JOURNAL OF BACTERIOLOGY, Sept. 1997, p. 5967–5970 Vol. 179, No. 18
0021-9193/97/$04.00+0

oxyR-Dependent Induction of *Escherichia coli grx* Gene Expression by Peroxide Stress

KAZUYUKI TAO*

Radioisotope Center, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Received 28 April 1997/Accepted 18 July 1997

The *Escherichia coli* OxyR protein is a transcriptional activator for a number of genes induced in response to low concentrations of hydrogen peroxide. To identify additional OxyR-regulated genes, I cloned a DNA fragment that shows promoter activity regulated by OxyR by direct selection of OxyR-binding DNA fragments. Analyses of the cloned fragment indicate that the *grx* gene, encoding glutaredoxin 1, is inducible by hydrogen peroxide in an *oxyR*-dependent fashion.

Reactive oxygen species such as hydrogen peroxide, superoxide anions, and hydroxyl radicals are generated during normal aerobic metabolism and as a consequence of environmental insults, and they are cytotoxic and mutagenic to cells. *Escherichia coli* and *Salmonella typhimurium* cells can adapt to this toxicity (4, 6, 18). Treatment of *E. coli* and *S. typhimurium* cells with nonlethal concentrations of H_2O_2 induces synthesis of about 50 proteins and makes the cells resistant to subsequent treatment with higher concentrations of H_2O_2 (3, 5). The *oxyR* gene encodes a bifunctional protein which acts as both a sensor of peroxide stress and a transcriptional activator for a regulon induced by the stress (3). *oxyR* deletion mutants cannot induce a subset of peroxide-inducible proteins, and they are hypersensitive to various oxidizing agents (3, 7). Furthermore, the spontaneous mutation frequency of aerobically grown *oxyR* deletion mutants is higher than that of *oxyR*$^+$ strains (17). Taken together these observations suggest the importance of the *oxyR* gene and the genes under its control for protecting cells from oxidative damage.

Several proteins have been identified as *oxyR*-regulated proteins on two-dimensional gels (3, 13), and some of them have been identified for known functions. They are catalase hydroperoxidase I (encoded by *katG*), glutathione reductase (*gor*), alkyl hydroperoxide reductase (*ahpCF*), and a nonspecific DNA-binding protein (*dps*) (1, 3, 7, 13). Recently, Mukhopadhyay and Schellhorn (14) demonstrated with gene fusions that OxyR activates transcription from the promoters of *hemF* (encoding coprophyrinogen III oxidase), *rcsC* (encoding a sensor regulator protein of capsular polysaccharide synthesis), and a gene encoding a putative f497 protein. To identify additional OxyR-regulated genes, I attempted to clone promoter DNA fragments whose expression is regulated by OxyR.

Since purified OxyR protein specifically binds to upstream regulatory regions of the genes under its control (19, 20), I tried to clone DNA fragments which would specifically bind to OxyR for identification of OxyR-regulated genes. Selection of DNA fragments was done by immune precipitation of protein-DNA complexes followed by amplification by PCR as described previously (11), with slight modifications. *E. coli* chromosomal DNA was digested with *Hae*III, *Hha*I, or *Sau*3AI, and the ends of the DNA fragments were made blunt with T4 DNA polymerase in the cases of *Hha*I and *Sau*3AI digestion. The blunt-ended DNA was ligated with double-stranded phosphorylated linker DNA consisting of linkers A (GAGTAGA ATTCTAATATCTC) and B (GAGATATTAGAATTCTAC TC) (11). To remove excessively ligated linkers, the DNA was digested by *Xho*I, whose recognition sequence is generated by self-ligation of the linkers. The linker-ligated DNA was incubated with purified OxyR protein (50 nM at final concentration) at room temperature for 30 min in 100 μl of binding buffer [20 mM Tris HCl (pH 7.5), 50 mM KCl, 0.2 mM dithiothreitol, 100 μg of poly(dI-dC)/ml, 10% glycerol]. Then, the mixture was transferred on ice and was diluted with 400 μl of ice-cold binding buffer. One microliter of rat anti-OxyR antiserum was added to the binding mixture. After a 30-min incubation, rabbit anti-rat immunoglobulin G antiserum was added to the mixture. After a further 30-min incubation, 100 μl of protein A-Sepharose 4B was added to the binding mixture and the mixture was incubated with rocking for 60 min. The OxyR protein-DNA complex bound to protein A-Sepharose was then collected by centrifugation and washed three times with 500 μl of binding buffer. Finally, the bound DNA was recovered by

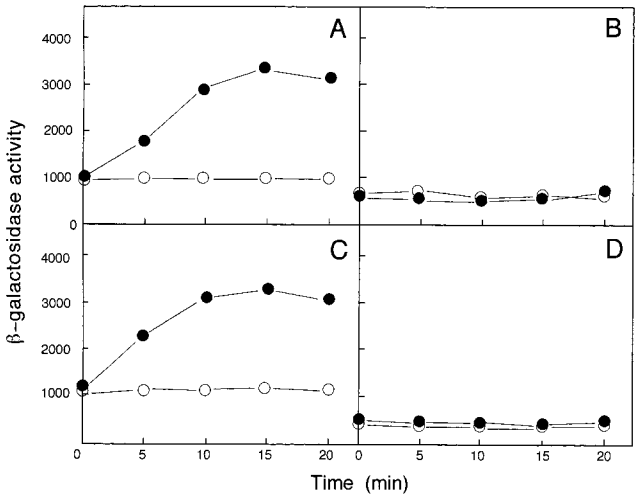

FIG. 1. Induction of the β-galactosidase activity encoded on pHA233 and pHA2331. Cells of *E. coli* RK4936/pHA233 (A), TA4112/pHA233 (B), RK4936/pHA2331 (C), and TA4112/pHA2331 (D) were grown to mid-log phase in Luria-Bertani medium containing 100 μg of ampicillin/ml. At time zero, H_2O_2 (100 μM at final concentration) was added to one portion of each culture. Samples were taken from H_2O_2-treated (filled circles) and untreated (open circles) cultures, and the β-galactosidase activities were measured. Activities of β-galactosidase are given in Miller units.

* Phone: 81-3-3812-2111. Fax: 81-3-3816-0422. E-mail: tao@ric.u-tokyo.ac.jp.

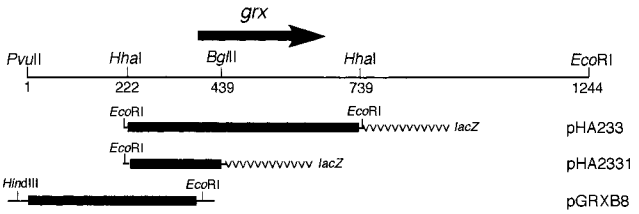

FIG. 2. Restriction cleavage map of the *grx* region and schematic representations of the plasmids used in this study. The coding region of the *grx* gene is shown by the arrow. Regions derived from the *E. coli* chromosome are represented by wide bars. Thin lines represent the regions derived from vectors or linker DNAs. Wavy lines indicate the *lacZ* region. For restriction enzyme cleavage sites, only relevant sites are shown. pGRXB8 is one of the deletion derivatives of pGRX100 (not shown in the figure), which carries the 1.2-kb *Pvu*II-*Eco*RI fragment of λ210 (12) containing the *grx* region.

incubating the immune complex bound to protein A-Sepharose in 100 μl of elution buffer (20 mM Tris HCl [pH 7.5], 2% sodium dodecyl sulfate, 0.3 M sodium acetate) at 60°C for 10 min. Recovered DNA fragments were amplified by PCR (25 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min) with *Taq* DNA polymerase and linkers A and B (50 pmol each) as primers. Amplified DNA fragments were subjected to a second cycle of selection and amplification. After the second amplification step, selective amplification of specific DNA fragments was observed (data not shown). The amplified DNA was digested by *Eco*RI, whose recognition sequence is present in the linker DNA, and cloned into the *Eco*RI site of a β-galactosidase operon fusion vector, pRS415 (16). Gel retardation analysis of the cloned DNA fragments indicated that most of them specifically bound OxyR (data not shown). Next, the hybrid plasmids carrying OxyR-binding fragments were introduced into an *E. coli* strain, RK4936, and the cells were examined for inducibility of the β-galactosidase activity encoded on hybrid plasmids in response to H_2O_2 (100 μM). β-Galactosidase activity of seven transformants carrying plasmids with different insert DNA fragments was found to be induced by H_2O_2 treatment. β-Galactosidase induction ratios of these clones ranged from 1.3- to 3.0-fold. Among these, pHA233 and pSA139 showed relatively high induction ratios (approximately threefold induction). Nucleotide sequence analysis of the insert DNA of pSA139 revealed that it carried a DNA fragment covering the *oxyS* promoter region, which had been shown to be induced by H_2O_2 in an *oxyR*-dependent fashion (21). In this study, another hybrid plasmid, pHA233, obtained from the *Hha*I-digested library and showing the highest induction ratio among the clones, was analyzed further.

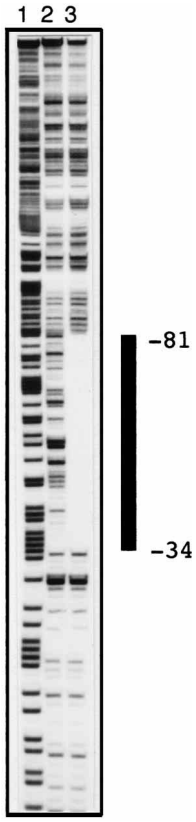

FIG. 4. DNase I footprinting of the *grx* promoter region. The probe DNA was subjected to partial digestion with DNase I in the presence (lane 3) or absence (lane 2) of OxyR protein (5 pmol), and the samples were analyzed by electrophoresis on a polyacrylamide gel containing 8 M urea and visualized by autoradiography. The Maxam-Gilbert G+A reaction of the same DNA was used as a size marker (lane 1). The protected region is indicated by the thick bar.

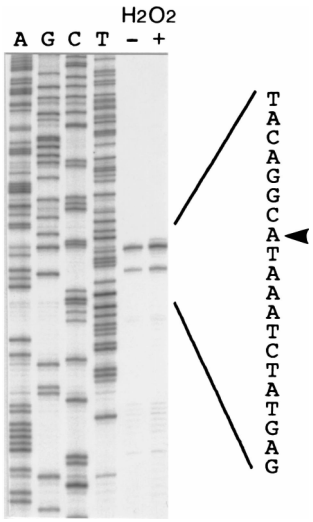

FIG. 3. Primer extension analysis of the *grx* transcripts. Total RNA was isolated from exponentially growing cells of RK4936/pHA233 with or without H_2O_2 treatment. A *grx*-specific primer (0.1 pmol) was hybridized to 10 μg of RNA and extended by reverse transcriptase. The products were electrophoresed on a polyacrylamide gel containing 8 M urea, followed by visualization by a Fujix Image Analyzer (model BAS1500). Dideoxy sequencing products of pHA233 primed with the same primer were electrophoresed on the same gel. The nucleotide sequence around the transcription start sites is shown on the right. The major transcription initiation site is indicated by the arrowhead.

As shown in Fig. 1A, the β-galactosidase activity of an *oxyR*$^+$ strain carrying pHA233 increased about threefold following treatment with H_2O_2. On the other hand, an *oxyR* deletion mutant (TA4112) carrying pHA233 did not induce β-galactosidase activity under the same conditions (Fig. 1B). These observations indicate that pHA233 carries a promoter DNA fragment which is regulated by OxyR.

To investigate the origin of the cloned DNA fragment, I determined the partial nucleotide sequence of the 0.5-kb *Eco*RI fragment of pHA233 from both ends. Comparison of the determined nucleotide sequence to *E. coli* nucleotide sequence databases revealed that the cloned fragment was identical to the 517-bp *Hha*I fragment containing the entire coding region of the *grx* gene, encoding glutaredoxin 1 (10, 15). Al-

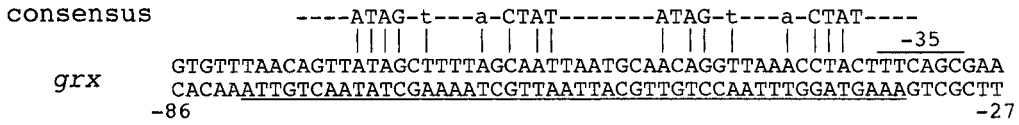

FIG. 5. Similarity of the nucleotide sequence of the OxyR binding site in the *grx* promoter to the proposed consensus sequence for OxyR binding (21). The region protected by OxyR in the bottom strand is underlined. The nucleotide sequences of both strands for the *grx* promoter region are shown.

though nucleotide sequence analysis of the fusion junction indicates that the *grx* gene is oriented in the same direction as the *lacZ* gene of the fusion vector, the fusion junction is downstream of the coding region (Fig. 2). Therefore, it is not clear whether the *grx* gene is regulated by OxyR. To address this point, I constructed another *grx′-′lacZ* fusion gene by cloning the 0.25-kb *Eco*RI-*Bgl*II fragment of pHA233 carrying the promoter and a part of the N-terminal coding region of *grx* into the *Eco*RI-*Bam*HI site of pMC1403 (2), resulting in pHA2331 (Fig. 2). As in the case of pHA233, the β-galactosidase activity encoded by pHA2331 increased about threefold with H_2O_2 treatment in an *oxyR*-dependent fashion (Fig. 1C and D), confirming that transcription from the *grx* promoter is regulated by OxyR.

To map the transcription start site of the *grx* gene, primer extension experiments were carried out (Fig. 3). Cells of RK4936 carrying pHA233 were grown to mid-log phase in Luria-Bertani medium, and half of the culture was treated with H_2O_2 for 10 min. Then, total RNA was isolated from the cultures by acid-phenol extraction. The ^{32}P-labeled synthetic oligonucleotide primer complementary to the *grx* transcripts (TCATCGCGTTCATTGCTCAATTTCTCAGCC) was hybridized to 10 μg of RNA and extended with avian myeloblastosis virus reverse transcriptase. Two extended products were observed for both RNA samples under the conditions employed, and about a twofold increase in the amount of both transcripts was observed for the H_2O_2-treated sample. The major and the minor transcripts start at A and C, 23 and 17 bases upstream of the *grx* initiation codon, respectively. As a putative promoter for the transcripts, TTcAgc (−35 to −30 from the major transcription start site) and gATcAT (−13 to −8) were assigned to the promoter −35 and −10 sequences, respectively.

Next, I determined the location of the OxyR binding site in the promoter region of *grx* by DNase I footprinting experiments. The 0.35-kb *Eco*RI-*Hin*dIII fragment of pGRX8 containing the *grx* promoter (Fig. 2) was labeled by [γ-^{32}P]ATP at the 5′ end of the *Eco*RI terminal and used as a probe. The probe DNA was incubated with or without purified OxyR protein and was partially digested with DNase I. As shown in Fig. 4, the OxyR protein protected the *grx* promoter from DNase I digestion from −34 to −81 with respect to the major transcription initiation site on the bottom strand. As has been observed previously for other *oxyR*-regulated promoters (21), the protected region is large (48 bp) and partially overlaps the putative promoter −35 sequence. In addition, the nucleotide sequence of the protected region in the *grx* promoter showed a high degree of homology (17 of the 20 bases matched) to the consensus motif for oxidized OxyR (Fig. 5) proposed by Toledano et al. based on results obtained by selection of binding sites from pools of random-sequence oligonucleotides (21). Together, these results support the proposed model for OxyR-DNA interaction.

Glutaredoxin 1 is a small dithiol protein originally identified as an alternative hydrogen donor for ribonucleotide reductase in an *E. coli* mutant lacking thioredoxin activity (8). Like thioredoxin, glutaredoxin 1 functions through reversible oxidation-reduction of two cysteine residues in the active center (9). Oxidized glutaredoxin 1 is reduced by glutathione, and oxidized glutathione is reduced by glutathione reductase and NADPH (9). Since expression of glutathione reductase is also regulated by OxyR, two components in the glutathione-dependent hydrogen donor system are induced by peroxide stress in an *oxyR*-dependent fashion. Under peroxide stress conditions, induction of glutaredoxin 1 together with glutathione reductase would be useful for maintaining an appropriate reducing environment for cells.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. **Altuvia, S., M. Almirón, G. Huisman, R. Kolter, and G. Storz.** 1994. The *dps* promoter is activated by OxyR during growth and by IHF and σ^S in stationary phase. Mol. Microbiol. **13:**265–272.
2. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. **143:** 971–980.
3. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell **41:**753–762.
4. **Demple, B.** 1991. Regulation of bacterial oxidative stress genes. Annu. Rev. Genet. **25:**315–337.
5. **Demple, B., and J. Halbrook.** 1983. Inducible repair of oxidative DNA damage in *Escherichia coli*. Nature **304:**466–468.
6. **Farr, S. B., and T. Kogoma.** 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **55:**561–585.
7. **Greenberg, J. T., and B. Demple.** 1988. Overproduction of peroxide-scavenging enzymes in *Escherichia coli* suppresses spontaneous mutagenesis and sensitivity to redox-cycling agents in *oxyR*$^-$ mutants. EMBO J. **7:**2611–2617.
8. **Holmgren, A.** 1976. Hydrogen donor system for *Escherichia coli* ribonucleoside-diphosphate reductase dependent upon glutathione. Proc. Natl. Acad. Sci. USA **73:**2275–2279.
9. **Holmgren, A.** 1985. Thioredoxin. Annu. Rev. Biochem. **54:**237–271.
10. **Höög, J.-O., H. von Bahr-Lindström, H. Jörnvall, and A. Holmgren.** 1986. Cloning and expression of the glutaredoxin (*grx*) gene of *Escherichia coli*. Gene **43:**13–21.
11. **Kinzler, K. W., and B. Vogelstein.** 1989. Whole genome PCR: application to the identification of sequences bound by gene regulatory proteins. Nucleic Acids Res. **17:**3645–3653.
12. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50:**495–508.
13. **Morgan, R. W., M. F. Christman, F. S. Jacobson, G. Storz, and B. Ames.** 1986. Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. Proc. Natl. Acad. Sci. USA **83:**8059–8063.
14. **Mukhopadhyay, S., and H. E. Schellhorn.** 1997. Identification and characterization of hydrogen peroxide-sensitive mutants of *Escherichia coli*: genes that require OxyR for expression. J. Bacteriol. **179:**330–338.
15. **Russel, M., and A. Holmgren.** 1988. Construction and characterization of glutaredoxin-negative mutants of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **85:**990–994.
16. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusion. Gene **53:**85–96.
17. **Storz, G., M. F. Christman, H. Sies, and B. N. Ames.** 1987. Spontaneous mutagenesis and oxidative damage to DNA in *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **84:**8917–8921.
18. **Storz, G., L. A. Tartaglia, S. B. Farr, and B. N. Ames.** 1990. Bacterial

defenses against oxidative stress. Trends Genet. **6:**363–368.
19. **Tao, K., K. Makino, S. Yonei, A. Nakata, and H. Shinagawa.** 1991. Purification and characterization of the *Escherichia coli* OxyR protein, the positive regulator for a hydrogen peroxide-inducible regulon. J. Biochem. **109:**262–266.
20. **Tartaglia, L. A., G. Storz, and B. N. Ames.** 1989. Identification and molecular analysis of *oxyR*-regulated promoters important for the bacterial adaptation to oxidative stress. J. Mol. Biol. **210:**709–719.
21. **Toledano, M. B., I. Kullik, F. Trinh, P. T. Baird, T. D. Schneider, and G. Storz.** 1994. Redox-dependent shift of OxyR-DNA contacts along an extended DNA-binding site: a mechanism for differential promoter selection. Cell **78:**897–909.