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Effect of Cyclic AMP on the Two Promoters of *Escherichia coli* Thioredoxin Gene

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Abstract : Thioredoxin is a multi-functional protein which is ubiquitous in microorganisms, animals and plants. Previously, expression of the *E. coli* thioredoxin gene was found to be negatively regulated by cAMP. In the present study, the effect of cAMP on two separate promoters of the *E. coli* thioredoxin gene was investigated. Cyclic AMP had a repressible effect on P1 and P1P2 promoter activity of the constructs. This effect was also observed in the *cya* strain. The P2 promoter construct gave very high β-galactosidase activity, and its expression was not affected by exogenous cAMP. It was assumed that a *cis*-acting negative element, probably the cAMP-CRP binding site, might have been deleted in the P1 promoter construct. Repression of the thioredoxin gene expression by cAMP appeared to be independent of ppGpp.
Key words : cyclic AMP, *Escherichia coli*, ppGpp, thioredoxin gene

Thioredoxins are small, ubiquitous oxidoreductase proteins with an exposed active site having the structure Trp-Cys-Gly-Pro-Cys. Since thioredoxin was originally discovered in *Escherichia coli* as a reducing agent for ribonucleotide reductase (Laurent *et al.*, 1964), it has been shown to participate in the reductions of sulfate (Porque *et al.*, 1970; Tsang and Schiff, 1976) and methionine sulfoxide (Porque *et al.*, 1970). The oxidized form contains a disulfide bridge which is reduced by thioredoxin reductase or ferredoxin-thioredoxin reductase (Droux *et al.*, 1987). The reduced thioredoxin is a non-specific protein reductase (Holmgren, 1979a; Holmgren, 1979b; Holmgren, 1984). The NADPH-dependent reduction of disulfide bond in DTNB (Moore *et al.*, 1964) or a protein such as insulin (Luthman and Holmgren, 1982) serves as a convenient assay of thioredoxin and thioredoxin reductase. In addition, *E. coli* thioredoxin acts as an essential subunit of phage T7 DNA polymerase (Mark and Richardson, 1976), and is required for the assembly of the filamentous phages M13 and f1 (Lim *et al.*, 1985; Russel and Model, 1985). In higher plants, thioredoxin is belived to be involved in the modulation of enzymatic activities by thiol redox control (Buchana *et al.*, 1979). Thioredoxin is known to be involve a in the regeneration of proteins inactivated by oxidative stress in endothelial cells (Fernando *et al.*, 1992; Davies *et al.*, 1995).

The gene encoding *E. coli* thioredoxin was designated as trxA and was mapped at 84 minutes on the genetic map of the *E. coli* K12 genome (Mark *et al.*, 1977). A HpaII fragment harboring the *E. coli trxA* gene was cloned and sequenced (Lim *et al.*, 1985b). It includes 281 bp upstream from the ATG at which translation is known to initiate and extends to the -35 region of the downstream *rho* gene promoter. The nucleotide sequences of thioredoxin are known in various organisms such as *Chromatium vinosum* (Johnson and Biemann, 1987), Anabaena sp. strain 7119 (Lim *et al.*, 1986), *Bacillus subtilis* (Chen *et al.*, 1989), rats (Tonissen *et al.*, 1989), humans (Wollman *et al.*, 1988), and chickens (Jones and Luk, 1988). Transcription of the *Rhodobacter sphaeroides* thioredoxin gene is regulated by high oxygen tension (Pasternak *et al.*, 1996), and human thioredoxin gene expression is enhanced through a cis-acting regulatory element responsive to the oxidative stress (Taniguchi *et al.*, 1996). In monkey tracheobronchial epithelial cells, the thioredoxin gene expression is transcriptionally up-regulated by retinol (An and Wu, 1992). Transcript levels of *Dictyostelium* thioredoxins were reported to be regulated during the developmental cycle (Wetterauer *et al.*, 1992). Low levels of the mRNAs could be detected during growth, whereas the message levels increased with maximal expression during aggregation, after the onset of development.

Expression of the *E. coli* thioredoxin was previously found to be repressed by cyclic AMP (Sa *et al.*, 1997).

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It was studied with *trxA-lac* translational fusion constructed in the vector pMC1403. In this report we investigated effects of cyclic AMP on the two promoters of *E. coli* thioredoxin gene, which were separately cloned.

Materials and Methods

Materials

Ampicillin, O-nitrophenyl β-D-galactopyranoside (ONPG), adenosine 3',5'-cyclic monophosphate (cyclic AMP; cAMP), and amino acids were purchased from Sigma Chemical Co. (St. Louis, USA). Bacto agar, Bacto yeast extract, and Bacto tryptone were obtained from Difco Laboratories (Detroit, USA). All other reagents were of analytical grade or the highest quality commercially available.

Bacterial strains and plasmids

The strains used in the present study are all derivatives of *E. coli K12* strains. Strain MC1061, used in most experiments, is *ksdR rglB araD139* Δ *(araABC-leu)* Δ*lacZ74 galU*. Strain CL100 (*cya thi pro his leu lacZ*) was also used for measurement of β-galactosidase activity expressed from the fusion plasmids (Sa *et al.*, 1995). Strain CL778 and CL779 are *lac* derivatives of strain CP78 and CP79, respectively, and were constructed by P1 transduction. Strain CP78 is *thi1 leuB hisB6 his65 argH46 ara13 gal3 malA1 xyl7 mtl2 tonA2 supE44*, and strain CP79 is an isogenic *relA2* derivative of strain CP78. The three fusion plasmids

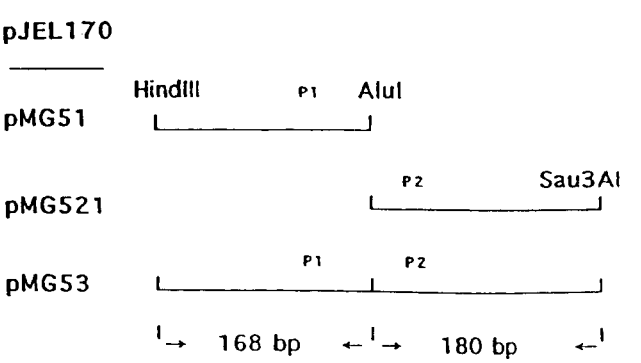

Fig. 1. Constructs containing *trxA* promoter inserts into pJEL 170. The plasmid pMG53 contains a 348 bp HindIII-Sau3AI fragment beginning 120 base pairs upstream of the -35 region of *trxA* P1 and includes 69 nucleotide pairs of the *trxA* coding region. Promoters *trxA* P1 and *trxA* P2 were cleaved by digestion with AluI located between the two tandem promoters in this insert. pMG51 consists of a 168 bp HindIII-AluI fragment cloned into the HindIII-SmaI sites of pJEL170. This fragment includes the -35 and -10 regions of *trxA* P1 and terminates before the -35 region of *trxA* P2. Plasmid pMG521 contains the complementary AluI-Sau3AI fragment including all of *trxA* P2 and was cloned into the SmaI-BamHI sites of pJEL170.

pMG51, pMG521 and pMG53 carry P1, P2, and P1P2 promoters of *E. coli* thioredoxin genes (Fig. 1), which were constructed in a single-copy plasmid pJEL170 (Larsen *et al.*, 1987) harboring promoterless *lacZ* gene.

Bacterial growth

The *E. coli* strains were routinely grown in Luria-Bertani broth (LB broth; 10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) or LB solid agar (LB broth plus 1.5% agar). They were also cultured in M9 minimal medium (Na_2HPO_4 7 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 1 g, 20% glucose 20 ml, 0.01 M $CaCl_2$ 10 ml, 0.1 M $MgSO_4$ 10 ml per liter) and solid minimal agar (M9 minimal medium plus 1.5% agar) after supplementation with required components. All *E. coli* strains used were cultured at 37°C. Ampicillin (50 μg/ml) was added to media, if necessary. Growth was monitored by measuring optical density at 600 nm with a double-beam spectrophotometer. Unless otherwise stated, cells were harvested during exponential growth.

Preparation of crude extract

Crude extract was prepared from bacterial cultures grown in LB broth or M9 minimal medium. Cells were harvested and resuspended in 0.1 M phosphate buffer (pH 7.3). The cells were disrupted by sonication, then centrifuged to remove cell debris and membrane-bound enzymes. Protein concentrations in crude extracts were determined by the method of Lowry *et al.* (1951).

Enzyme assays

β-Galactosidase activity in crude extracts was determined according to a slight modification of the method of Miller (1972). For high β-galactosidase activity, the absorbance change at 420 nm was directly scanned after the preparation of a reaction mixture. For low -galactosidase activity, the reaction mixture was incubated for an appropriate time, then the absorbance at 420 nm was measured.

Results and Discussion

Thioredoxin is a very conserved and compact protein, which is found in every organism and known to have various functions in the cells. The *trxA* gene encoding *E. coli* thioredoxin was cloned and its nucleotide sequence was determined (Lim *et al.*, 1985). Northern analysis and S1 mapping showed that *E. coli trxA* gene contained two different promoters. Their presence was confirmed by dissecting the two promoters into a promoter-cloning vector. In a previous paper (Sa *et al.*, 1997), we have reported that the expression of the *E. coli* thioredoxin gene was negatively regulated by cAMP.

It was obtained from studies using *trxA-lac* fusion constructed in pMC1403. In the same article, the expression of the chromosomal *trxA* gene appeared to be reduced by exogenous cAMP. However, we couldn't understand the effects of cAMP on the two separate *trxA* promoters. This paper describes the effects of cAMP on the two promoters of *E. coli* thioredoxin gene.

Strength of the two separate promoters

In the nucleotide sequence of the *E. coli trxA* gene, an AluI site was found to locate between the tandem promoters. This site was used to dissect the two promoters. Plasmids pMG51, pMG521, and pMG53 (Fig. 1) harbor P1, P2, and P1P2 promoters, respectively, in a single-copy fusion vector pJEL170 (Lim *et al.*, 1997). Their expression was monitored by measuring β-galactosidase activity. The three fusion plasmids were introduced into the *lacZ* strain MC1061, and the resulting strains were cultured in M9 minimal medium with glucose as a unique carbon source. In the extract prepared from the exponential cultures, β-galactosidase assays were performed. The results are shown in Table 1. Strain MC1061/pMG521 showed a much higher specific activity than strains MC1061/pMG51 and MC1061/pMG53. This indicates that only P2 promoter has a stronger promoter activity than the tandem P1P2 promoter. It might be due to the fact that the plausible cAMP-CRP binding site overlapped with P1 promoter had been deleted in plasmid pMG521. Then, the absence of the cAMP-CRP site might remove the repression effect of cAMP in the P2 promoter. Deletion of an unknown negative cis-acting element in plasmid pMG521 could be also assumed. Although deletion of the negative effect in pMG521 is considered, P2 promoter seems to be stronger than P1 promoter. This was based on the fact that about 59% P1P2 promoter activity would be obtained from the P2 promoter in the original location (Table 1).

Table 1. Two *traxA* promter activities in exponential phase of *E. coli* MC1061 containing various *trxA-lac* fusion plasmids

Plasmid[a]	*trxA* promoter	β-Galactosidase specific activity (ΔOD_{410}/min/mg protein)	Relative activity (%)
pMG53	P1P2	1.15	100
pMG51	P1	0.47	41
pMG521	P2	3.55	309

[a]Strain MC1061 with pMG51 containing *trxA* p1 promoter, pMG521 containing *trxA* P2 promoter, and pMG53 containing *trxA* P1P2 promoter were grown in M9 minimal medium with glucose, and during its exponential growth, the cells were harvested. The β-galactosidase activity was determined at 25°C by the spectrophotometric assay using O-nitrophenyl-β-galactopyranoside (ONPG) as a substrate.

Effect of the exogenous cAMP

The effect of exogenously added cAMP on β-galactosidase synthesis in *cya*$^+$ *crp*$^+$ strain MC1061 cells carrying pMG51, pMG521 or pMG53, which had exponentially grown in M9 minimal medium, was examined (Fig. 2). The specific acitivities of β-galactosidase in strains

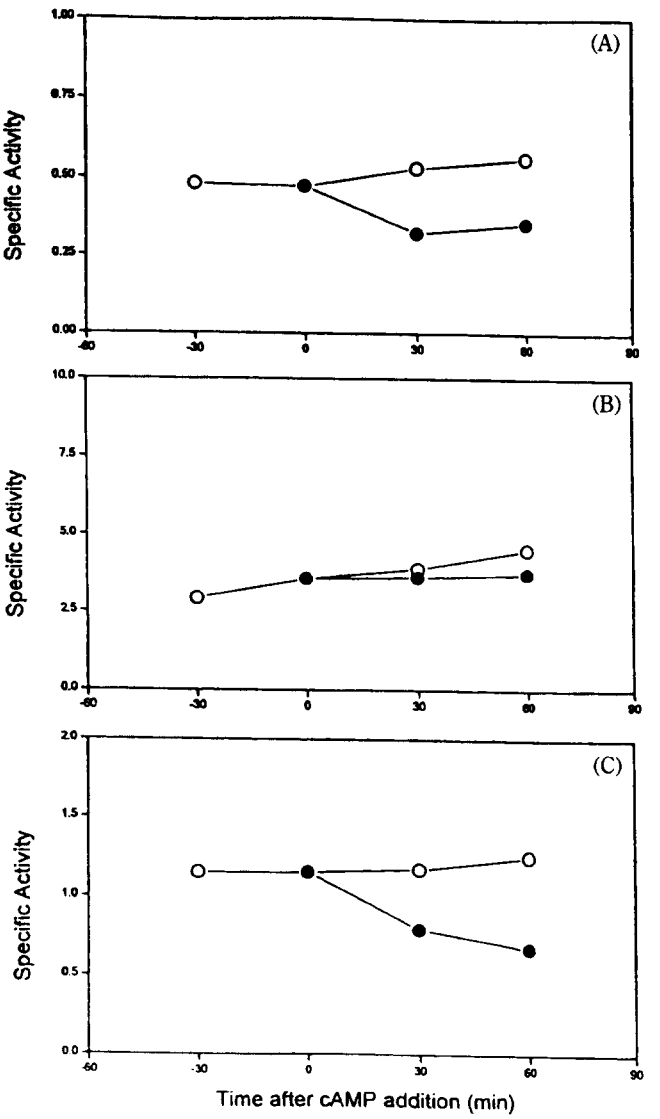

Fig. 2. Time-course effects of exogenous cAMP on the β-galactosidase synthesis in *cya*$^+$ *crp*$^+$ strain MC1061 cells harboring *trxA-lac* fusion plasmids. Strain MC1061 with pMG51 containing *trxA* P1 promoter (panel A), pMG521 containing *trxA* P2 promoter (panel B), and pMG53 containing *trxA* P1P 2 promoter (panel C) were grown in M9 minimal medium with glucose, and during its exponential growth, the culture was split at time 0. Open and closed circles indicated β-galactosidase synthesis in the presence (●—●) and absence (○—○) of 1 mM cAMP. The β-galactosidase activity was determined by the spectrophotometric assay using ONPG as a substrate, and its specific activity was expressed in ΔOD_{410}/min/mg protein. Reaction mixture contained 112 mM β-mercaptoethanol, 1 mM $MgCl_2$, 2.27 mM ONPG, and cell extract in 0.1 M phosphate buffer (pH 7.3).

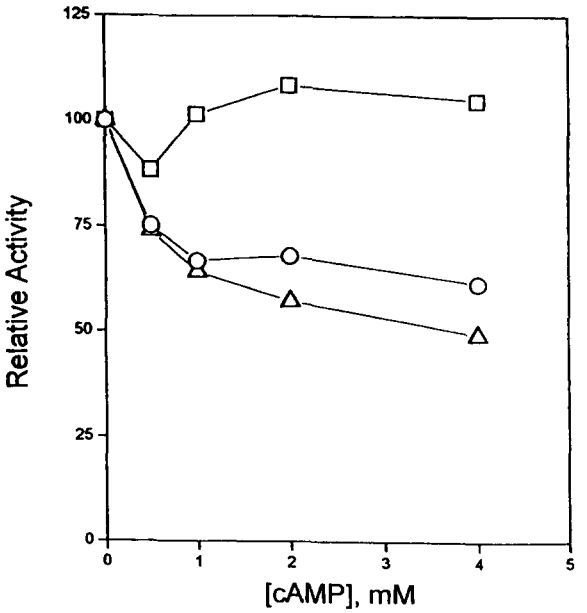

Fig. 3. Concentration effects of cAMP on the expression of the *trxA* promoters in *cya*$^+$ *crp*$^+$ strain MC1061 grown in M9 minimal medium. Strain MC1061 with pMG51 containing *trxA* P1 promoter (△—△), pMG521 containing trxA P2 promoter (□—□), and pMG53 containing *trxA* P1P2 promoter (○—○) were grown in M9 minimal medium with glucose, and during its exponential growth, the culture was split. Cyclic AMP was added at various concentrations, and cells were harvested at 30 minutes after the addition of cAMP. The assay procedure was described in the legend of Fig. 3, and β-galactosidase specific activity was expressed in ΔOD_{410}/min/mg protein.

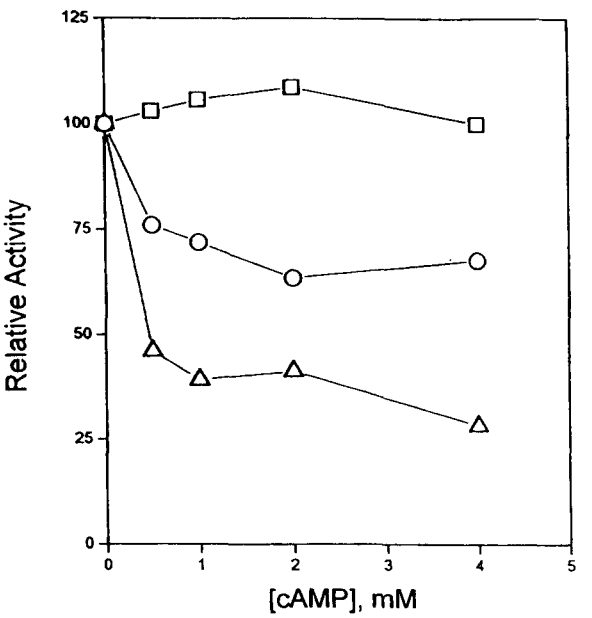

Fig. 4. Effect of cAMP on the expression of the *trxA* promoters in *cya*$^-$ *crp*$^+$ strain CL100 grown in M9 minimal medium. Strain CL100 with pMG51 containing *trxA* P1 promoter (△—△), pMG521 containing *trxA* P2 promoter (□—□), and pMG53 containing *trxA* P1P2 promoter (○—○) were grown in M9 minimal medium with glucose, and during its exponential growth, the culture was split. Cyclic AMP was added at various concentrations, and cells were harvested at 30 minutes after the addition of cAMP. The assay procedure was described in the legend of Fig. 3, and β-galactosidase specific activity was expressed in ΔOD_{410}/min/mg protein.

MC1061/pMG51 and MC1061/pMG53 significantly decreased at both 30 and 60 minutes after the additon of 1 mM cAMP (Fig. 2A and Fig. 2C). However, strain MC1061/pMG521 did not show a cAMP effect (Fig. 2B). This was confirmed using various concentrations of cAMP as shown in Fig. 3. These results suggest that P1 and P1P2 construct are susceptible to repression due to cAMP, but P2 construct is not. Similar results were obtained when the *cya*$^-$ *crp*$^+$ strain CL100 was used as a host (Fig. 4). The P2 only construct was not responsive to the cAMP (0.5-4 mM). The repressive effect of the cAMP in the case of the P1 construct was much stronger than P1P2 construct. However, specific activities were highest in the P2 construct. These results suggest that the P1 and P1P2 constructs still have a cAMP-CRP binding site, whereas the P2 construct does not. However, P2 may be susceptible to the repression by cAMP in its original location. This also corresponds with the results shown in Table 1.

cAMP repression in the absence of ppGpp

The *E. coli trxA* expression was found to be under the control of ppGpp (unpublished results). Strain CL778 and its isogenic *relA* derivative, CL779, are *lacZ* transductants of CP78 and CP79, respectively, and were used as hosts for the transformation of pMG51, pMG 521, and pMG53. The *relA* strain is unable to accumulate ppGpp. The 6 plasmid-containing strains were grown in M9 minimal medium and their extracts were used for β-galactosidase assay (Table 2). Specific activities in the extracts prepared from the *relA*$^+$ strain CL

Table 2. Two *trxA* promoter activities in exponential phase of *E. coli* CL778 (*relA*$^+$) and CL779 (*relA*$^-$) containing various *trxA-lac* fusion plasmids

Plasmid[a]	*trxA* promoter	β-Galactosidase activity (ΔOD_{410}/min/mg protein)	
		CL778 (*relA*$^+$)	CL779 (*relA*$^+$)
pMG51	P1	0.61	0.34
pMG521	P2	8.25	2.89
pMG53	P1 P2	1.61	1.29

[a]Strains CL778 and CL779 with pMG51 containing *trxA* P1 promoter, pMG521 containing *trxA* P2 promoter, and pMG53 containing *trxA* P1P2 promoter were grown in M9 minimal medium with glucose, and during its exponential growth, the cells were harvested. The β-galactosidase activity was determined at 25°C by the spectrophotometric assay using ONPG as a substrate, and its specific activity was expresses in ΔOD_{410}/min/mg protein.

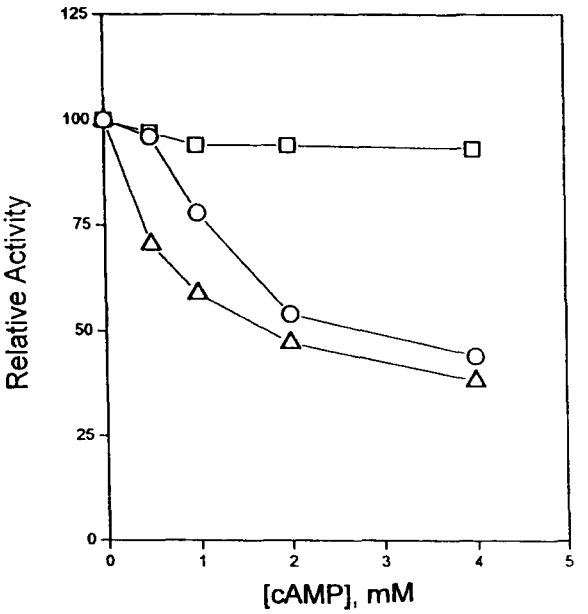

Fig. 5. Effects of exogenous cAMP on the β-galactosidase synthesis in the *relA*⁻ strain CL779 containing *trxA-lac* fusion plasmids. Strain CL779 with pMG51 containing *trxA* P1 promoter (△—△), pMG521 containing *trxA* P2 promoter (□—□), and pMG53 containing *trxA* P1P2 promoter (○—○) were grown in M9 minimal medium with glucose, and during its exponential growth, the culture was split. Cyclic AMP was added at various concentrations, and cells were harvested at 30 minutes after the addition of cAMP. The assay procedure was described in the legend of Fig. 3, and β-galactosidase specific activity was expressed in ΔOD_{410}/min/mg protein.

778 cultures were significantly higher than those from the *relA* strain CL779. These results confirmed that expression of the *trxA* gene was regulated by ppGpp. To find out the relationship between cAMP- and ppGpp-control in the expression of *the E. coli trxA* gene, various concentrations of cAMP were added into the exponential cultures of CL779 strains harboring three promoter constructs. Af- ter 30 minutes, β-galactosidase was measured in their extracts (Fig. 5). The repression effect of exogenous cAMP was observed in the *relA* strain CL779 (Fig. 5). This might indicate that the repressive effect of cAMP on the *trxA* expression is not dependent on the presence of ppGpp. Therefore, our results suggest that at least two separate control mechanisms are involved in the expression of *E. coli* thioredoxin gene. Their physiological role remains elusive.

The present results prove that the *E. coli* trxA P1 and P1P2 promoter are regulated by cAMP in a negative manner. They also propose that the P2 promoter is also repressed by cAMP or other negative regulators. However, repression of the P2 promoter by cAMP is more probable because plasmid pMG521 does not contain its plausible cAMP-CRP binding site. In the original location (Fig. 6) the plausible cAMP-CRP binding site is

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  1
GGGCTTCTACAACCTTCGGATGCAGGGCGAAGTCGGAAAACTTCTGTT

 49                                          ↓
CTGTTAAATGTGTTTTGCTCATAGTGTGGTAGAATATCAGCTTACTAT
-35 P1                    -10 P1              -35 P2

 97                                  ↓
TGCTTTACGAAAGCGTATCCGGTGAAATAAAGTCAACCTTTAGTTGGT
                    -10 P2                    SD

145
TAATGTTACACCAACAACGAAACCAACACGCCAGGCTTATTCCTGTGG

193
AGTTATAT ATG AGC GAT AAA ATT ATT CAC CTG ACT GAC
SD       Met Ser Asp Lys Ile Ile His Leu Thr Asp

5'-TGTGANATNNNTCANA-3'     Consensus
5'-TGTGTTTTGC TCATA-3'     E. coli trxA
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Fig. 6. The nucleotide sequence on the regulatory region of the *E. coli trxA* gene. The two sites for transcriptional initiation are indicated by arrows, and the corresponding -10 and -35 regions for the promoters are indicated by underlining. The initiation codon for the extended thioredoxin is marked by a strike through. The numbers indicate the first nucleotide of each row. A unique AluI site, AGCT, is seen on the -35 region of the P2 promoter. The location of a plausible binding site for cAMP-CRP complex is marked by doubly underlining. The bottom side shows its resemblance to the consensus sequence.

found to exist very close to P2 promoter. Thioredoxin is implicated in a variety of physiological processes, and then it may be susceptible to various regulatory mechanisms at a gene level. It has been demonstrated that thioredoxin genes are regulated by oxygen tension, oxidative stress, and retinol in various organisms. On the other hand, UV irradiation can induce the expression the thioredoxin gene (Yodoi *et al.*, 1992; Nakamura *et al.*, 1994). Negative regulation of the thioredoxin gene by cAMP may be linked to cell growth because rapid growth of *E. coli* cells results in a high level of thioredoxin. Furthermore, it may play a role in many functions of thioredoxins, such as DNA replication, electron source for biosynthetic pathways, regulation of CO_2 fixation, or stimulation of resting cells. In recent years, it has been found that thioredoxins may be involved in the mechanism controlling transcriptional factors (Abate *et al.*, 1990; Deiss and Kimchi, 1991). Therefore, regulation of the thioredoxin gene has become a more important interest than before.

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