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Cloning and Nucleotide Sequence of the *trxA* Gene of *Escherichia coli* K-12

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The *Escherichia coli* K-12 *trxA* gene, the gene encoding thioredoxin, has been cloned and sequenced. The DNA sequence includes 280 base pairs upstream and 46 base pairs downstream of the coding region. The downstream sequence contains the −35 region of the promoter of the *rho* gene. Northern analysis of the *trxA* mRNA and S1 nuclease mapping indicate the presence of two promoters for the *trxA* gene. Initiation from either promoter results in an mRNA containing two potential translation initiation codons, one of which could initiate synthesis of a protein 18 amino acids longer than the mature *trxA* gene product. The 3′ end of the gene, including the last eight codons, contains a stable stem-loop structure (ΔG = −12.9 kcal) typical of a rho-independent transcription termination signal.

Thioredoxin isolated from *Escherichia coli* B is an 11,700-dalton protein which contains an active site for oxidation-reduction by active cystine disulfide (22) that is reduced by the enzyme thioredoxin reductase (30). In vitro, thioredoxin has been shown to participate in the reduction of ribonucleotide (22), methionine sulfoxide (4, 34), sulfate (34, 43), and protein disulfides (15). In addition, thioredoxin functions as an essential subunit of the bacteriophage T7 DNA polymerase (28) and serves an essential function in filamentous bacteriophage f1 and M13 assembly (23, 37, 38).

The primary sequence of the 108 amino acid residues of thioredoxin from *E. coli* B (14) as well as the three-dimensional structure at a 2.8-Å (0.28-nm) level (19) has been determined previously. Thioredoxin is composed of five β sheets and four α helices, with 75% of the residues involved in secondary structure.

Mutants defective in thioredoxin activity, *trxA*, were isolated in *E. coli* B as mutants that were unable to support bacteriophage T7 replication (17). The *trxA* gene was found to be nonessential for *E. coli* growth (17). A *trxA* mutation in *E. coli* K-12 was isolated and mapped to 85 min of the *E. coli* chromosome (27). Since there are 10,000 molecules of thioredoxin per cell (17), the gene encoding thioredoxin would appear to be one of the highly expressed genes of *E. coli*.

In this report, the cloning and sequencing of *trxA* is presented. The protein sequence derived from the DNA sequence of an *E. coli* K-12 strain reported here is shown to differ from the protein sequence reported for an *E. coli* B strain by two inversions of two amino acids; however, comparison of the protein sequencing results (14, 18) with the protein sequence (14) indicates that an error in presenting the protein sequence from an *E. coli* B strain accounts for these differences. Northern analysis of the *trxA* mRNA as well as S1 nuclease mapping indicates that there are two promoters for *trxA*.

MATERIALS AND METHODS

Enzymes and reagents. *Hpa*II was obtained from New England BioLabs, Inc. Other restriction endonucleases, T4 DNA ligase, bacterial alkaline phosphatase, polynucleotide kinase, *E. coli* DNA polymerase I Klenow fragment, and S1 nuclease were obtained from Bethesda Research Laboratories, Inc. All enzymes were used as specified by the manufacturer.

[γ-^{32}P]ATP (7,000 Ci/mmol) and [α-^{32}P]dATP (800 Ci/mmol) were obtained from New England Nuclear Corp.

Bacterial strains. Strain BH2012 was constructed by Lim et al. (23). Its genotype is *araD139? galU galK hsdR rpsL metE46 argH1 trxA2 ilvC*::Tn*5*. Strains JA200(pLC44-7) and JA200(pLC36-14) were obtained from B. Bachmann. The construction of plasmids pLC44-7 and pLC36-14 was described by Clarke and Carbon (7, 8).

Thioredoxin assays. The thioredoxin activity in cells in the late exponential phase of growth was determined either by a methionine sulfoxide reductase coupled assay (11) or by a spectrophotometric assay with 5,5′-dithiobis(2-nitrobenzoic acid) (41). Supernatants obtained after sonication were heated at 80°C for 10 min to inactivate interfering enzymes such as glutathione reductase. The resulting heated extracts were used for the thioredoxin assays.

The protein content of the unheated extracts was determined by a modification (13) of the method of Lowry et al. (24).

Purification of plasmid DNA and DNA restriction fragments. Plasmid DNA was isolated and purified from fresh overnight cultures by the alkaline lysis method described by Maniatis et al. (26), except that phenol-chloroform (1:1) extraction was performed three times and chloroform extraction was performed once before ethanol precipitation.

DNA restriction fragments were recovered from 1% agarose gel by DEAE-cellulose paper elution, *n*-butanol extraction, and ethanol precipitation (9).

DNA sequence analysis. Subclones were made by using M13mp10 and M13mp11 (29) and were sequenced by the dideoxy chain termination method (40) with a synthetic 17-base universal primer.

Isolation of RNA. RNA was isolated from *E. coli* cells in the late exponential phase of growth by the diethyl pyrocarbonate method (42), except that three phenol-chloroform (1:1) extractions were performed before the RNA was precipitated with ethanol.

Northern transfer. Total *E. coli* RNA was separated by electrophoresis on 1.75% agarose containing 6% formaldehyde (35). A GeneScreen Plus (New England Nuclear Corp.) hybridization transfer membrane was used for RNA blotting as specified by the manufacturer.

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TABLE 1. Thioredoxin activities of strain BH2012 containing various *trxA*-containing plasmids

Strain	Plasmid	Thioredoxin activity[a]	Relative activity
MC1061	None	0.014	1.0
BH2012	None	0	0
BH2012	pLC44-7	0.070	5.0
BH2012	pCJF1	0.092	6.6
BH2012	pCJF2	0.431	30.8
BH2012	pCJF4	0.382	27.3
BH2012	pCJF5	0.181	12.9
BH2012	pCJF6	0.065	4.6
BH2012	pCJF7	0.033	2.3
BH2012	pCJF8	0.113	8.1
BH2012	pCJF9	0.007	0.5

[a] Thioredoxin activity is expressed as ΔOD_{412} (optical density at 412 nm) per minute per milligram of protein.

M13 hybridization probes were made from M13mp10-6 and M13mp11-6 by the procedure of Hu and Messing (20). These single-stranded phage each contain a strand of the *Pst*I-*Bam*HI fragment transferred from pCJF6. The hybridizations were carried out at 42°C for 24 h. Autoradiography with preflashed Kodak XAR-5 X-ray film was performed for various lengths of time at −70°C.

S1 nuclease mapping. The 390-base-pair (bp) *Bam*HI-*Ava*II fragment of pCJF5 was isolated and end labeled with polynucleotide kinase. DNA-RNA hybridization and S1 nuclease treatment were performed by the Berk-Sharp procedure (2) as modified by Weaver and Weissmann (45).

RESULTS

Identification of the *trxA* gene. The *trxA* gene was reported to be located at about the 85-min region of the *E. coli* chromosome (27). Lim et al. (23) recently showed that the *trxA* gene mapped closer to the *ilv* operon than reported earlier. Since Neidhardt et al. (31) reported that plasmids pLC44-7 and pLC36-14 complement *ilv* mutations, plasmids pLC44-7 and pLC36-14 were candidates for carrying the *trxA* gene. Using two-dimensional gel electrophoresis of the protein products encoded by pLC44-7 in maxicells, Neidhardt et al. (31) also showed that pLC44-7 encoded a protein that migrated in a similar way to thioredoxin. Strains JA200(pLC44-7) and JA200(pLC36-14) were assayed and found to have a higher level of thioredoxin activity than did other $trxA^+$ strains (data not shown). To confirm the presence of the *trxA* gene in pLC44-7, this plasmid was introduced into strain BH2012 by conjugation. As expected, all ilv^+ transconjugants selected were found to contain high thioredoxin activity [see strain BH2012(pLC44-7) in Table 1 as an example]. While this study was in progress, Lunn et al. (25) independently reported the overproduction of thioredoxin by a cloned *trxA* gene, although the details of the cloning were not reported. As a result of the cloning of the *trxA* gene reported here, Lim et al. (23) were able to show that the *fip* gene cloned by Russel and Model (37) was actually an allele of the *trxA* gene. Russel and Model (38) also reported that *fip* was a thioredoxin allele.

Subcloning of the *trxA* gene. Five DNA restriction fragments were produced from *Pst*I digestion of pLC44-7. These fragments were ligated to the *Pst*I-cleaved and phosphatase-treated plasmid pBR325. The resulting ligation mixture was used to transform the *trxA* mutant strain BH2012. The ampicillin-resistant (Amp^r) transformants selected could be classified into one of the two groups based on colony size. All larger colonies were found to contain thioredoxin activity and to contain a 3.5-kilobase *Pst*I fragment of plasmid pLC44-7. These transformants also regained the ability to support bacteriophage T7 growth. One of these hybrid plasmids was designated pCJF1, and a thioredoxin assay of strain BH2012 carrying pCJF1 is presented in Table 1.

The 3.5-kilobase *Pst*I fragment of plasmid pCJF1 was isolated and partially digested with *Hpa*II, and the resulting fragments were inserted into the *Acc*I site of plasmid pUC13. The larger colonies selected from the Amp^r transformants were shown to be able to support bacteriophage T7 growth. The hybrid plasmids from these transformants were found to have *Hpa*II inserts of different sizes, 900 bp (pCJF2), 650 bp (pCJF4 and pCJF5), and 460 bp (pCJF6). Figure 1 shows the structures and restriction maps of these inserts. Plasmids pCJF4 and pCJF5 were found to have the same insert in a different orientation. Table 1 shows the thioredoxin activities of strain BH2012 containing different hybrid plasmids. Plasmids pCJF2, pCJF4, and pCJF5 in strain BH2012 showed 31-, 37- and 13-fold higher activities, respectively, than that of strain MC1061, the $trxA^+$ parent of strain BH2012. Plasmid pCJF6, the plasmid with the shortest insert, has significantly reduced thioredoxin activity. The insert of plasmid pCJF6 was transferred into the *Bam*HI-*Hin*dIII site of plasmid pUC18 to reverse its orientation compared with the promoter of the *lac* gene. This plasmid was designated pCJF7. Plasmid pCJF7 in strain BH2012 was found to have only half the thioredoxin activity of plasmid pCJF6 (Table 1). This suggested that plasmids pCJF6 and pCJF7 may not have the entire promoter of the *trxA* gene and that the expression of thioredoxin in pCJF6 may be from the promoter of the *lac* gene. Two plasmids, pCJF8 and pCJF9, were constructed by deleting the *Hin*dIII-*Hin*cII and the *Bam*HI-*Hin*cII region from pCJF4 and pCJF5, respectively (Fig. 1). The *Hin*dIII and *Bam*HI ends were filled in with Klenow fragment of DNA polymerase I before blunt end ligation. These hybrid plasmids contain inserts 15 bp shorter than those in pCJF6 and pCJF7. Since plasmids pCJF8 and pCJF9, which differ only in their orientation with respect to the *lac* promoter, have a 16-fold difference in thioredoxin activity (Table 1), we conclude that they do not contain the promoter of the *trxA* gene and that the thioredoxin activity of plasmid pCJF8 is expressed from the *lac* promoter of pUC13.

Nucleotide sequence of the *trxA* gene. Figure 2 presents the nucleotide sequence of the 657-bp *Hpa*II partially digested DNA containing the *trxA* gene. An open reading frame that extends from the ATG at nucleotide 228 to the stop codon (TAA) at nucleotide 609 could encode a protein whose primary structure is very similar to that of the known amino acid sequence of thioredoxin from the B strain (14), except that the N-terminal methionine is removed. Compared with the protein sequence of thioredoxin (14) from a B strain, the gene product encoded from the *E. coli* K-12 gene has amino acids Val_{16}-Leu_{17} and Gly_{71}-Ile_{72} inverted. Examination of the primary protein sequencing data (14, 18) indicates that the protein sequence given in Fig. 5 of reference 14 does not agree with the data presented in Fig. 4 of reference 14 and Fig. 2 of 18. Rather, the data presented in references 14 and 18 agree with the protein sequence of the protein product encoded by the DNA sequence of the *trxA* gene from *E. coli* K-12.

A Shine-Dalgarno sequence was found four nucleotides 5′ to the initiation codon. It contains six out of nine nucleotides complementary to the 3′ end of 16S RNA. In the same reading frame, another ATG codon centered at 228 with a

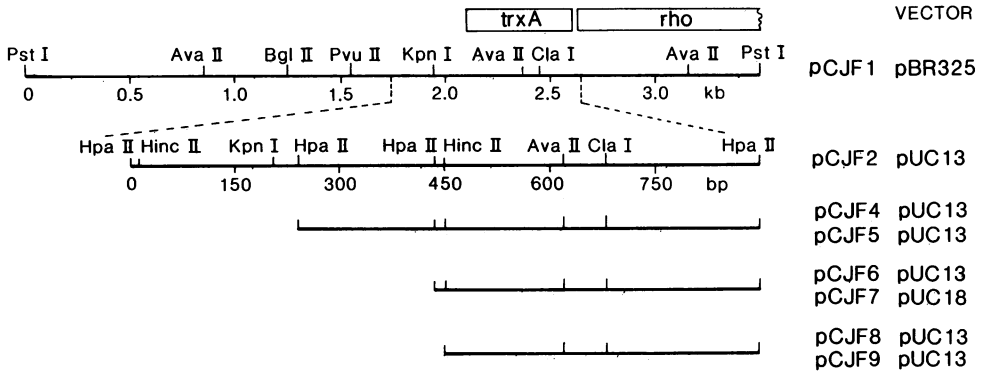

FIG. 1. Construction of various *trxA* plasmids. Plasmids pCJF2, pCJF4, pCJF6, and pCJF8 contain the *trxA* gene in the same orientation as the *lac* gene of vector DNA, and plasmids pCJF5, pCJF7, and pCJF9 are in the reverse orientation.

Shine-Dalgarno sequence only two nucleotides away was found (Fig. 2). A protein product initiated at this ATG codon has not been reported.

When compared with the known nucleotide sequence of the *rho* gene, the DNA sequence (Fig. 2) is seen to contain the −35 region of the *rho* gene at about 35 nucleotides 3′ to the stop codon of the *trxA* gene (33). This fact suggests that the transcription termination of the *trxA* gene must be located between nucleotides 610 and 645 and that the *trxA* gene and *rho* genes have the same direction of transcription. One stable stem-loop structure ($\Delta G = -12.9$ kcal) centered at nucleotide 601 (Fig. 3) was found. This stem-loop contains eight codons including the stop codon, which is unusual. It is possible that this stem-loop structure functions as a rho-independent transcription termination signal (36).

The downstream sequence of the *trxA* gene differs from the reported upstream sequence of the *rho* gene at two positions. At position 619 we report a G, which is absent in

```
    1
CCGGCTACGTCACGACCCGCCAGCGTCACGAAGGGCCAGTGCCTGAATGGGCGTACAGTTATGAAACCCTTTTTTTTCAA

   81
GGGCTTCTACAACCTTCGGATGCAGGGCGAAGTCGGAAAACTTCTGTTCTGTTAAATGTGTTTTGCTCATAGTGTGGTAG
                                          -35 P1                          -10 P1
  161
AATATCAGCTTACTATTGCTTTACGAAAGCGTATCCGGTGAAATAAAGTCAACCTTTAGTTGGTTAATGTTACACCAACA
      -35 P2                      -10 P2          ↑          SD
  241
ACGAAACCAACACGCCAGGCTTATTCCTGTGGAGTTATAT Met Ser Asp Lys Ile Ile His Leu Thr Asp
                                    SD       ATG AGC GAT AAA ATT ATT CAC CTG ACT GAC
  311
Asp Ser Phe Asp Thr Asp Val Leu Lys Ala Asp Gly Ala Ile Leu Val Asp Phe Trp Ala
GAC AGT TTT GAC ACG GAT GTA CTC AAA GCG GAC GGG GCG ATC CTC GTC GAT TTC TGG GCA
  371
Glu Trp Cys Gly Pro Cys Lys Met Ile Ala Pro Ile Leu Asp Glu Ile Ala Asp Glu Tyr
GAG TGG TGC GGT CCG TGC AAA ATG ATC GCC CCG ATT CTG GAT GAA ATC GCT GAC GAA TAT
  431
Gln Gly Lys Leu Thr Val Ala Lys Leu Asn Ile Asp Gln Asn Pro Gly Thr Ala Pro Lys
CAG GGC AAA CTG ACC GTT GCA AAA CTG AAC ATC GAT CAA AAC CCT GGC ACT GCG CCG AAA
  491
Tyr Gly Ile Arg Gly Ile Pro Thr Leu Leu Leu Phe Lys Asn Gly Glu Val Ala Ala Thr
TAT GGC ATC CGT GGT ATC CCG ACT CTG CTG CTG TTC AAA AAC GGT GAA GTG GCG GCA ACC
  551
Lys Val Gly Ala Leu Ser Lys Gly Gln Leu Lys Glu Phe Leu Asp Ala Asn Leu Ala ###
AAA GTG GGT GCA CTG TCT AAA GGT CAG TTG AAA GAG TTC CTC GAC GCT AAC CTG GCG TAA
  611
GGGAATTTGCATGTTGGGTGCCCCGTCGCTAAAAACTGGACGCCCGG
                                  -35 rho
```

FIG. 2. Nucleotide sequence of the *E. coli* K-12 *trxA* gene. The nucleotides are numbered 5′ to 3′ from the upstream *Hpa*II cleavage site. Two transcription initiation sites are indicated by vertical arrows, and their −10 and −35 regions are indicated by underlining. The ribosome binding site for the *trxA* reading frame corresponding to the purified protein is marked by underlining and the designation SD. Another ATG codon having its appropriate Shine-Dalgarno sequence is enclosed by a dotted box. The region of dyad symmetry is depicted by facing arrows. The −35 region of the promoter of the *rho* gene is indicated by a box and the designation −35 rho. The numbers above the sequence indicate the first base of each row.

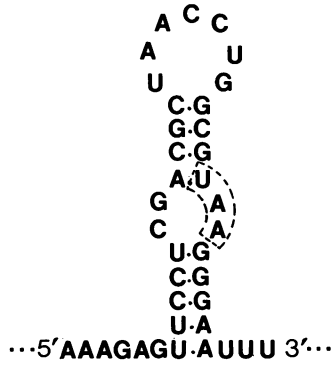

FIG. 3. A possible stem-loop structure of the 3′ end of the *trxA* mRNA, which is the probable rho-independent transcription termination signal. The ΔG of this structure is −12.9 kcal. The stop codon is indicated by a box.

the reported sequence (33); also, the reported sequence (33) has a C between positions 625 and 626 of our sequence. Thus there is one nucleotide addition and one nucleotide deletion between the two sequences.

Transcription initiation and mRNA size. Since plasmids pCJF8 and pCJF9 appear to lack a functional promoter but contain sufficient coding information to produce biologically active thioredoxin when placed under the control of the *lac* promoter, the initiation of transcription must be near this region. To define the region of the *trxA* gene where transcription is initiated, the 5′ terminus of the mRNA was determined by S1 nuclease mapping. A 390-bp *Bam*HI-*Ava*II fragment of pCJF5 was isolated, and the 5′ end was labeled by polynucleotide kinase and hybridized to total cellular RNA isolated from strain MC1061. The hybridized molecules were treated with S1 nuclease to digest single-stranded nucleic acid. The product was run on a denaturing sequencing gel, with a sample of a sequencing reaction used as size markers. Figure 4 indicates that there are two different-sized *trxA* mRNAs, which are respectively initiated at 74 and 114 nucleotides upstream of the ATG codon (Fig. 4). For both initiations, the appropriate −10 and −35 regions could be found and are indicated in Fig. 2. Both −35 regions are rich in A · T pairs, whereas the −10 regions for both initiations, TGTGGTA and TCCGGTG, display limited homology with the consensus sequence. Two single promoter-containing fragments, the *Hin*dIII-*Alu*I fragment for promoter 1 and the *Alu*I-*Sau*3A fragment for promoter 2 from pCJF4, were separately cloned into the promoter-cloning vector pK0100, and expressions from both promoters were confirmed (C.-J. Lim and J. A. Fuchs, unpublished data). A Northern analysis also suggested the existence of two sizes of mRNA (Fig. 5). Total cellular RNA was separated by size by gel electrophoresis, and the *trxA* mRNAs were identified by hybridization to M13mp10-6 and M13mp11-6 probes (M13mp10-6 andMp13mp11-6 contain the single-stranded *Pst*I-*Bam*HI fragment of pCJF6 in opposite orientations). Comparison of the size of the *trxA* mRNA transcripts with the standards indicates that the transcripts are approximately 400 to 440 and 415 to 480 nucleotides in length. The size of the transcripts together with the S1 nuclease mapping indicates that the transcription of the *trxA* gene would terminate very close to the stop codon.

DISCUSSION

The *E. coli* K-12 *trxA* gene, the gene encoding thioredoxin, has been cloned and its DNA sequence has been

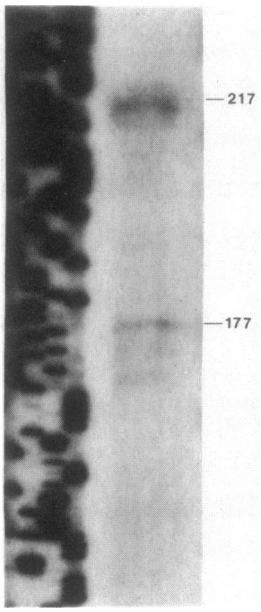

FIG. 4. S1 nuclease mapping of *trxA* mRNA. Total cellular RNA from strain MC1061 was used. The *Bam*HI-*Ava*II fragment, 390 bp in length, from plasmid pCJF5 was end labeled by polynucleotide kinase and used to map the transcripts. The major protected bands are marked.

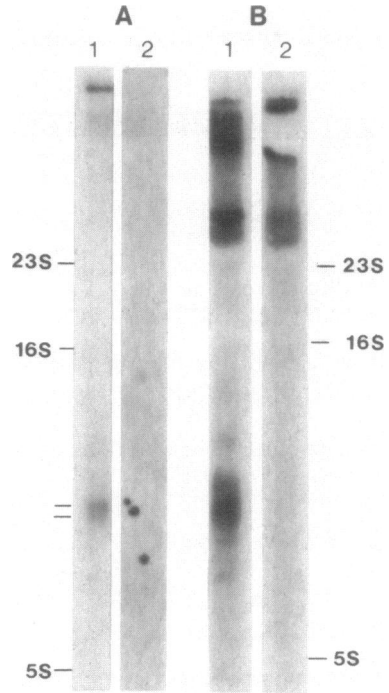

FIG. 5. RNA transfer blot hybridization. Total cellular RNA was prepared from strains MC1061 and BH2012(pCJF5) and used in A and B, respectively. Two M13 hybridization probes were made from M13mp10-6 and M13mp11-6, which contain the *Pst*I-*Bam*HI fragment transferred from pCJF6, and separately used for hybridizations in lanes 1 and 2. The upper dark regions in B correspond to hybridization with plasmid DNA.

determined. The DNA sequence includes 280 bp upstream and 46 bp downstream of the coding region. The downstream sequence contains the −35 region of the promoter of the *rho* gene, the gene encoding the transcription termination factor Rho, which was sequenced by Pinkham and Platt (33). The 35 nucleotides between the stop codon of *trxA* and the −35 region of the *rho* promoter would be expected to contain the transcription termination of *trxA*. The data reported here indicate that termination must occur close to the stop codon. The structure presented in Fig. 3 would be a good candidate. This small region may represent an interesting region to use to define the details of transcription termination. From the nucleotide sequence, the following conclusions can be made: (i) the amino acid sequence of *E. coli* B thioredoxin as reported previously (14) is incorrect, and when corrected the amino acid sequence derived from the DNA sequence of *E. coli* K-12 is identical to that of *E. coli* B thioredoxin except that the N-terminal methionine is removed; (ii) the gene order, *ilv-rep-trxA-rho*, on about 85 min of the *E. coli* chromosome map is confirmed (3); (iii) a missense mutant thioredoxin, *trxA7007*, which is defective in bacteriophage T7 DNA replication and contains an aspartic acid instead of Gly-92 (16) was caused by a GC-to-AT transition; (iv) the unknown polypeptide with an approximate molecular weight of 12,000 to 14,000 produced in maxicells by p39 (33), the plasmid containing the *rho* gene, is thioredoxin.

The significance of the two promoters for the *trxA* gene is unknown. In most cases when more than one promoter is found, the promoters are differentially regulated (1, 10, 39, 44). One exception is the *amp* gene of pBR322, which contains two promoters, only one of which appears to be derived from a natural plasmid; the second promoter was added to *amp* by fusion of the chimeric plasmid (5). The *trxA* gene is not known to be regulated. The two promoters could be involved in high-level expression of the *trxA* gene, although other highly expressed genes contain only one promoter with the exception of an operon encoding the ribosomal protein S21, DNA primase, and sigma factor (6).

We pointed out another ATG codon, which contains its own Shine-Dalgarno sequence, in the same reading frame at about 50 nucleotides 5′ to the ATG codon for thioredoxin. The distance between the ATG codon and its Shine-Dalgarno sequence, two nucleotides, would not explain why this ATG codon could not be used for translation initiation, since a distance of two nucleotides in the β-lactamase gene (12) is sufficient for translation. The codons between the two ATG codons consist of 13 out of 15 nonoptimal codons that can be tested (21). The peptide encoded in this region does not appear to contain residues characteristic of a signal peptide. It is interesting that thioredoxin has been reported to be present at two different cellular locations at different stages of growth of a culture (32). However, no evidence exists to indicate that the longer translation product is ever made. This may be an example where the Shine-Dalgarno sequence, although required for translation, is not sufficient for initiation of translation (12), or alternatively the longer translation product is made and rapidly processed either as an unusual type of signal peptide or simply removed by proteolytic cleavage.

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