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Multiprobe RNase Protection Assay Analysis of mRNA Levels for the *Escherichia coli* Oxidative DNA Glycosylase Genes under Conditions of Oxidative Stress

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***Escherichia coli* formamidopyrimidine DNA glycosylase (Fpg), MutY DNA glycosylase, endonuclease VIII, and endonuclease III are oxidative base excision repair DNA glycosylases that remove oxidized bases from DNA, or an incorrect base paired with an oxidized base in the case of MutY. Since genes encoding other base excision repair proteins have been shown to be part of adaptive responses in *E. coli*, we wanted to determine whether the oxidative DNA glycosylase genes are induced in response to conditions that cause the type of damage their encoded proteins remove. The genes *fpg*, *mutY*, *nei*, and *nth* encode Fpg, MutY, endonuclease VIII, and endonuclease III, respectively. Multiprobe RNase protection assays were used to examine the transcript levels of these genes under conditions that induce the SoxRS, OxyR, and SOS regulons after a shift from anaerobic to aerobic growth and at different stages along the growth curve. Transcript levels for all four genes decreased as cells progressed from log-phase growth to stationary phase and increased after cells were shifted from anaerobic to aerobic growth. None of the genes were induced by hydrogen peroxide, paraquat, X rays, or conditions that induce the SOS response.**

In *Escherichia coli*, as in other prokaryotes and eukaryotes, a form of DNA repair called base excision repair removes oxidatively damaged bases from DNA (for reviews see references 14 and 60). Oxidatively damaged bases result from attack by oxygen free radicals generated during normal oxidative metabolism and by exposure to exogenous agents such as X rays and redox-generating chemicals. Base excision repair proteins called DNA glycosylases hydrolyze the N-glycosylic bond between the damaged or incorrect base and the sugar, leaving an abasic site or a strand break, depending on the type of glycosylase, which is then acted on by other proteins to complete the repair process.

Formamidopyrimidine DNA glycosylase (Fpg) and MutY DNA glycosylase work together to protect cells from the mutagenic effects of the common oxidative damage 7,8-dihydro-8-oxoguanine (8-oxoG) (41). Fpg removes 8-oxoG from 8-oxoG-C pairs, giving the repair DNA polymerase a chance to put in G (10, 58). If 8-oxoG is not removed before DNA replication occurs, it can mispair with A. MutY removes A in 8-oxoG-A mispairs (41, 42). Failure of this process results in a GC → TA transversion. The DNA glycosylases endonuclease III (endo III) and endo VIII have overlapping substrate specificities and recognize and remove a wide range of oxidized pyrimidines. Some of these oxidized pyrimidines, such as thymine glycol, act as blocks to DNA polymerase and are lethal to cells (34, 44); oxidized cytosines such as uracil glycol, 5-hydroxyuracil, and 5-hydroxycytosine pair with A and are premutagenic, leading to GC→AT transitions (32, 48, 49).

Using reverse transcription-PCR, we have previously shown that all four oxidative DNA glycosylase genes are transcribed as part of operons (18, 19) and have determined transcription initiation and termination sites by RNase protection and primer extension. *fpg* is the terminal gene in an operon with the gene order *radC*, *rpmB*, *rpmG*, and *fpg* (19). RadC has been suggested to play a role in growth medium-dependent, *recA*-dependent repair of DNA single-strand breaks after X-irradiation and in postreplication repair after UV irradiation (17). *rpmB* and *rpmG* encode the ribosomal proteins L28 and L33, respectively (36). This operon has transcription initiation sites upstream of *radC*, in the *radC* coding region, and immediately upstream of *fpg*. There is a strong attenuator in the *rpmG-fpg* intergenic region and three transcription termination sites downstream of *fpg*. There is an additional site in the *radC-rpmB* intergenic region that corresponds either to a transcription initiation site or to an RNase E or RNase III cleavage site. *mutY* (MutY) is the first gene in an operon with the gene order *mutY*, *yggX*, *mltC*, and *nupG* (19). *yggX* encodes a protein of unknown function; *mltC* encodes membrane-bound lytic transglycosylase C, which has been shown to have peptidoglycan hydrolase activity (15); and *nupG* encodes a high-affinity nucleoside transport protein (64). This operon has transcription initiation sites upstream of *mutY*, in the *mutY* coding region, and immediately upstream of *nupG*. There also appear to be attenuators in the *yggX-mltC* and *mltC-nupG* intergenic regions. *nth* (endo III) is the terminal gene in an operon with seven open reading frames that encode proteins of unknown function (18). The six open reading frames immediately upstream of *nth* show homology to the genes *rnfA*, *rnfB*, *rnfC*, *rnfD*, *rnfG*, and *rnfE* from *Rhodobacter capsulatus*. The *rnf* genes are required for nitrogen fixation in *R. capsulatus* and have been predicted to make up a membrane complex involved in electron transport to nitrogenase (53). The *nth* operon has transcription initiation sites upstream of the first and second open reading frames and a single transcript termination site downstream of *nth*. *nei* (endo VIII) is the terminal gene in an operon with four open reading frames that encode proteins of unknown function (18). This operon has two confirmed tran-

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scription initiation sites upstream of the first open reading frame and two transcript termination sites downstream of *nei*.

When cells are exposed to low doses of a toxic agent, they often become less sensitive to the effects of subsequent higher doses. Adaptive responses were first observed in bacteria and have since been observed in yeast, plants, and mammals (11). Two regulons, the SoxRS regulon and the OxyR regulon, enable *E. coli* to adapt to oxidative stress (1, 13, 47). The SoxRS regulon is turned on in response to $O_2^{\cdot -}$ and induces the expression of proteins specific for removing $O_2^{\cdot -}$ from the cell and minimizing the damaging effects of $O_2^{\cdot -}$ (1, 47). The OxyR regulon is turned on in response to H_2O_2 and induces proteins specific for removing H_2O_2 from the cell and minimizing the damaging effects of the presence of H_2O_2 (9, 45).

There have been few studies on the regulation of the oxidative DNA glycosylases in *E. coli*. It has been shown that cells exhibit increased Fpg enzyme activity when shifted from anaerobic to aerobic growth conditions and when exposed to the $O_2^{\cdot -}$-generating compound paraquat (31, 35). This response still occurs in mutants defective in SoxR and SoxS, demonstrating that *fpg* (Fpg) is not part of the SoxRS regulon (31, 35). It has also been shown that, under anaerobic growth conditions, Fpg enzyme activity increases in strains deficient in the global regulators Fur, Fnr, and ArcA (35). Possible Fur, Fnr, and ArcA binding sites have been identified in the *fpg* promoter region, suggesting that these proteins may play a negative regulatory role in *fpg* regulation. There have been no reported studies on MutY regulation. There is not observed increase in endo VIII enzyme levels after the administration of H_2O_2, paraquat, or agents that induce the SOS response or in *oxyR* or *soxR* mutants constitutive for the H_2O_2- and $O_2^{\cdot -}$-inducible responses, respectively (40). There have been no reported studies on endo III regulation.

In this study we wanted to determine whether *fpg*, *mutY*, *nth*, and *nei* are induced as part of an adaptive response to oxidative stress in *E. coli*. We also investigated whether these genes are part of the stationary-phase regulon, which controls the induction of several genes involved in protection against oxidative stress (33, 39), and whether they are induced after a shift from anaerobic to aerobic growth. Our results indicate that *fpg*, *mutY*, *nth*, and *nei* transcript levels decrease as cells progress from log-phase growth to stationary phase and increase after cells are shifted from anaerobic growth to aerobic growth. These genes do not appear to be induced by H_2O_2, paraquat, or X rays, nor are they induced as part of the SOS response.

MATERIALS AND METHODS

Bacterial strains. *E. coli* GC4468 [DE(*argF-lac*)*169* λ⁻ IN(*rrnD-rrnE*)*1 rpsL179*(*strR*)], KL16 (λ⁻ *relA1 spoT1 thi-1*), and KL16-99 (KL16 *recA1*) were obtained from the Yale University *E. coli* Genetic Stock Center. Strain DJ901 (GC4468 Δ*soxRS901*) was kindly supplied by Bruce Demple, Harvard School of Public Health. Strain GC122 (GC4468 *rpoS13*::Tn*10*) was kindly supplied by Herb Schellhorn, McMaster University. Strains QC1732 (GC4468 Δ*fur*::*kan*), QC2085 (GC4468 Δ*arc*::*tet*), and QC2086 (GC4468 Δ*fnr zdc-235*::Tn*9*) were kindly supplied by Danièle Touati, University of Paris. Strain BW402 (KL16 *nth-1*::*kan*) was kindly supplied by Bernard Weiss, Emory University. Strain CSH11 (KL16 *mutY*::mini-*tet*) was kindly supplied by Jeffrey Miller, University of California, Los Angeles. Strains SW2-8 (KL16 *nei*::*cm*), SW2-F (KL16 *fpg*::*amp*), and SW2-F8 (KL16 *fpg*::*amp nei*::*cm*) were made in this laboratory as previously described (3). Strains UC574 (*arg56 nad113 ara81*) and UC1247 (UC574 *oxyR*::*kan*) were kindly provided by Carmen Pueyo, University of Cordoba.

Growth conditions and RNA isolation. *E. coli* cultures (1 ml) were grown overnight in Luria-Bertani (LB) broth with shaking at 250 rpm. The overnight cultures were diluted 1/100 in fresh LB broth and were grown until they reached the desired optical density at 600 nm (OD_{600}). Anaerobic cultures were grown in a Forma Scientific anaerobic chamber with 10% hydrogen, 5% carbon dioxide, and 85% nitrogen. LB broth was equilibrated in the anaerobic chamber for 2 days before use, and the colonies used in overnight cultures were streaked and grown in the anaerobic chamber. Aliquots of cells were taken at different times after exposure to chemical agents or the desired growth conditions (see figure legends), and the cells were spun down and snap frozen in liquid nitrogen. The cell pellets were stored at −70°C until the RNA was isolated. Cells were grown overnight in the presence of the appropriate antibiotic, with the exception of QC1732, QC2085, and QC2086, which were plated in the presence of the appropriate antibiotic but which were grown in LB broth without selection. Total RNA was isolated with a Qiagen RNeasy kit according to the manufacturer's recommendations. After elution from the RNeasy column, the RNA was treated with DNase, extracted twice with acid pH phenol, and extracted once with chloroform-isoamyl alcohol. The RNA was precipitated with ammonium acetate and ethanol, washed in 75% ethanol, and resuspended in RNase-free water.

RPAs. RNase protection assays (RPAs) were performed with an Ambion RPA II kit. RNA antisense probes were transcribed with a template containing a T7 phase promoter. The antisense probe template was prepared by PCR with genomic DNA as the template and primer sets with the T7 phage promoter incorporated into the downstream primer. PCR was performed with 50-μl reaction mixtures containing Stratagene *Pfu* DNA polymerase and Idaho Technologies 1× buffer with 3 mM $MgCl_2$ and 200 μM concentrations of each deoxynucleoside triphosphate on an Idaho Technologies Air Thermo-Cycler. PCR products were analyzed on a 1% agarose gel; then, the products were cut out, eluted in water, dried under vacuum with centrifugation, and resuspended in 20 μl of water. The template was transcribed with 5 U of Ambion T7 RNA polymerase in a reaction mixture containing Ambion 1× transcription buffer, 1 μl of template, 500 μM ATP, 500 μM CTP, 500 μM GTP, 12.5 μM [α-^{32}P]UTP (800 Ci/mmol; 40 mCi/ml), and water in a final reaction volume of 5 μl. The reaction mixture was incubated at 37°C for 45 min and then run on a 5% polyacrylamide gel to purify the probe. The sizes of the RNA probes were staggered so they could be distinguished from each other in multiprobe RPAs (Fig. 1). *E. coli* RNA (10 μg) was hybridized overnight with the labeled RNA probes (25,000 cpm of each probe used in the assay) at 47°C in hybridization buffer. Unhybridized probe was digested with 0.5 U of RNase A and 20 U of RNase T1 in digestion buffer, the RNases were inactivated, and the remaining RNA was precipitated. The pellet was resuspended in formamide gel loading buffer, and the sample was run on a 5% polyacrylamide gel. The intensity of the protected products was quantitated by phosphorimager analysis, and the results are reported as counts normalized for the number of U residues in the protected product.

RESULTS

Multiprobe RPAs were performed to measure the transcript levels of *fpg*, *mutY*, *nth*, *nei*, and the appropriate control genes, under various conditions. The RNA antisense probes for the genes of interest were designed to be different lengths so the protected products could be resolved when run on a 5% polyacrylamide gel. Each probe was designed to anneal starting at the A of the AUG start site for each RNA transcript, and the lengths of the full-length probes are shown in Fig. 1. Each probe (25,000 cpm) was hybridized overnight with 10 μg of *E. coli* RNA, and the unhybridized probe was digested with RNase A-RNase T1. The RNA antisense probes have 16 bases that will not hybridize to the transcript, so the protected product is 16 bases shorter than the full-length probe. The probes were transcribed in reactions with [α-^{32}P]UTP, so the amounts of protected product were normalized for the number of U residues before the levels of transcript for different genes were compared to each other. Figure 1 shows the number of U residues in each protected product.

Transcript levels of the oxidative DNA repair glycosylase genes decrease as cells progress from logarithmic to stationary phase. Aliquots of *E. coli* GC4468 were removed when cell cultures reached OD_{600}s of 0.2, 0.4, 1.0, 1.65, and 1.78. On the growth curve for GC4468, OD_{600} readings of 0.2 and 0.4 are found during logarithmic growth; an OD reading of 1.0 is reached in late log or early stationary phase, and OD_{600} readings of 1.65 and 1.78 occur during stationary phase. The transcript levels for *fpg*, *mutY*, *nth*, *nei*, and *katE* were measured at the different OD readings. *katE* encodes hydroperoxidase II and is part of the stationary-phase regulon which is under the control of the alternative sigma factor σ^S encoded by *rpoS* (46, 52). Genes that are part of this regulon are upregulated as cells enter stationary phase. As expected, the *katE* transcript level increased at an OD of 1.0 and reached a maximal level of 38-fold induction at an OD of 1.65 (Fig. 2). The transcript levels for *fpg*, *mutY*, and *nth* were highest at an OD of 0.2 and

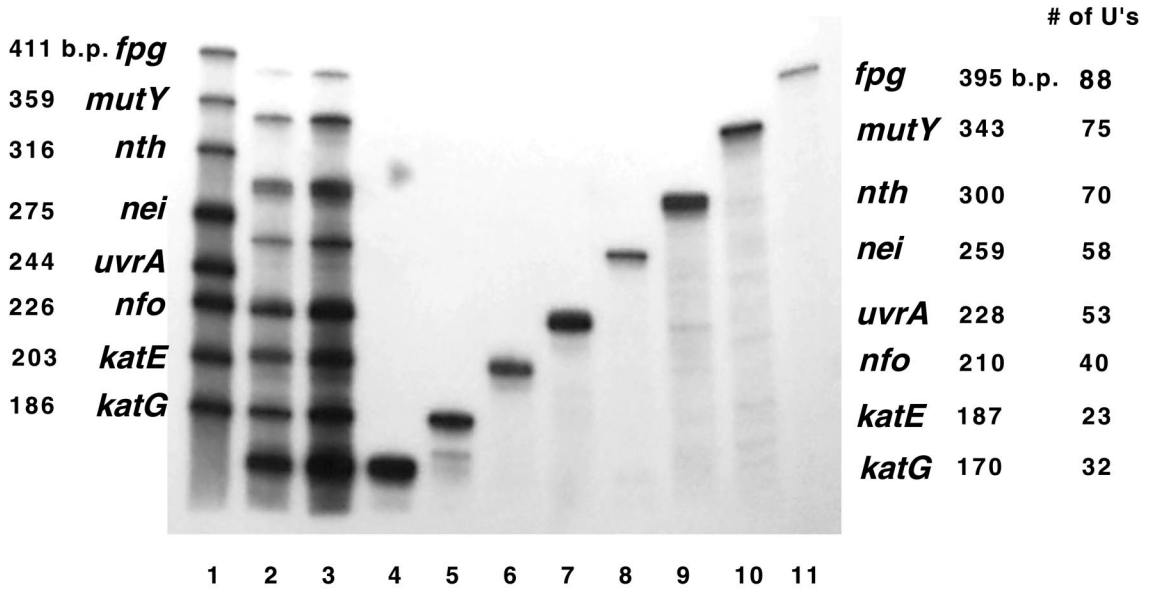


FIG. 1. Full-length antisense RNA probes and protected products from multiprobe RPAs. Lane 1, full-length probes (to the left of lane 1 are the name of the gene corresponding to each probe and size of each probe); lanes 2 and 3, protected products after overnight hybridization of 10 μg (lane 2) and 20 μg (lane 3) of *E. coli* RNA with 25,000 cpm of each full-length probe and then digestion with RNase A-RNase T1; lanes 4 to 11, protected products resulting from hybridization of 25,000 cpm of each individual probe with 20 μg of RNA. To the right are the name of the gene corresponding to each product, the size, and the number of U residues in each protected product.

decreased at subsequent times. Transcript levels for *nei* remained approximately level up to an OD of 1.0 but decreased when cells entered stationary phase. The decreases in transcript levels from the initial to the final measurement for *fpg*, *mutY*, *nth*, and *nei* were 6-, 10.4-, 10.3-, and 4.4-fold, respectively. At an OD of 0.2, and normalized for the number of U residues, the levels of *mutY* and *nth* transcripts were the highest and were approximately the same; the levels of *fpg* and *nei* transcripts were 4- and 3.3-fold lower, respectively. RNA probes for *uvrA*, *nfo*, and *katG* were also included in the experiment (they are positive controls for other conditions). The *uvrA* transcript level remained approximately the same until late log phase before increasing a total of 2.3-fold in stationary phase. The *nfo* transcript level remained approximately the same until late log phase before increasing a total of 1.4-fold in stationary phase. A twofold increase in *katG* transcripts was observed up to an OD of 1.65 before the level started to decrease. To determine if there was any effect of σ^S on *fpg*, *mutY*, *nth*, and *nei* transcript levels, samples were taken at OD readings of 0.2 and 1.78 from cultures of isogenic wild-type and *rpoS* mutant cells. The transcript levels for *fpg*, *mutY*, *nth*, and *nei* at both stages of growth in the wild-type and mutant strains were approximately the same; however, *katE* levels, which increased 38-fold in wild-type cells, decreased 1.9-fold in the *rpoS* mutant (not shown). These results indicate that the decrease in transcripts seen for *fpg*, *mutY*, *nth*, and *nei* is not the result of repression by a regulated σ^S gene.

fpg is the only gene of the four with a promoter controlling only its own transcription, and we wanted to determine whether the sixfold decrease in the *fpg* transcript seen in stationary phase was due to a decrease in transcription from its own promoter or to upstream regulatory events. RPAs were performed using a probe that anneals to the *fpg* promoter region and RNA from log-phase and stationary-phase cells. The RPA with the *fpg* probe resulted in products of 239, 109, and 94 bp (Fig. 3, lanes 2 and 3). The 239-bp product corresponds to transcript readthrough from the upstream genes (19) and was 13.3-fold more abundant in early log phase than in stationary phase. The 94-bp product corresponds to the transcript terminating at an attenuator between the upstream genes and *fpg* (19) and was present in equal amounts in early log phase and stationary phase, indicating that the amount of attenuation at this site did not shift. The 109-bp product corresponds to a transcript originating at the *fpg* promoter (19) and was present in a 1.4-fold-greater amount in early log phase than in stationary phase. It appears that the decrease in the *fpg* transcript is due only in a small part to decreased transcription from the *fpg* promoter and is primarily due to a decrease in transcript readthrough from the upstream genes.

Transcript levels of *nth* are increased in *fpg* and *fpg nei* mutants during logarithmic growth. Transcript levels for the oxidative DNA glycosylase genes in the wild type and *fpg*, *mutY*, *nth*, *nei*, and *fpg nei* mutants were compared (Fig. 4). All cells were harvested at an OD of 0.5. Levels of *fpg* transcript were relatively equal in the strains tested with the exception of the *fpg* and *fpg nei* mutants, where no transcripts above background levels were observed. The *fpg*, *nei*, and *fpg nei* mutants were all made by insertion-deletion mutations (3), and the *mutY* mutant was made by an insertion in the promoter region. Levels of the *mutY* transcript in the *fpg*, *nth*, *nei*, and *fpg nei* mutants were slightly elevated compared to that in the wild type. In the *mutY* mutant no transcripts above background level were observed. Levels of *nth* transcripts in wild-type and *nei* and *mutY* mutant backgrounds were similar. However, the levels of the *nth* transcript in the *nth* mutants were approximately 2.5-fold greater than that in the wild type. This increase is presumably due to an increase in message stability from the kanamycin resistance gene inserted into *nth* (63). Interestingly, in the *fpg* and *fpg nei* mutants, transcript levels of *nth* were increased 2.4-fold and 2.0-fold, respectively. Levels of *nei* transcript were relatively equal in wild-type and *nth*, *fpg*, and *mutY* mutant backgrounds. In *nei* and *fpg* nei mutants, no *nei* tran-

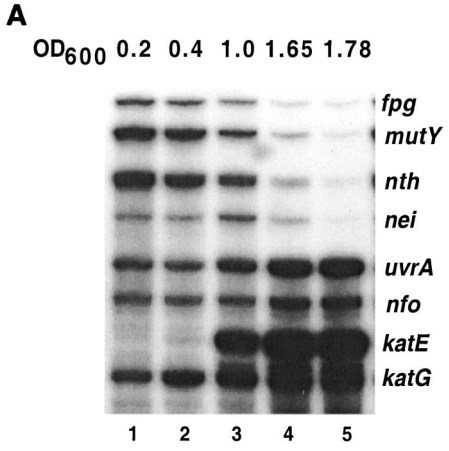
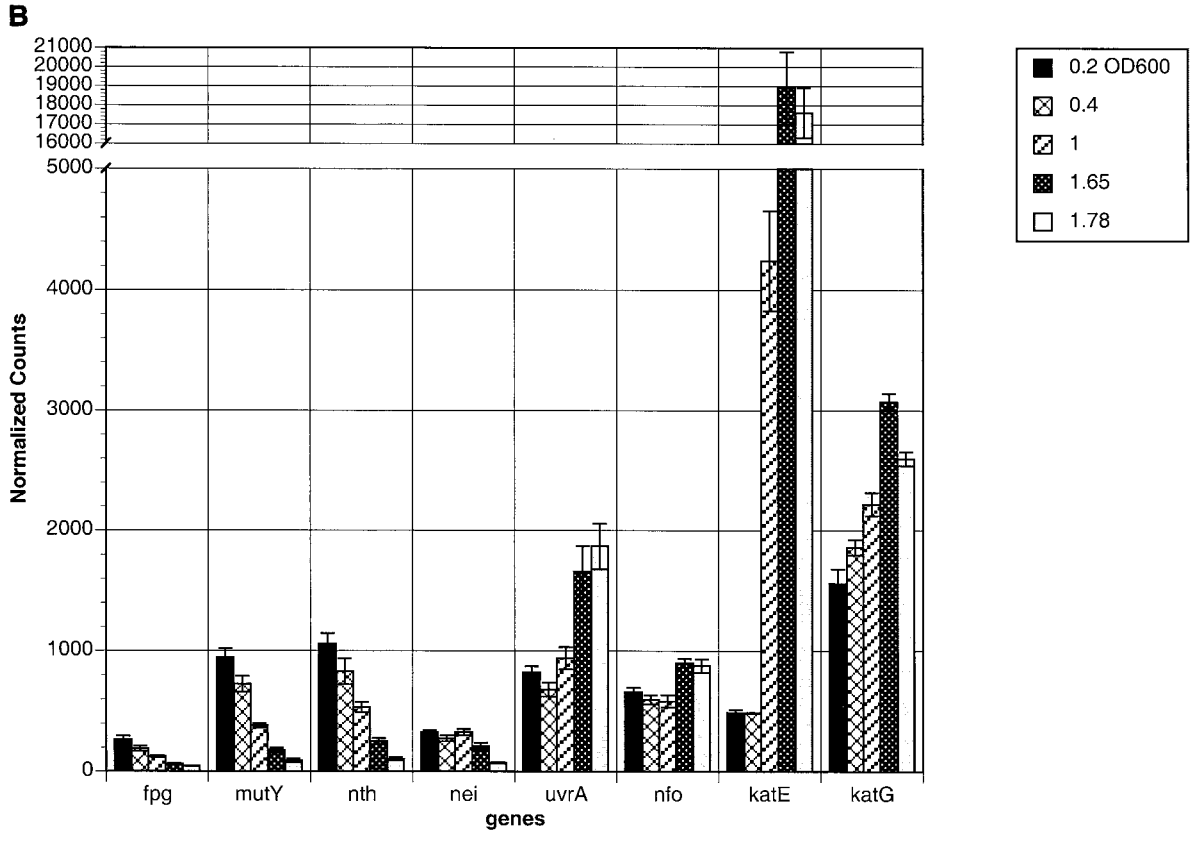


FIG. 2. Levels of transcript for each gene at OD_{600}s of 0.2, 0.4, 1.0, 1.65, and 1.78. (A) Representative multiprobe RPA for the growth phase experiment. Shown are levels of transcript for each gene at the tested OD_{600}s. The gene corresponding to each protected product is listed on the right. Overnight cultures of *E. coli* were diluted to an OD_{600} of 0.02 in fresh LB growth medium, and then samples were taken at the listed OD_{600}s. Cells were immediately spun down and snap frozen in liquid nitrogen. The isolated RNA (10 μg) was hybridized overnight with 25,000 cpm of each probe and was then digested with RNase A-RNase T1. The samples were then run on a 5% polyacrylamide gel. (B) Multiprobe RPA results were quantitated on a phosphorimager and are reported as counts normalized for the number of U residues in each protected product. The following values are means ± standard errors of the means ($n = 3$), reported from lowest to highest OD: *fpg*, 266 ± 28.0, 187 ± 21.8, 123 ± 7.58, 60.5 ± 5.59, and 44.0 ± 1.86 counts; *mutY*, 945 ± 73.8, 726 ± 65.5, 380 ± 18.1, 176 ± 19.3, and 90.7 ± 13.1 counts; *nth*, 1,060 ± 85.6, 829 ± 107, 533 ± 43.1, 250 ± 25.0, and 103 ± 11.3 counts; *nei*, 325 ± 19.5, 275 ± 26.2, 327 ± 25.2, 209 ± 28.5, and 73.2 ± 7.71 counts; *uvrA*, 823 ± 50.9, 682 ± 59.2, 941 ± 91.4, 1,660 ± 213, and 1,870 ± 191 counts; *nfo*, 659 ± 35.6, 597 ± 36.8, 585 ± 51.3, 899 ± 36.5, and 876 ± 55.5 counts; *katE*, 490 ± 24.0, 486 ± 4.90, 4,240 ± 413, 18,900 ± 1,910, and 17,600 ± 1,300 counts; *katG*, 1,560 ± 120, 1,860 ± 66.3, 2,220 ± 96.1, 3,070 ± 71.8, and 2,600 ± 59.1 counts.



script was detected above background levels. No differences in the levels of any transcripts between mutant and wild-type cells were found when the cells were harvested at an OD_{600} of 0.2 or 1.7 (data not shown).

Transcript levels of the oxidative DNA repair glycosylase genes increase after a shift from anaerobic to aerobic growth. Cultures of *E. coli* GC4468 were grown anaerobically overnight, diluted 1/50 (OD_{600} of ~0.01) in fresh LB medium, and then again grown anaerobically until an OD_{600} of ~0.125 was reached and the first sample was taken. The cell cultures were then shifted to a rotary shaker in a 37°C warm room, and samples were taken 5, 20, and 60 min after the shift to aerobic conditions. Transcript levels for *fpg*, *mutY*, *nth*, and *nei* more than doubled at 5 min after the shift from anaerobic to aerobic growth (Fig. 5). *fpg* and *nei* transcript levels increased 4.2- and 3.3-fold, respectively, at 20 min after the shift and started to decline by 60 min. *mutY* and *nth* transcript levels continued to increase after the shift from anaerobic to aerobic growth for total increases of 3.8- and 5.6-fold, respectively. Transcript levels of *uvrA*, *nfo*, and *katE* all increased two- to threefold by 20 min before starting to decline (not shown). Interestingly, the level of the *katG* transcript was very high before the shift to aerobic growth (24.6-fold higher than that of the *uvrA* transcript, which was 1.9-fold lower than that of the *katG* transcript in early aerobic growth) (data not shown). The level of the *katG* transcript increased 2.3-fold 5 min after the shift to aerobic growth before decreasing a total of 22-fold by 60 min.

In order to determine whether the 4.2-fold increase in *fpg*

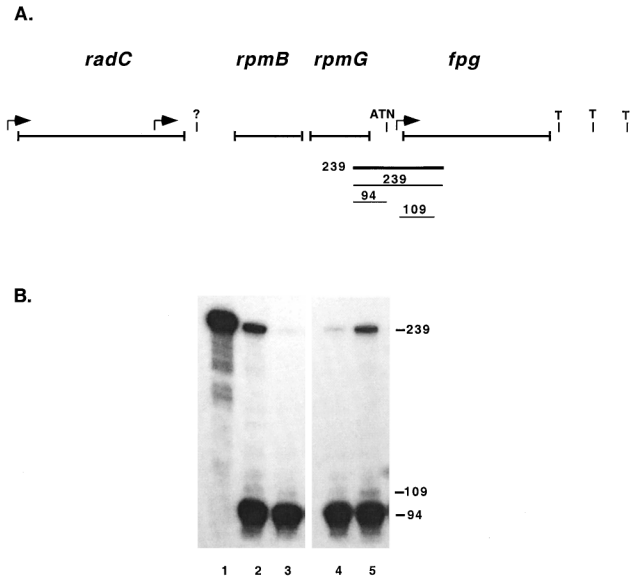


FIG. 3. Measurement of *fpg* transcript originating from the *fpg* promoter and from upstream. (A) Features of the *fpg* operon. Arrows, mapped transcription initiation sites; ATN (attenuator), mapped termination site that also allows transcript readthrough; T, termination sites; ?, either transcription initiation site or RNase E or RNase III cleavage site; thick line 239, approximate annealing location of the probe used in the RPA; thin lines, products obtained from the RPA (sizes are indicated). The probe is 239 nucleotides long and anneals from 90 bp 3′ to the *fpg* start codon to 52 bp 5′ to the *rpmG* stop codon. (B) Lane 1, full-length probe; lanes 2 and 3, results obtained with RNA from cells at OD_{600} 0.2 (lane 2) and 1.78 (lane 3); lanes 4 and 5, results obtained with RNA from anaerobically grown cells (lane 4) and cells 20 min after a shift to aerobic growth (lane 5). Numbers beside panels are in base pairs.

transcript seen after a shift from anaerobic to aerobic growth was due to a increase in transcription from its own promoter or

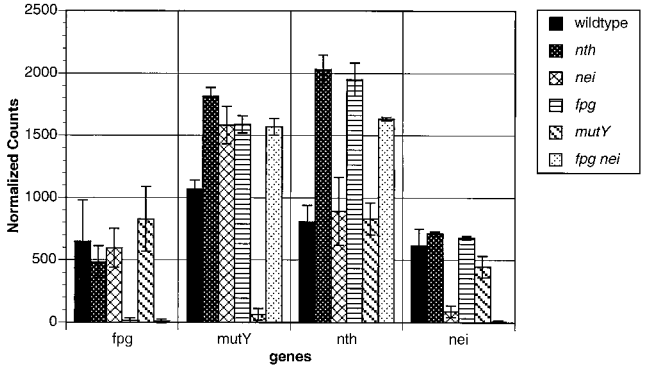


FIG. 4. Levels of transcript for each gene in various base excision repair mutant backgrounds. Overnight cultures of each strain were diluted to an OD_{600} of 0.02 in fresh LB growth medium and grown to an OD_{600} of 0.5. Cells were immediately spun down and snap frozen in liquid nitrogen. The isolated RNA (10 μg) was hybridized overnight with 25,000 cpm of each probe and was then digested with RNase A-RNase T1. Samples were then run on a 5% polyacrylamide gel. Multiprobe RPA results were quantitated on a phosphorimager and are reported as counts normalized for the number of U residues in each protected product. The following values are means ± standard errors of the means ($n = 3$), reported in the following order: wild type and *nth*, *nei*, *fpg*, *mutY*, and *fpg nei* mutants. *fpg* probe, 646 ± 334, 596 ± 154, 484 ± 127, 17.0 ± 18.1, 828 ± 259, and 9.00 ± 14.6 counts; *mutY* probe, 1,069 ± 71.2, 1,583 ± 150, 1,812 ± 72.6, 1,589 ± 69.5, 65.0 ± 45.2, and 1,569 ± 67.0 counts; *nth* probe, 805 ± 134, 891 ± 271, 2,031 ± 113, 1,950 ± 132, 831 ± 129, and 1,632 ± 13.9 counts; *nei* probe, 614 ± 135, 89.0 ± 47.1, 711 ± 16.2, 678 ± 13.9, 446 ± 87.0, and 10.0 ± 9.29 counts.

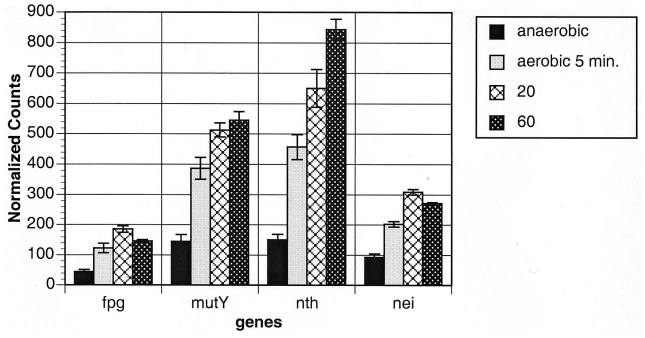


FIG. 5. Levels of transcript for each gene in anaerobically grown cells and cells 5, 20, and 60 min after a shift to aerobic growth. Anaerobically grown overnight cultures of *E. coli* were diluted to an OD_{600} of 0.01 in fresh LB broth and then were again grown anaerobically until an OD_{600} of 0.125 was reached and the first sample was taken. The cell cultures were then shifted to a rotary shaker, and samples were taken 5, 20, and 60 min after the shift. Cells were immediately spun down and snap frozen in liquid nitrogen. Multiprobe RPA results were quantitated on a phosphorimager and are reported as counts normalized for the number of U residues in each protected product. The following values are means ± standard errors of the means ($n = 3$, reported in the following order: anaerobic growth and 5, 20, and 60 min of aerobic growth. *fpg*, 43.9 ± 7.10, 122 ± 16.5, 185 ± 10.7, and 146 ± 3.73 counts; *mutY*, 145 ± 21.7, 386 ± 36.1, 513 ± 23.1, and 546 ± 27.7 counts; *nth*, 150 ± 19.1, 457 ± 40.5, 651 ± 61.6, and 844 ± 32.6 counts; *nei*, 92.6 ± 10.5, 202 ± 8.93, 309 ± 8.87, and 271 ± 4.20 counts.

to upstream regulatory events, RPAs were performed using the probe that anneals to the *fpg* promoter region and RNA from anaerobically grown cells and cells 20 min after the shift to aerobic growth (Fig. 3, lanes 4 and 5). The 239-bp product corresponding to transcript readthrough from the upstream genes was 4.4-fold less abundant in the anaerobically grown cells than in the cells 20 min after the shift to aerobic growth. The 94-bp product corresponding to the attenuated product was present in equal amounts. The 109-bp product corresponding to the transcript originating at the *fpg* promoter was present in a twofold-lesser amount in the anaerobically grown cells than in the cells 20 min after the shift. It appears that part of the increase in *fpg* transcript after a shift from anaerobic to aerobic growth is due to upregulation at its own promoter.

A previous study showed that Fpg activity increased in anaerobically grown cells that were mutant for *arcA* (aerobic respiration control), *fur* (ferric uptake regulation), and *fnr* (fumarate nitrate reductase) (35). ArcA and Fnr are involved in anaerobic activation and repression of numerous genes (25, 54, 56), and Fur represses transcription of genes involved in iron uptake (2). We examined transcript levels for *fpg*, *mutY*, *nth*, and *nei* in anaerobically grown isogenic wild-type cells and *arcA*, *fur*, and *fnr* mutants. Transcript levels were measured 2, 3, 4, and 6 h after a dilution from an overnight culture. No increase in transcript levels over those of the wild type was seen with any of the mutants at any of the time points, suggesting that ArcA, Fur, and Fnr are not acting as transcriptional repressors for these genes during anaerobic growth (data not shown).

Transcription of the oxidative DNA repair glycosylase genes is not induced by agents that produce the damage that gene products repair. A variety of agents known to produce damage that gene products repair were examined, along with the appropriate control genes, to determine whether they induce *fpg*, *mutY*, *nth*, or *nei*. None of the following agents yielded more than a slight (less than twofold) increase in transcript levels for *fpg*, *mutY*, *nth*, or *nei*: 10 μM H_2O_2, 300 μM paraquat, 100 Gy of X rays, and 50 μg of nalidixic acid/ml (Table 1).

TABLE 1. Effect of agents that produce DNA damage[a]

Treatment	Gene	Mean level (counts) ± SEM (n = 3) at:			
		Pretreatment	5 min	20 min	60 min
10 μM H_2O_2	*katG*	928 ± 99.3	19,100 ± 455	2,960 ± 525	1,980 ± 176
	fpg	214 ± 4.44	150 ± 13.3	178 ± 46.0	99.5 ± 5.74
	mutY	414 ± 18.7	347 ± 43.9	289 ± 51.5	248 ± 37.4
	nth	378 ± 26.3	265 ± 34.9	188 ± 31.6	221 ± 42.4
	nei	170 ± 6.35	122 ± 17.1	211 ± 46.4	200 ± 15.1
100 Gy of X rays	*uvrA*	535 ± 53.5	1,790 ± 303	4,770 ± 600	
	katG	1,420 ± 114	28,200 ± 2,060	5,240 ± 583	
	fpg	252 ± 50.1	192 ± 30.5	449 ± 32.4	
	mutY	742 ± 49.1	678 ± 74.8	577 ± 25.2	
	nth	921 ± 43.1	513 ± 88.5	444 ± 38.5	
	nei	269 ± 36.1	245 ± 34.5	339 ± 35.3	
300 μM paraquat	*nfo*	862 ± 47.1	8,500 ± 1,200	10,400 ± 977	7,010 ± 255
	fpg	172 ± 14.6	308 ± 37.6	184 ± 20.4	74.2 ± 11.5
	mutY	393 ± 31.6	423 ± 52.8	316 ± 38.3	198 ± 12.0
	nth	572 ± 42.4	484 ± 47.4	424 ± 64.5	362 ± 45.7
	nei	261 ± 8.73	319 ± 34.1	330 ± 33.3	214 ± 10.7
50 μg of nalidixic acid/ml	*uvrA*	2,030 ± 197	10,000 ± 943	10,900 ± 865	5,520 ± 245
	fpg	402 ± 23.0	540 ± 23.8	366 ± 19.3	311 ± 23.2
	mutY	982 ± 63.2	337 ± 19.0	592 ± 22.7	439 ± 143
	nth	1,210 ± 53.6	658 ± 53.3	714 ± 51.0	195 ± 47.7
	nei	782 ± 66.3	373 ± 29.0	496 ± 43.7	345 ± 9.52

[a] Cells were treated as indicated and irradiated in 2-ml aliquots in a 35- by 10-mm culture dish with stirring. The X rays were produced by a Philips XRG3000 X-ray generator. Samples were taken just before treatment and 5, 20, and 60 min after treatment. Cells were immediately spun down and snap frozen in liquid nitrogen. Multiprobe RPA results were quantitated on a phosphorimager and are reported as counts normalized for the number of U residues in each protected product.

H_2O_2 can undergo a Fenton-like reaction in the presence of Fe^{2+}, generating $OH^{\cdot}$, a powerful oxidant (24). Since iron can localize along the phosphodiester backbone of nucleic acids, DNA is a target of $OH^{\cdot}$. *katG* encodes hydroperoxidase I and is part of the *oxyR* regulon, which is turned on in the presence of H_2O_2 (9). The level of *katG* transcript increased 20.5-fold 5 min after treatment of cells with 10 μM H_2O_2 and then decreased at 20 and 60 min (Table 1). There was no increase in the transcript levels of *fpg*, *mutY*, *nth*, or *nei*. The experiment was also performed with an *oxyR* mutant, and the pattern of transcript levels across the times was the same with the exception that the *katG* transcript only increased a total of 2.7-fold (data not shown).

Treatment of cells with ionizing radiation also generates $OH^{\cdot}$ (62). When cells were treated with 100 Gy of X rays, *katG* transcript levels increased 19.8-fold 5 min after treatment and *uvrA* transcript levels increased 8.9-fold 20 min after treatment (Table 1). The level of *fpg* transcript showed a small increase at 20 min (1.8-fold); the levels of *mutY*, *nth*, and *nei* transcripts did not increase.

Paraquat is a redox cycling drug that generates $O_2^{\cdot -}$ (29). The $O_2^{\cdot -}$ radical does not react directly with DNA (4, 6, 37, 50); however it can be dismutased to H_2O_2, which can lead to $OH^{\cdot}$ formation as described above. It can also damage iron-sulfur proteins, leading to release of iron into the cytosol, where it catalyzes the oxidation of DNA in conjunction with H_2O_2 (30, 38). *nfo* encodes the base excision repair protein endo IV and is part of the SoxRS regulon, which is induced in response to $O_2^{\cdot -}$-generating agents such as paraquat (8). The level of *nfo* transcript increased 9.9-fold at 5 min after treatment of the cells with 300 μM paraquat and reached a maximum of 12-fold induction at 20 min (Table 1). The level of *fpg* transcript increased 1.8-fold at 5 min before decreasing, the *nei* transcript level increased 1.3-fold at 20 min before decreasing, and *mutY* and *nth* transcript levels did not increase. Under the same conditions, the levels of *nfo* transcript did not increase when a *soxRS* mutant strain was treated with paraquat (data not shown).

The SOS response is turned on in response to treatment of cells with UV irradiation, chemicals such as nalidixic acid, and ionizing radiation and requires the activity of RecA (55). Transcript levels in cells treated with 50 μg of nalidixic acid/ml were measured. *uvrA* encodes the nucleotide excision repair protein UvrA and is induced as part of the SOS response (59). The level of *uvrA* transcript increased a maximum of 5.4-fold 20 min after treatment of cells with nalidixic acid and was lower at 60 min (Table 1). The *fpg* transcript level increased 1.3-fold 5 min after treatment, and the amounts of *mutY*, *nth*, and *nei* transcripts decreased. The experiment was also performed with a *recA* mutant, and the pattern of transcript levels at the different times was the same with the exception that the *uvrA* transcript only increased 1.5-fold (data not shown).

E. coli cells were also grown to stationary phase (OD_{600} of 1.7) and treated with 10 μM H_2O_2, 300 μM paraquat, or 100 Gy of X rays. No more than a 1.2-fold increase was seen in *fpg*, *mutY*, *nth*, and *nei* transcript levels with any of the conditions at 5, 20, and 60 min after treatment (data not shown).

DISCUSSION

We have used multiprobe RPAs to measure the transcript levels of *fpg*, *mutY*, *nth*, *nei*, and the appropriate control genes under various conditions. Transcript levels decreased 5- to 10-fold for the four genes of interest as cells progressed from log-phase growth to stationary phase (Fig. 2) and increased about 4-fold after cells were shifted from anaerobic to aerobic growth (Fig. 5).

Of the four genes, only *fpg* has its own promoter in addition

to upstream promoters, thus allowing for the possibility of regulation of *fpg* without the upstream genes. However, during the progression from log phase to stationary phase the decrease in *fpg* transcript levels was primarily due to a decrease in transcript readthrough from upstream (Fig. 3). Since the decrease in the oxidative DNA glycosylase transcripts in stationary phase was not *rpoS* related, it is not known whether the decrease is due to repression by another regulator or to a general decrease in transcription of non-stationary-phase-specific genes. For example, Rsd (regulator of sigma D) was identified as an RNA polymerase σ^{70}-associated protein found in stationary-phase *E. coli* that has an inhibitory activity on σ^{70} transcription in vitro (27). The intracellular levels of Rsd start to increase during the transition from growth to stationary phase (27). Thus Rsd may be involved in the replacement of RNA polymerase sigma subunit σ^{70} with σ^{S} during the transition from exponential growth to stationary phase (28). The transcription initiation sites for the four operons containing the oxidative DNA glycosylase genes are all preceded by predicted σ^{70} promoters (18, 19). If indeed σ^{70} is sequestered by Rsd as cells transition from exponential growth to stationary phase, then σ^{70}-regulated genes, as ours appear to be, will be downregulated.

The transcript levels of *fpg*, *mutY*, and *nei* in the single-mutant backgrounds and in the *fpg nei* double mutant were similar to wild-type levels in early log and mid-log phase and in stationary phase. However, *nth* transcript levels increased severalfold in *fpg* and *fpg nei* mutants in cells grown to mid-log phase (Fig. 5) but not in cells in early log phase or in stationary phase. This is consistent with observations that cell extracts prepared from mid-log-phase *fpg nei* mutants show a 5- to 10-fold increase in cleavage, relative to wild-type extracts, of oligonucleotides containing either thymine glycol, 5-hydroxycytosine, or 5-hydroxyuracil lesions, whereas extracts of *fpg nth* mutants do not (Z. Hatahet, personal communication). Thymine glycol, 5-hydroxycytosine, and 5-hydroxyuracil are substrates for endo III (60). The increase in *nth* transcript level in an *fpg* mutant background, taken together with the increase in cleavage of substrates for endo III, suggests that in *fpg* mutants there is an increase in endo III activity, presumably resulting from either an increase in *nth* expression or an increase in mRNA stability.

Why might the transcript levels for the oxidative DNA glycosylase genes be high in exponential phase and low in stationary phase? If the changes in transcript levels are specific for the glycosylases, rather than an indirect consequence, it might be that the levels of these glycosylases are at their highest during early log phase because, in exponentially growing *E. coli*, both $O_2^{\cdot -}$ and H_2O_2 are generated by the auto-oxidation of components of the respiratory chain (21, 23). There is a 10-fold increase in the rate of H_2O_2 generation during the exponential phase of aerobic growth (21). The increased concentration of H_2O_2 could be associated with oxidative DNA damage since there is a 1.9- to 3.4-fold-higher spontaneous mutation frequency in exponentially growing wild-type *E. coli* cells than in stationary-phase cells (20). Since cells are experiencing more oxidative stress during exponential growth, it may make sense to have higher levels of enzymes that repair oxidative DNA damage present during this time. In possible disagreement with this, it has been calculated that the rate of production of the common oxidative damage 8-oxoG in the DNA of starved cells is threefold greater than in the DNA of growing cells (7). It has been shown that the mismatch repair protein MutL becomes limiting for mismatch repair during stationary phase, and it has been speculated that this could allow cells to regulate their potential to evolve (22). In fact, *E. coli* cultures grown to stationary phase give rise to mutants with the ability to prevail under limiting conditions (65). It is possible that the decrease in mismatch repair during stationary phase contributes to the generation of mutants with a growth advantage in stationary phase, and since the levels of the oxidative DNA glycosylases decrease in stationary phase, a decrease in base excision repair may play a role here as well. Creation of a hypermutable state due to lower levels of DNA repair may help to generate populations of cells that are better able to survive the environmental challenges they experience. Alternatively, until cells begin dividing again there may be no reason to have these repair systems fully functioning. Mismatch repair and base excision repair systems are responsible for repair of premutagenic lesions (43, 60, 61), and premutagenic lesions cannot become mutagenic without DNA replication.

When cells were shifted from anaerobic to aerobic growth, the transcript levels of the oxidative DNA glycosylases increased (Fig. 4), possibly in response to the resumption of aerobic respiration, which generates free radicals, placing the cells under oxidative stress. Alternatively, the increase in transcript levels may be due to an increased growth rate rather than to oxidative stress since the highest transcript levels for the four genes were seen in early log phase when cells are dividing rapidly. The *fpg* promoter appeared to play a greater role in the transcript increase seen in the shift from anaerobic to aerobic growth than in the transcript decrease seen during progression into stationary phase (Fig. 3). It has previously been shown that Fpg activity increases in anaerobically grown cells that are mutant for *arcA*, *fur*, and *fnr* and that there are possible consensus sequences for the products of these genes in the *fpg* promoter region (35). These results suggested that ArcA, Fur, and Fnr act as repressors of Fpg during anaerobic growth. However, we failed to see an increase in levels of the *fpg* transcript in *arcA*, *fur*, and *fnr* mutants at any time during anaerobic growth. Since, in the previously reported results, enzyme activity was examined, it is possible that the increases in enzyme activity occurred at a posttranscriptional level. There was also no increase in *mutY*, *nth*, or *nei* transcripts in these mutants, suggesting that ArcA, Fur, and Fnr do not play a role in the transcriptional regulation of the oxidative DNA glycosylase genes during anaerobic growth.

fpg, *mutY*, *nth*, and *nei* were not induced by H_2O_2, paraquat, X rays, or nalidixic acid. Although the single oxidative DNA glycosylase mutants are not sensitive to the cytotoxic effects of oxidizing agents and ionizing radiation (5, 12), they are mutators (3, 26, 41) due to the formation of spontaneous oxidative DNA lesions of the type formed by oxidizing agents. Also, *nth nei* double mutants, defective in both pyrimidine-specific DNA glycosylases, are hypersensitive to hydrogen peroxide (51, 60) and ionizing radiation (26). Thus, it was unexpected that transcription of the oxidative DNA glycosylase genes was not induced in response to these agents. A previous study reported 2.4- and 4.4-fold responses in Fpg activity in cells about 30 min after treatment (35) for 3 h with 100 and 500 μM paraquat, respectively (31, 35). We saw a 1.8-fold increase in *fpg* transcript levels 5 min after treatment with 300 μM paraquat, but the transcript levels returned to the pretreatment level by 20 min after treatment. Although a 1.8-fold increase in transcript levels could account for a 4-fold increase in enzyme activity, it seems unlikely since Fpg activity did not begin to increase until 30 min after treatment with paraquat. It is possible that the reported increase in Fpg enzyme activity is due to a posttranscriptional event.

It is interesting that the transcription of the oxidative DNA glycosylases does not appear to be upregulated by the treatments that produce the damage the enzymes recognize. This is

especially true since the enzymes responsible for the next step in the pathway, the apurinic endonucleases exonuclease III (*xth*) and endo IV (*nfo*), are significantly upregulated by the KatF and SoxRS pathways, respectively (16, 57). It should be noted that exonuclease III and endo IV directly recognize a number of cytotoxic lesions produced by oxidizing agents (60). It is possible that the levels of endogenous base damage are so significant that high constitutive levels of the oxidative DNA glycosylases are necessary for genome maintenance and that the increased levels of damage produced by treatment with oxidizing agents are low compared to the high level of background lesions.

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