCl, 0.01 M MgSO₄, 0.05 M β-mercaptoethanol) and 0.3 ml of O-nitrophenyl-β-D-galactoside (4 mg/ml). The mixture was kept for various intervals of time at room temperature, and the reaction was terminated by the addition of 0.55 ml of 1 M Na₂CO₃. The mixture was centrifuged to remove cell debris, and the absorbance at 420 nm was measured. The value was multiplied by 1000 to give the β-galactosidase activity.
Accurate Gene Expression under Oxidative Stress

Purification and Assay of the Guanylate Kinase—Guanylate kinase was partially purified from strains BT369 (wild-type) and BT3701 (ts0-2), according to a procedure described previously (19). To extract the enzyme from beads to which the enzyme was bound, 300 μl of elution buffer (10 mM glutathione in 50 mM Tris-HCl, pH 8.0) was applied, and the mixture was kept at room temperature for 10 min. The enzyme preparation was concentrated by a Microcon YM3 kit (Amicon) and was stored in a buffer containing 50 mM Hepes-KOH, pH 7.4, 50 mM KCl, 10% glycerol, and 1 mM DTT.

The reaction was carried out with 5 ng of enzyme preparation and 2.5 mM GMP in 20 μl of a reaction mixture containing 50 mM Hepes-KOH (pH 7.4), 2.5 mM ATP, 40 mM MgCl₂, and 40 mM (NH₄)₂SO₄. After the reaction, GMP and GDP were separated by HPLC using a TSKgel DEAE-25W column (4.6 × 250 mm; Tosho Corp.) in an isocratic solution of 0.12 M sodium phosphate (pH 6.0) containing 40% acetonitrile (19). The amount of GDP produced from GMP was used to express the enzyme activity.

Purification and Assay of RNA Polymerase—Logarithmically growing cells (1 g) were collected and lysed in 10 ml of buffer A (50 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 0.1 mM EDTA, 0.1 mM DTT). The lysate was subjected to sonication for 2 min twice on ice. After centrifugation, the supernatant (10 ml) was mixed with 0.35 ml of 10% Polymen P (Sigma-Aldrich). The resulting precipitate was collected and washed with buffer B (10 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 0.1 mM EDTA, 0.1 mM DTT, 0.5 mM NaCl, 5% glycerol, 1 mM PMSE). RNA polymerase was eluted from the precipitate by mixing with solution B containing a higher concentration of NaCl (1 M) and 50% ammonium sulfate. After the fraction was dissolved in 7 ml of buffer B containing 0.15 M NaCl, Amicon Ultra-15 centrifugal filter devices (Millipore, Billerica, MA) were used to remove the ammonium sulfate. The fraction (7 ml) was mixed with a double-stranded DNA-cellulose (Sigma-Aldrich), and the DNA-cellulose recovered was washed with buffer B containing 0.15 M NaCl. A partially purified RNA polymerase was then obtained by eluting with buffer B containing 0.5 M NaCl, and the preparation was kept in buffer A containing 0.1 M NaCl and 50% glycerol at −20 °C.

32P-Labeled 8-oxo-GTP was prepared and purified using a MonoQ column as described previously (19). An in vitro transcription assay was performed at 30 °C in a reaction mixture containing 10 mM Tris-HCl, pH 7.9, 4 mM MgCl₂, 0.1 mM DTT, 0.4 mM ATP, 0.4 mM UTP, 0.4 mM CTP, 50 μM GTP, and the Bluescript SK DNA was used as a template.

RESULTS

Partial Phenotypic Suppression of the lacZ Amber Mutation by 8-Oxo-Gua—Taddei et al. (16) established the specific role of MutT protein in preventing transcriptional errors caused by 8-oxo-Gua by using the lacZ amber system developed by Cupples and Miller (20). In the present study, we have extended this system to allow for further identification of the genes required for accurate gene expression under oxidative stress. As illustrated in Fig. 1, 8-oxo-Gua may be introduced to a site of the amber codon in messenger RNA, resulting in the formation of a small amount of active β-galactosidase protein in BT370 (lacZam mutT⁻) cells. In the original model (16), it was assumed that the “GAG codon (where “G represents an 8-oxo-Gua-containing nucleotide) thus formed might pair with an anticodon sequence, 3’-CUC-5’, of mRNA, which would code for glutamic acid. Detailed surveys of tRNA genes in the tRNA sequence library (21) revealed that E. coli K12 has no such tRNA carrying the 3’-CUC-5’. Instead, the anticodon sequence of glutamic acid tRNA, 3’-CUU-5’, can pair with the 5’-GAG-3’ because the U at the 5’-position of the anticodon sequence works as a wobble base (22).

In the present study, we introduced a base substitution at nucleotide position 1456 of the lacZ gene to yield an amber codon. As a result of the suppression of the “GAG codon, an active enzyme molecule would carry glutamic acid as the 468th amino acid, whereas the wild-type enzyme possesses glutamine at this site. This would allow for the discrimination of the enzymes formed by the suppression from the wild-type proteins. We confirmed that the protein carrying glutamic acid at position 486 has essentially the same specific activity as the wild-type enzyme (data not shown). If the partial phenotypic suppression observed with BT370 cells was caused by 8-oxo-Gua insertion into the site of the nonsense codon, we suppose that increased levels of 8-oxo-Gua insertion into the messenger RNA and/or higher degrees of stability of such RNA in cells would further enhance the levels of phenotypic suppression, as illustrated in Fig. 1.

The photographs shown in the bottom of Fig. 1 demonstrate the colors of colonies formed on LB plates containing X-gal and IPTG. The CR63 lacZ+ cell cultures yielded dark blue-colored colonies, whereas BT369 lacZam cells produce white colonies. Colonies of BT370 lacZam mutT⁻ cells exhibited a faint blue color, reflecting the fact that a small amount of active β-galactosidase protein was produced. BT370X⁻, a mutant derived from the precipitate by mixing with solution B containing a higher concentration of NaCl (1 M) and 50% ammonium sulfate.
from BT370, which carries an additional mutation in a certain gene X, produces colonies with an increased blue color. Based on these color changes, we can detect mutants having mutations in genes that are required for controlling the gene expression under oxidative stress.

**Isolation and Characterization of Mutants Exhibiting High Levels of Phenotypic Suppression under Oxidative Stress**—Fig. 2A shows the protocol used for the isolation of such mutants from strain BT370 lacZam mutT::Cm<sup>T</sup>. The BT370 cells were subjected to penicillin screening at 42 °C for 120 min and then plated on LB containing 250 μg/ml of X-gal and 100 μM IPTG. After incubation at 30 °C for 48 h, the plates were examined, and colonies with a darker blue color were selected as candidates containing such mutant clones. Fig. 2B shows the appearance of the colonies on the plate, with the mutant clone indicated by an arrow. We adopted this isolation procedure, assuming that mutations may arise in genes essential for growth. Such naturally arising mutants may be concentrated during growth at 42 °C in the presence of ampicillin, whereas wild-type cells as well as mutants having mutations in non-essential genes may not survive.

The mutants thus isolated were subjected to the quantitative determination of the β-galactosidase activity, the results of which are summarized in Fig. 2C. When cells were grown under aerobic conditions, the mutant clones showed increased levels of β-galactosidase activity, which were significantly higher than those of the parental strain, BT370 lacZam mutT<sup>T</sup>. On the other hand, the BT369 lacZam cells yielded only a small level of activity. When an excess amount of MutT protein was supplied by introducing a mutT<sup>T</sup> plasmid into BT370X<sup>T</sup> cells, these high levels of expression of the lacZ gene were almost completely suppressed, which would support the notion that the phenotypic suppression is caused by 8-oxo-Gua. Additional support for this notion comes from the finding that these increased levels of β-galactosidase activity diminished during the growth of the cells under anaerobic conditions, where no oxidation of guanine is expected to occur. No appreciable amount of active β-galactosidase was detected in any of the BT370X<sup>T</sup> cells carrying mutations in various genes when the cells were grown under anaerobic conditions.

By using this procedure, we isolated 77 mutant clones from 20 independently grown cultures. Among them, 48 clones exhibited distinct temperature sensitivity for growth; these cells can produce colonies at 30 °C but not at 42 °C. To identify the genes in which the mutations had arisen, we performed the complementation analyses using the library of Kohara’s λ clones. The library consists of 476 clones, each of which carries a part of a defined region of the *E. coli* chromosome (18). We performed complementation tests by using a mixture of 10 λ clones that covered adjacent chromosome regions. When a positive result was obtained, we proceeded to the next round of complementation testing, in which each of the single clones derived from the original set of 10 clones was used for the analysis. In this way, we could identify the chromosomal region where the respective mutations existed.

We selected mutants that showed relatively high levels of β-galactosidase activity and further proceeded to the identification of the responsible gene mutations. Based on the *E. coli*
gene map (23), we were able to identify genes that were located in an affected region and made a sequence determination for each of the genes. The sequences of wild-type and mutant clones were compared, and the mutation that had arisen in the mutant was finally identified. As a result, it was revealed that mutations in five genes caused increased levels of phenotypic suppression, as measured by the $\beta$-galactosidase activity. The sites and the types of mutations and the resulting amino acid substitutions present in the corresponding protein products are summarized in Table 1. In this table, the results of the rpoB mutation, obtained by a different analysis, are also included.

**A Mutation in the Gene Encoding Guanylate Kinase**—A more detailed analysis of the ts0-2 mutant was then performed. The temperature-sensitive growth characteristic of strain ts0-2 was complemented by two $\lambda$ clones, 571 and 572. As illustrated in Fig. 3A, there are four known genes in this region (24). To identify the mutated gene, we performed DNA sequencing of all of these genes. As a result, a mutation was found in the gmk gene, which codes for guanylate kinase. A single base substitution from C to T at position 119 probably caused an amino acid change from threonine to isoleucine at the 40th position. There were no alterations in the DNA sequences of the other three genes.

It was demonstrated that the *E. coli* guanylate kinase, which catalyzes the conversion of GMP to GDP, is unable to phosphorylate 8-oxo-GMP, thus preventing utilization of oxidized guanine nucleotides for nucleic acid synthesis (19). Because there is a possibility that the mutated enzyme of ts0-2 might acquire the capacity to phosphorylate 8-oxo-GMP, we purified the guanylate kinase enzyme from wild-type and ts0-2 cells for comparison.

**TABLE 1**

Mutations arising in genes involved in enhanced phenotypic suppression of lacZ amber

| Strain | Mutant | Gene | Phage clone(s) complemented | $\beta$-Galactosidase activity | Mutation | DNA | Protein |
|--------|--------|------|-----------------------------|-------------------------------|----------|-----|---------|
| BT3701 | ts0-2  | gmk  | 571, 572                    | 17.6                          | C119T    | G119T| Thr40Ile |
| BT3704 | ts0-42 | rpoC | 533                         | 8.4                           | G2699A   | G2699A| Gly900Asp|
| BT3706 | ts0-45 | rpoC | 533                         | 15.5                          | G2699A   | G2699A| Gly900Asp|
| BT3707 | ts1-2  | dnaB | 636                         | 13.6                          | G82A     | G82A | Asp28Asn |
| BT3714 | ts2-19 | msbA | 217                         | 6.6                           | G1135A   | G1135A| Val379Met |
| BT3722 | ts4-5  | rpoC | 533                         | 9.9                           | G808A    | G808A| Ala270Thr |
| BT3728 | ts4-18 | dnaN | 565                         | 10.6                          | G3760A   | G3760A| Glu1254Lys |
| BT3750 | Rd-3   | rpoB | 9.9                         | 15.0                          | G470A    | G470A| Gly157Asp |
|        |        |      |                              |                               | A1538C   | A1538C| Gln513Pro |

**FIGURE 3.** A mutant with a base substitution in the gene encoding guanylate kinase. A, the results of an analysis of the mutation by complementation and DNA sequencing. The top black bar represents a part of the *E. coli* chromosome, above which the nucleotide number is shown by kilobase. The number in parenthesis is the genetic site, which was based on the report by Berlyn et al. (23). Kohara’s $\lambda$ phage clones used for the analysis are shown by dark gray lines. In the area shown in yellow, the following four genes are present: ligB, coding for NAD$^+$-dependent DNA ligase; gmk, coding for guanylate kinase; rpoZ, encoding the RNA polymerase subunit; and spoT, encoding (p)ppGpp synthetase. By DNA sequencing, a mutation was found in the gmk gene (red bar), whereas no apparent mutations were detected in the other three genes, shown in light blue. The amino acid alteration predicted from the DNA sequence is shown with red letters. B, the guanylate kinase activity in the partially purified preparations derived from wild-type (●) and ts0-2 mutant (○) bacteria. The assay was conducted at both 42 and 30 °C.
son purposes. The reactions with the enzymes were carried out at pH 7.4 in the presence of ATP and Mg$^{2+}$, and the reaction products were analyzed by HPLC. As shown in Fig. 3B, the mutant enzyme exhibited no or little activity at both 30 and 42 °C, whereas the wild-type enzyme showed a high level of activity at both temperatures. Although we performed the assay at lower temperatures (e.g., at 22 °C), only a little activity was detected in the ts0-2 enzyme preparation. This confirmed that the mutation had arisen in a critical region of the enzyme, although the substrate specificity of the mutant enzyme was still unclear.

Mutations in the rpoC Gene Encoding the β′ Subunit of RNA Polymerase—The temperature-sensitive growth of three mutants, ts0-42, ts0-45, and ts4-5, was suppressed by infection with phage clone 533 but not with 532. As shown in Fig. 3A, nine genes, including rpoC, were present in an area covered by λ clone 533 but not 532, as shown in yellow. B, SDS-PAGE of the partially purified preparations of RNA polymerases from BT370 (shown as wild type), BT3706 (rpoC ts0-45), and BT3750 (rpoB Rif$^{	ext{3}}$). SDS-polyacrylamide gels were stained with silver, and bands for subunit proteins are shown by arrowheads. C, the results of the Western blot analyses of the β′ subunit. Partially purified preparations of RNA polymerase were used for the Western blotting analysis using an anti-RNA polymerase β′ prime antibody (Santa Cruz Biotechnology, Inc.). The numbers indicate the relative quantity of the β′ subunit. D, incorporation of $^{32}$P-labeled GTP and 8-oxo-GTP into RNA by RNA polymerase preparations derived from various bacterial strains. Partially purified preparations derived from the strains described above were used. The radioactivity incorporated into the RNA was measured, and the values for GTP and 8-oxo-GTP are shown by green and red bars, respectively. The ratios of 8-oxo-G/G are shown above the red bars.

FIGURE 4. Mutants with mutations in the rpoC gene coding for the β′ subunit of RNA polymerase. A, characterization of the mutations. The procedures were essentially the same as those described in the legend to Fig. 3A. Nine genes, including rpoC, were present in an area covered by λ clone 533 but not 532, as shown in yellow. B, SDS-PAGE of the partially purified preparations of RNA polymerases from BT370 (shown as wild type), BT3706 (rpoC ts0-45), and BT3750 (rpoB Rif$^{	ext{3}}$). SDS-polyacrylamide gels were stained with silver, and bands for subunit proteins are shown by arrowheads. C, the results of the Western blot analyses of the β′ subunit. Partially purified preparations of RNA polymerase were used for the Western blotting analysis using an anti-RNA polymerase β′ prime antibody (Santa Cruz Biotechnology, Inc.). The numbers indicate the relative quantity of the β′ subunit. D, incorporation of $^{32}$P-labeled GTP and 8-oxo-GTP into RNA by RNA polymerase preparations derived from various bacterial strains. Partially purified preparations derived from the strains described above were used. The radioactivity incorporated into the RNA was measured, and the values for GTP and 8-oxo-GTP are shown by green and red bars, respectively. The ratios of 8-oxo-G/G are shown above the red bars.
**Analyses of rpoB Mutants**—It is known that the β and the β′ subunits of bacterial RNA polymerase are important for the recognition and loading of substrate nucleotides (25, 26). Although rpoC mutants having amino acid substitutions in the β′ subunit were obtained, no mutant with a mutation in the rpoB gene that encodes the β′ subunit was found in our mutant collection. It has been shown that some mutations in the rpoB gene of *E. coli* cause increased resistance to an antibiotic, rifampicin (27–29). We thus selected rifampicin-resistant (Rifr) mutants from BT370 cells and examined their abilities to suppress the lacZ amber mutation according to the protocol shown in Fig. 5A. In this way, we were able to isolate three independent Rifr mutants exhibiting high levels of phenotypic suppression, as based on the β′-galactosidase activity under aerobic conditions. The assay was performed as described in the legend to Fig. 2C. D, β′-galactosidase activity under anaerobic conditions.

**Mutations in Genes Related to DNA Replication and Membrane Structure**—We have extended our analyses to different categories of mutants that have mutations in genes related to DNA replication and membrane structure. These include ts1-2, ts4-18, and ts2-19, mutations of which were found in the dnaB, dnaN, and msbA genes, respectively. As summarized in Fig. 6, each of these mutants carries a single base substitution in the respective gene sequence. It has been shown that the dnaB gene codes for replicative DNA helicase (30), whereas the dnaN gene encodes the β′ subunit of DNA polymerase III (31, 32). On the other hand, the msbA codes for ATP-binding transport protein, which is mainly located in the inner part of the cell membrane (33, 34). It is uncertain, however, how these protein elements function to regulate gene expression specifically under oxidative stress.

To address this topic, we examined the processes of RNA and DNA syntheses in these mutants. Fig. 7 shows the result of these analyses, in which RNA and DNA syntheses were followed by the incorporation of [3H]uridine and [3H]thymidine into the acid-insoluble fraction, respectively. As expected, the ts1-2 and ts4-18 mutants with mutations in the dnaB and dnaN genes showed decreased levels of DNA synthesis at 42 °C, whereas the ts0-45 mutant with a rpoC mutation exhibited a somewhat

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**FIGURE 5. Isolation and characterization of cells with a mutation in the rpoB gene.** A, the isolation procedure. BT370 cells were plated on rifampicin-containing X-gal- IPTG plates, and light blue-colored colonies were isolated for examination. Three mutants, Rifr-2, Rifr-3, Rifr-4, were derived from independently grown cultures. B, the DNA sequence of the rpoB gene derived from wild-type and Rifr-3 cells. The results obtained with Rifr-2 and Rifr-4 were essentially the same. C, β′-galactosidase activity under aerobic conditions. The assay was performed as described in the legend to Fig. 2C. D, β′-galactosidase activity under anaerobic conditions.
decreased level of RNA synthesis. With ts2-19, which has an alteration in the membrane structure, no significant effect of temperature was observed with regard to both the RNA and DNA syntheses. It can be inferred from these findings that those mutations may affect some process of gene expression through interactions with other proteins.

DISCUSSION

Errors in RNA synthesis can cause the accumulation of abnormal proteins, and a number of cellular functions are involved in preventing such outcomes. In aerobically growing cells, in which reactive oxygen species are produced as side products of oxygen utilization, the guanine in nucleic acids and also in the precursor nucleotides can be converted to the oxidized form, 8-oxo-Gua. Because 8-oxo-Gua can pair with both adenine and cytosine, the misincorporation of 8-oxo-Gua into RNA would cause alterations in gene expression. As the first line of defense against such outcomes, the MutT protein degrades 8-oxo-Gua-containing ribonucleotides, 8-oxo-GTP and 8-oxo-GDP, to 8-oxo-GMP, thereby preventing the misincorporation of 8-oxo-Gua into RNA (16, 14). Following this MutT-driven process, many other mechanisms would work to promote and ensure accurate gene expression. These include (a) preventing the utilization of 8-oxo-GMP, formed by the action of the MutT as well as by oxidation of GMP, (b) reducing the rate of misincorporation of 8-oxo-Gua into RNA, and (c) preventing 8-oxo-Gua-containing RNA from entering into the translation process. The elucidation of these processes is important for improving our understanding of the cellular mechanisms responsible for accurate gene expression under oxidative stress, and the present study was designed and executed to achieve this goal.

In the present study, we have searched for genes whose mutations would cause enhanced degrees of 8-oxo-Gua-related phenotypic suppression under the conditions where the MutT protein was dysfunctional. With the aid of this selection procedure, we were able to isolate candidate genes from the whole E. coli gene library and further identify the responsible mutations via

FIGURE 6. Analysis of mutant having mutations in the other genes. The procedure used was the same as those described for Fig. 3. A, analysis of the ts1-2 mutant, which carries a mutation at position 1135 of the dnaB gene. B, analysis of ts4-18, which carries a mutation at position 470 of the dnaN gene. C, analysis of ts2-19, which carries a mutation at 808 position of the msbA gene.
sequencing. The genes identified in this way included gmk and rpoC, which are known to be related to the above-mentioned mechanisms a and b, respectively. We further found that some Rif mutants, which have base substitutions in the rpoB gene, can also be characterized as those involved in category b. Mutants belonging to category c were not found in the present study, perhaps due to our isolation procedure, in which prospective temperature-sensitive mutants were concentrated by penicillin screening. Mutants belonging to category c, which might grow normally at 42 °C, cannot be isolated by this procedure. It should be kept in mind that E. coli mutants deficient in the PNP protein, which binds specifically to RNA carrying 8-oxo-Gua, exhibit normal growth even at elevated temperatures (35). Therefore, it will be necessary to develop a procedure that allows for the systematic isolation and characterization of such types of mutants.

In the biosynthetic pathway of guanine-containing ribonucleotides, GMP is formed first and is then phosphorylated to GDP and further to GTP (Fig. 8). The enzyme responsible for the first step is GMP synthase, which catalyzes the ATP-driven amination of xanthylate (36). The GMP thus formed is phosphorylated to GDP by the action of guanylate kinase (37). This enzyme has a strict substrate specificity and is unable to phosphorylate 8-oxo-GMP, an oxidized form of GMP (19). In this way, the reutilization of 8-oxo-GMP, formed by the action of the MutT protein, is prevented, and thus, the guanylate kinase can act as one of the gatekeepers against oxidative stress. In the present study, it was found that a specific mutation that had arisen in gmk, the structural gene encoding the guanylate kinase enzyme, caused an increased level of phenotypic suppression of the amber codon in the tester lacZ gene. Because the assay was performed in the absence of MutT, it is possible that the mutation might alter the ability of cells to utilize 8-oxo-GMP for RNA synthesis. This hypothesis, however, cannot be adequately tested in the present study, because the mutant enzyme is too unstable to assay in vitro. It has been pointed out that the threonine to isoleucine substitution found in the mutant enzyme is located very close to the substrate binding site (see the inset of Fig. 8), and we may need to generate more mutants to establish the discriminative role of guanylate kinase against 8-oxo-Gua.

In the cellular nucleotide pool, 8-oxo-GDP may be produced by the oxidation of GDP by reactive oxygen species. The 8-oxo-GDP thus formed can be converted to 8-oxo-GTP by nucleoside diphosphate kinase and adenylate kinase, both of which catalyze the conversion of GDP to GTP and do not discriminate between 8-oxo-GDP and normal GDP (19). The 8-oxo-GTP
produced in this way and also by the oxidation of GTP can be used for RNA synthesis (see Fig. 8). This leads to questions about how efficiently the RNA polymerase enzyme can discriminate 8-oxo-GTP from normal GTP. If certain amino acid substitutions occur in the substrate recognition binding sites of RNA polymerase, such mutants might exhibit altered levels of read-through at the 8-oxo-Gua-containing codon of messenger RNA. It is noteworthy that in our collection of mutants exhibiting enhanced levels of phenotypic suppression, some carried mutations in the rpoC gene and also in the rpoB gene.

It has been shown previously that the RNA polymerase core enzyme of E. coli is composed of two α subunits and one each of the β, β′, and ω subunits, with the β and the β′, encoded by the rpoB and the rpoC genes, respectively, possessing the substrate binding sites (25). Because the rpoC mutants isolated in this study exhibited temperature-sensitive growth, the amino acid residues that were altered in the mutants might play essential roles in the catalytic activity. Indeed, the amino acid residues altered in the mutants, Asp278, Gly900, and Glu1254, are located in an evolutionarily conserved region (38). Among them, Gly900 is located in the trigger loop (Lys911–Phe1145), which plays a vital role in nucleotide binding and elongation (26, 39). It is possible that a substitution of Gly900 might influence the movement of the trigger loop and allow RNA polymerase to load an incorrect ribonucleotide onto the template, which could result in a lower fidelity of transcription. As shown in the inset of Fig. 8, Glu1254 is located close to Gly900, which raises the possibility that Gly900 works together with Glu1254 to ensure correct transcription.

It is tempting to speculate that mutations in the rpoB gene could also show a similar read-through phenotype, because the β and β′ subunits of RNA polymerase provide a place for substrate loading and work together to synthesize RNA by reading the transcriptional strands of DNA. As shown in Fig. 8, the identified mutation of rpoB turned out to be at Gln513, which is located in the channel near the active center for RNA synthesis. It is possible that Gln513 plays a role in monitoring the correct interactions between the template DNA and ribonucleoside triphosphates. Because the same single mutation was found in the three independently isolated mutants, Gln513 may play a critical role in ensuring accurate transcription. It was recently reported that some of RifR mutants, whose mutations are associated with the β subunit of RNA polymerase, showed transcriptional slippage during elongation (29). The Gln513 mutation belongs to this class, implying that the increased level of phenotypic suppression observed might be caused by slippage. Taken together, the read-through mutants of the rpoB and rpoC gene might influence the mode of incorporation of 8-oxo-Gua into RNA through protein conformational changes. To address this possibility, we have examined the abilities of RNA polymerase enzymes derived from the wild type and various rpoC and rpoB mutants to utilize 8-oxo-GTP as a substrate. However, no significant differences were observed in these strains.

The mechanisms underlying the transcriptional fidelity revealed here with E. coli may be principally applicable to those for higher organisms, although the mammalian systems are more complex than those found in bacteria. For instance, human cells possess at least four distinct MutT-related enzymes, each exhibiting different substrate specificities toward 8-oxo-Gua-containing nucleotides (40). Human MTH1 and MTH2 preferentially degrade the 8-oxo-Gua-containing nucleotide triphosphate, 8-oxo-dGTP, whereas MTH3 and NUDT5 are specifically active on its diphosphate counterpart, 8-oxo-dGDP. Unlike the E. coli MutT, which is capable of degrading 8-oxo-Gua-containing ribonucleotides as efficiently as their deoxyribonucleotide counterparts (14), MTH1 and MTH2 are less active on 8-oxo-Gua-containing ribonucleotides. On the other hand, MTH3 degrades 8-oxo-GDP and 8-oxo-dGDP with almost the same efficiency. In the case of guanylate kinase, the situation is much simpler because only one molecular species of guanylate kinase exists in both E. coli and in mammalian cells. The molecular sizes and the gross structures of the enzymes from prokaryotes and eukaryotes are essentially similar, and the critical amino acid substitution site found in the E. coli enzyme in the present study is also located near the active site of the mammalian enzyme (41, 42). For the RNA polymerase, mammalian cells possess three distinct classes of enzymes, RNA polymerase I, II, and III (25, 43). Among them, RNA polymerase II, which is responsible for the synthesis of most of messenger RNAs, is structurally similar to the E. coli RNA polymerase. These enzymes are composed of two α subunits and one each of the β, β′, and ω subunits, with the β and the β′ subunits possessing the substrate binding sites. When the mutation sites revealed with the E. coli enzyme are superimposed on the structure of the mammalian enzyme, these sites are placed in critical regions of the mammalian enzyme. Therefore, although there are many differences in the architecture of prokaryotic and eukaryotic cells, the findings obtained with E. coli may provide useful hints to understand the response to oxidative stress in mammalian cells.

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