JOURNAL OF BACTERIOLOGY, July 1985, p. 238–242
0021-9193/85/070238-05$02.00/0

Vol. 163, No. 1

Direct Cloning of the *trxB* Gene that Encodes Thioredoxin Reductase

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Received 13 February 1985/Accepted 9 April 1985

A strain was constructed which contains mutations in the genes encoding thioredoxin (*trxA*) and thioredoxin reductase (*trxB*) such that filamentous phage f1 cannot grow. The complementation of either mutation with its wild-type allele permits phage growth. We used this strain to select f1 phage which contain a cloned *trxB* gene. The location of the gene on the cloned fragment was determined, and its protein product was identified. Plasmid subclones that contain this gene overproduce thioredoxin reductase.

The facilitated assembly of filamentous phage f1 requires two phage gene products (27; R. E. Webster and J. Lopez, *in* S. Casjens, ed., *Virus Structure and Assembly*, in press) and depends absolutely on host-provided thioredoxin (the product of the *trxA* or *fip* gene [21, 24]) and at least partially on thioredoxin reductase (the product of the *trxB* gene [5, 24]).

Thioredoxin was first identified as a hydrogen donor for ribonucleotide reductase in *Escherichia coli* (13). It is a ubiquitous small protein that contains two cysteines as part of a highly conserved hexapeptide active site (7, 9). Oxidized thioredoxin is reduced by NADPH-thioredoxin reductase (19); reduced thioredoxin is a powerful general reductant of protein disulfides and has been implicated in regulatory processes in animal cells, in which it activates a glucocorticoid receptor (6), and in plant cells, in which it activates a variety of enzymes in response to light (4, 10).

Thioredoxin reductase was identified as the hydrogen donor for thioredoxin (19) and seems to be highly specific. Electrons are believed to flow from NADPH to a flavin adenine dinucleotide molecule bound to each subunit of the dimeric enzyme and from the flavin adenine dinucleotide to the redox-active disulfide active site of thioredoxin reductase (25, 26, 28). A mutation in the thioredoxin reductase gene has been isolated (5); the gene appears not to be required by *E. coli* under normal growth conditions.

Thioredoxin is not essential in *E. coli* (8, 23), but it has been found to be absolutely required for two processes: it is a subunit of the phage T7 DNA polymerase (17) and is thus essential to T7 growth, and it is required for filamentous phage assembly (14, 23, 24). Its precise role in assembly is not yet understood, but it appears to involve an interaction between thioredoxin and one of the two filamentous phage-encoded morphogenetic proteins, the product of gene *I*.

It is not yet known whether the requirement for the *trxA* protein in these two phage systems reflects any requirement for the redox potential of the thioredoxin system. Active T7 DNA polymerase can be reconstituted in vitro from reduced (but not oxidized) thioredoxin in the absence of any additional reducing agent (2). This suggests that cycling between oxidized and reduced thioredoxin is not required for T7 DNA replication. T7 grows normally on a *trxB* mutant strain (5, 24), implying that a thioredoxin reductase-independent reducing system generates sufficient reduced thioredoxin for T7 growth. A recent report (14) concluded from plating data that the *trxB* mutation does not affect filamentous phage, but we have shown (24) that even though f1 forms normal plaques at unit efficiency on a *trxB* host the yield is reduced by an order of magnitude. This f1 dependence on thioredoxin reductase could be due to a larger quantitative requirement for reduced thioredoxin in filamentous phage assembly than in T7 DNA replication, or it might reflect a necessity for cycling via the thioredoxin system in the former but not the latter case. It is also possible that thioredoxin reductase (and thioredoxin) play a fortuitous structural role in filamentous phage assembly.

As part of an effort to elucidate the role of the thioredoxin system in filamentous phage assembly, we isolated the *trxB* gene from *E. coli* by cloning it into a filamentous phage vector. The isolation and characterization of the *trxB* clone are described.

MATERIALS AND METHODS

Bacterial and phage strains. The *E. coli* K-12 strains used in this work are described in Table 1.

Wild-type f1 and derivatives R226, a gene *I* one-codon insertion mutant (3), and R229, an f1 derivative with a unique *Eco*RI cloning site located in a nonessential region of the genome (29), are from our laboratory collection. CGF4, which has a unique *Hin*dIII site inserted at the *Eco*RI site of R229, was from Gerald Vovis (Collaborative Genetics, Inc.).

Plasmid pIN-II-A1 is an expression vector developed by Nakamura and Inouye (20). It is derived from pBR322 and contains the *bla* gene, the *E. coli* lipoprotein (*lpp*) promoter followed by the *lac* UV5 promoter, a portion of the *lpp* coding region, several unique cloning sites, and a region from the 3′ end of the *lpp* gene.

DNA manipulations. Plasmid and replicative form phage DNA was prepared as described (22). Restriction endonuclease digestions, alkaline phosphatase treatment, and ligations were performed by standard means (16). DNA digests were analyzed on ethidium bromide-containing agarose gels, and transfections and transformations were done by the method of Mandel and Higa (15).

The CGF4-*E. coli* library was prepared by ligating *Hin*dIII-digested total *E. coli* DNA from K91 to phage CGF4 replicative form DNA that had been digested to completion with *Hin*dIII and treated with alkaline phosphatase. Competent K38 cells were transfected with the ligation mixture, and 25,000 plaques were washed off the transfection plates and pooled. The phosphatase treatment was effective since control transfections with ligated vector alone gave only 1/10 as many plaques.

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TABLE 1. *E. coli* K-12 strains

Strain	Genotype	Construction (source or reference)
K38	HfrC *sup*$^+$ (λ)	Our laboratory strain; S26 (Garen)
K91	HfrC *sup*$^+$	K38 cured of λ
K963	K91 *srl-300*::Tn*10 recA56*	P1 transduction from JC10240 (A. J. Clark)
K37	K38 *supD*	Our laboratory strain; S26-RIE (Garen)
A179	K38 *trxA*::*kan*	*fip*::*kan* (23)
A237	K38 *trxB zbj-1230*::Tn*10*	*trxB* from BH76 (5); *zbj-1230*::Tn*10* from *E. coli* Genetic Stock Center strain 6392 introduced into BH76, then into K38 by two step P1 transduction.
A219	K37 *trxA*(Am) *ilvY*::Tn*10* *zie-1*::Tn*5*	(24); *trxA*(Am) from JM109 (18)
A245	*trxA*(Am) *supD zie-1*::Tn*5* *ilv*$^+$ (Tet^s)	Spontaneous revertant of A219
A238	*trxA*(Am) *supD zie-1*::Tn*5* *trxB zbj-1230*::Tn*10*	P1 transduction of A245
A280	K37 *trxB zbj-1230*::Tn*10*	P1 transduction
K311	F$^-$ *minA minB gal thr leu thi lacY rpsL*	p678-54 λ^s (1)

Transposon mutagenesis of the cloned *trxB* gene. Mutagenesis was performed by a modification of the procedure of Kleckner et al. (11). K963(pPMR13) cells, which contain *trxB* cloned in a plasmid, were infected at a multiplicity of about 0.2 by λNK467, a Tn*5*-containing *O*am phage that cannot replicate in this *sup*$^+$ host. Kanamycin-resistant colonies were selected, and plasmid DNA was prepared from about 5×10^5 pooled colonies. This DNA was used to transform strain A237, selecting for kanamycin and ampicillin resistance. Transformants were screened for the presence of an inactive *trxB* gene by cross-streaking colonies against phage R226. R226 cannot plaque on the *trxB* mutant strain (24); *trxB*$^+$ plasmids restore the ability of strain A237 to support phage R226 plaque formation.

Preparation of crude extracts and thioredoxin reductase assays. Cultures (1 liter) were grown to mid-log phage. Cells (ca. 3.5 g) were harvested, washed once in 50 mM Tris hydrochloride–50 mM NaCl (pH 7.4), suspended in 9 ml of 50 mM Tris hydrochloride–1 mM EDTA (pH 7.4), and broken by sonication. All subsequent steps were carried out at 4°C. Nucleic acids were precipitated by the addition of 3.6 ml of a 5% solution of streptomycin sulfate, and the mixture was centrifuged successively for 20 min at 30,000 × *g* and 2 h at 100,000 × *g*. Ammonium sulfate (0.66 g/ml of supernatant) was added slowly with stirring to the second supernatant. The resulting precipitate was collected by centrifugation and suspended in 3 ml of 30 mM Tris hydrochloride, and the solution was then dialyzed against 30 mM Tris hydrochloride–1 mM EDTA (1 liter, three changes) over a period of 18 h. The dialysate was used in the assays. Thioredoxin reductase was further purified from crude extracts of K38(pPMR13) cells by chromatography on Sephadex A-25 with a linear NaCl gradient (0.05 to 0.5 M) in 0.05 M Tris hydrochloride (pH 7.4) and subsequent gel filtration on Sephadex G-100. Pooled fractions showing peak thioredoxin reductase activity from the G-100 column contained thioredoxin reductase as a major component, although they were by no means homogeneous (data not shown).

Thioredoxin reductase activity was measured colorimetrically by the reduction of (bis) dithionitrobenzoic acid as described by Moore et al. (19) except that the amount of purified thioredoxin added was as shown in the legend to Fig. 2.

Other procedures. Minicells were purified from strain K311 and labeled with [^{35}S]methionine exactly as described (22). Proteins were electrophoresed on sodium dodecyl sulfate-10 or 12.5% polyacrylamide gels prepared by the method of Laemmli (12).

RESULTS

Isolation of the cloned *trxB* gene. Neither wild-type nor mutant alleles of *trxB* have an easily selectable phenotype. For example, although the rate of production and yield of f1 phage is reduced 10-fold in a thioredoxin reductase mutant strain (24), this level of progeny phage production is sufficient for f1 to form plaques at unit efficiency. By combining a mild *trxA* mutation with the *trxB* mutation, we constructed a host on which filamentous phage f1 will not form plaques. Relief of either the *trxA* or the *trxB* mutation is sufficient to restore phage growth.

The thioredoxin (*trxA*) mutation in strain JM109 isolated by Mark et al. (18) is an amber mutation that arose from a glutamine codon (24). Strains that carry this amber mutation in a *sup*$^+$ background do not support f1 growth, while a *trxA*(Am) *supD* (serine-inserting) derivative allows f1 plaque formation at normal efficiency at 37°C but not at 42°C. This amber mutation, when suppressed by *supD*, served as the mild *trxA* mutation. When the *trxA*(Am) and the *trxB* mutations are combined in a *supD* host, as in strain A238, the efficiency of plaque formation is less than 10^{-8} at 37°C. This host was used to select f1 phage derivatives that contained a functional copy of the wild-type *trxB* gene. The filamentous phage-*E. coli* library (CGF4-*E. coli*$_{HindIII}$) plated with an efficiency of 10^{-5} on the double mutant strain A238. Phage selected on A238 were isolated and retested on A238 and on A179, a thioredoxin null strain (23). Several of the isolates plated on A179 as well as on A238. These were presumed to contain a cloned *trxA* gene and were discarded. Replicative form DNA was prepared from cells infected by three phage isolates that grew only on A238. Each contained an insert of about 6.4 kilobases (kb). Further analysis with additional restriction enzymes indicated that the three isolates contained identical 6.4-kb *Hin*dIII inserts and that both orientations were represented (R363, R364). A restriction map of the cloned segment is diagrammed in Fig. 1A.

***trxB* plasmid-containing cells overproduce thioredoxin reductase.** The 6.4-kb insert was subcloned into pIN-II-A1 (20), creating pPMR13 (Fig. 1B). To confirm that the *trxB* gene had, in fact, been cloned, crude extracts from three different strains, K38(pPMR13), K38, and A237 (*trxB*), were assayed for thioredoxin reductase activity.

The thioredoxin reductase activity in crude extracts from cells carrying pPMR13 was about 25 times that of K38, the *trxB*$^+$ control strain (Fig. 2). This activity was dependent on the addition of substantial amounts of purified thioredoxin. Extracts of *trxB* cells have less activity than those from *trxB*$^+$ cells.

Localization of the *trxB* gene. The *trxB* gene was subcloned from the original 6.4-kb fragment to a 1.7-kb fragment in two steps. First, a *Bgl*II digest of phage R363 replicative form DNA was ligated into the unique *Bam*HI site of pIN-II-A1 (20). Ampicillin-resistant *trxB*$^+$ transformants of A237, a *trxB* mutant strain, were identified by their ability to support

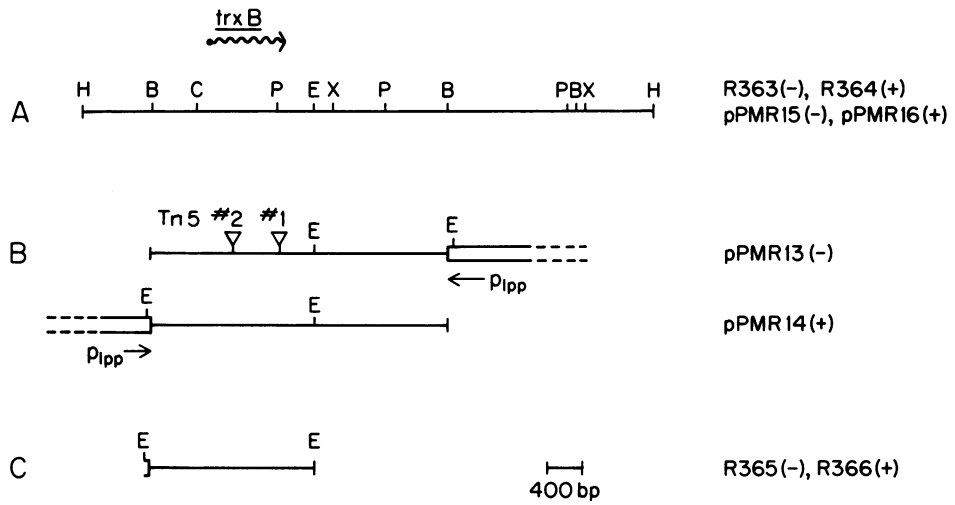

FIG. 1. Restriction map of the *E. coli* DNA fragment containing the *trxB* gene. (A) The restriction map of the 6.4-kb fragment cloned in phage CGF4 (R365 and R366) or in plasmid pIN-II-A1 (pPMR15 and pPMR16) was deduced from analysis of both partial and double restriction enzyme digests. (B) Both orientations of the *trxB*$^+$ 3.2-kb *Bgl*II fragment cloned into the *Bam*HI site of pIN-II-A1 are shown, keeping the insert aligned as in panel A. (C) The *trxB*$^+$ 1.7-kb *Eco*RI fragment from pPMR14 cloned into R229 in both orientations. +, *trxB* gene oriented in the same direction as transcription of the f1 genome (in the phage clones) and as the *lpp/lac* promoter (in the plasmid clones); −, the opposite orientation. H, *Hin*dIII; P, *Pvu*II; C, *Cla*I; B, *Bgl*II; X, *Xho*I; and E, *Eco*RI sites. The inverted triangles indicate the locations of Tn*5* elements that transposed into the cloned *trxB* gene (see the text). The wavy arrow indicates orientation of *trxB*.

the growth of the f1 gene *I* mutant R226 (3) in a cross-streak test.

R226 is one of several f1 gene *I* mutants that are supersensitive to perturbations in the thioredoxin system; they fail to form plaques on various *trxA* (*fip*) missense mutants under conditions that are permissive for both wild-type f1 and mutants in any of the other eight phage genes (21), and they also fail to form plaques on a *trxB* mutant strain (24). The plasmid subclones (pPMR13 and pPMR14) that enabled strain A237 to support R226 plaque formation contained a 3.2-kb insert fragment, as indicated in Fig. 1B.

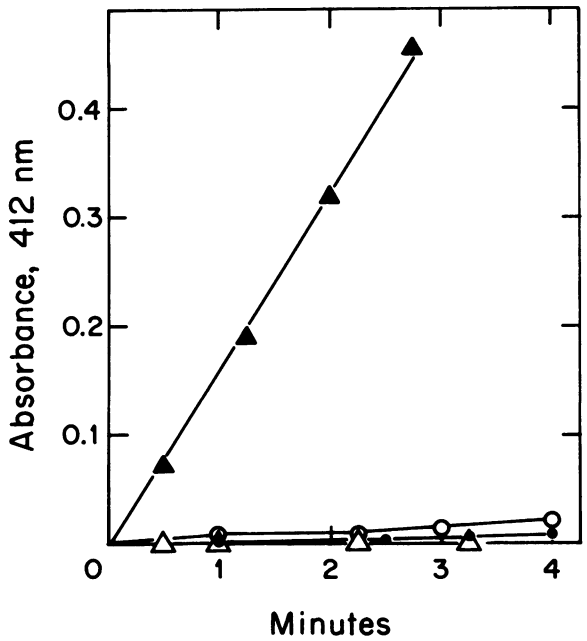

FIG. 2. Thioredoxin reductase activity in lysates from plasmid-containing cells. Thioredoxin reductase was assayed in crude cell extracts (see the text) of K38(pPMR13) (▲), K38 (○), and A237 (*trxB*) (●) in the presence of 11.2 μg of purified thioredoxin (in a 0.5-ml reaction volume) or in the K38(pPMR13) extract in the absence of added thioredoxin (△).

Second, pPMR14 DNA was digested with *Eco*RI and ligated into the *Eco*RI site of the f1 derivative, R229. Phage that contained a functional *trxB* gene were selected directly by transfecting A238 cells, which are nonpermissive for f1$^+$ and R229. These phages contained a 1.7-kb insert as diagrammed in Fig. 1C. Thus, the *trxB* gene is located on a 1.7-kb *Bgl*II-*Eco*RI fragment. Both orientations of this fragment are recovered (R365 and R366). Since only one strand of filamentous phage DNA is transcribed, and since the *trxB*-containing fragment is cloned in a nontranscribed region of the phage genome, this 1.7-kb fragment must also include the *trxB* promoter.

Identification of the *trxB* product and orientation of the gene. Plasmids pIN-II-A1, pPMR15 and pPMR16 (derivatives containing the original 6.4-kb *Hin*dIII fragment cloned in pIN-II-A1 in both orientations), and pPMR13 and pPMR14 were introduced into the minicell-producing strain K311. Minicells were purified and labeled with [^{35}S] methionine, and the cell lysates were electrophoresed on a sodium dodecyl sulfate-10% polyacrylamide gel (Fig. 3). An extra protein band was present in lysates derived from the *trxB*-containing plasmids. This protein comigrated with the major band in a thioredoxin reductase preparation kindly provided by A. Holmgren (data not shown). Its apparent molecular weight, 32,000, is in good agreement with the molecular weight of the dimeric native enzyme which has been estimated to be 65,800 (25). No other insert-specific gene products were detected, even when the lysates were run on acrylamide gels capable of resolving proteins as small as 6,000 daltons.

A band that comigrated with the plasmid-encoded 32,000-dalton radioactive protein was also detected in the Coomassie blue staining pattern of whole cells containing any of the *trxB*$^+$ plasmids. No distinct band of this mobility could be discerned in lysates from cells that lacked a *trxB*$^+$ plasmid.

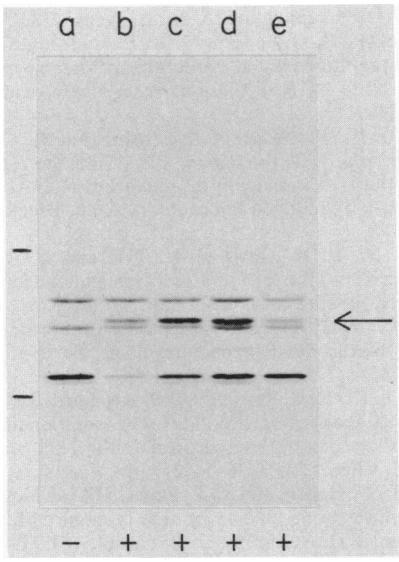

FIG. 3. Plasmid-encoded proteins produced in minicells. Minicells were purified from K311 derivatives containing pIN-II-A1 (lane a) (the vector plasmid), pPMR15 (b), pPMR16 (c), pPMR14 (d), or pPMR13 (e) and labeled with [^{35}S]methionine as described (22). The labeled samples were suspended and heated in sample buffer and electrophoresed on a sodium dodecyl sulfate-10% acrylamide gel (12). The positions of molecular weight standards are indicated to the left of the figure; marker proteins were ovalbumin (43,000 daltons) and α-chymotrypsinogen (25,700 daltons). The arrow indicates the 32,000-dalton polypeptide found in lanes b through e but not a. +, *trxB* complementation by the plasmid; −, absence of complementation.

To confirm that *trxB* complementation was due to the 32,000-dalton product and to determine the orientation of the *trxB* gene, transposon mutagenesis was performed. Tn*5* was introduced into a pPMR13-containing strain via a nonreplicating lambda phage (NK467), and about 5×10^5 Kan^r isolates were selected and pooled. Plasmid DNA prepared from this pool was used to transform strain A237, selecting for kanamycin and ampicillin resistance. Isolates that had lost *trxB*-complementing activity were identified by cross-streaking them against R226. Plasmid DNA was prepared from 12 such *trxB*$^-$ isolates. Suprisingly, plasmid from 10 of these 12 strains no longer contained the Tn*5* element and appeared identical to pPMR13 in size and restriction pattern. Because they had lost *trxB* function, we presume that the Tn*5* initially located within the cloned *trxB* gene had, in each case, excised imprecisely and relocated in the chromosome. The reason for this apparent instability is not known.

Plasmid DNA from the remaining two of the Amp^r Kan^r *trxB* mutant transformants retained the Tn*5* element. The location of the transposon in each plasmid was determined by restriction enzyme analysis as described (22) and is indicated in Fig. 1B. The two mutant plasmids were introduced into K311, and the products from purified minicells were analyzed as before.

pPMR13 *trxB*::Tn*5*-2 encodes a truncated polypeptide of 11,000 to 13,000 daltons, while the truncated product from pPMR13 *trxB*::Tn*5*-1 is about 29,000 daltons (Fig. 4). Both lack the 32,000-dalton band. Thus, the 32,000-dalton protein is the product of the *trxB* gene, and the *trxB* is oriented as indicated by the wavy arrow at the top of Fig. 1. The end points of the gene (i.e., the wavy arrow) were extrapolated from the map position of the Tn*5* insertions and the size of the intact protein and the truncated polypeptides.

DISCUSSION

Efficient filamentous phage assembly depends on both thioredoxin reductase and thioredoxin. Although strains with mutations in thioredoxin reductase plaque f1 normally (14, 24), this lesion does affect phage yield (24). When a *trxB* mutation is combined with a thioredoxin mutation which, by itself, has little effect on phage growth at 37°C, the plating efficiency of f1 on such a doubly mutant host is less than 10^{-8}. This suggests that filamentous phage require a substantial amount of reduced thioredoxin. In the presence of normal levels of wild-type thioredoxin this requirement can be partially met in the absence of a fully functional thioredoxin reductase gene, either by residual thioredoxin reductase activity or by another transhydrogenase. There is not enough reduced thioredoxin, however, to support the growth of phage gene *I* mutants like R226. The combination of a mutant thioredoxin altered both in amount and amino acid sequence because of the amber mutation suppressed by *supD* and the absence of normal thioredoxin reductase must lower the level of reduced thioredoxin below that which even wild-type f1 can tolerate. We used this property to design a host-vector system in which a functional *trxB* gene could be selected directly. This was an efficient selection; all

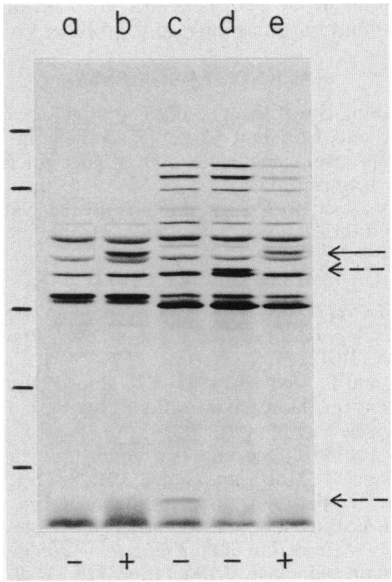

FIG. 4. Localization of the *trxB* gene and determination of the direction of transcription by transposon mutagenesis. Minicells were purified from K311 derivatives containing pIN-II-A1 (lane a), pPMR13 (b), pPMR13 *trxB*::Tn*5*-2 (c), pPMR13 *trxB*::Tn*5*-1 (d), or a *trxB*$^+$ Tn*5*-containing pPMR13 control plasmid (e). Labeling and sample preparation were as described in the legend to Fig. 3. The samples were electrophoresed on a sodium dodecyl sulfate-12.5% acrylamide gel (12). The positions of molecular weight standards are indicated to the left of the figure; marker proteins were bovine serum albumin (68,000 daltons), ovalbumin (43,000 daltons), α-chymotrypsinogen (25,700 daltons), β-lactoglobin (18,400 daltons), and lysozyme (14,300 daltons). The unbroken arrow indicates full-length thioredoxin reductase, and the dashed arrows indicate the truncated derivatives. +, *trxB* complementation by the plasmid; −, absence of complementation.

phage which formed plaques on the doubly mutant strain (A238) carried either the *trxA* or the *trxB* gene.

Cells carrying cloned copies of the *trxB* gene overproduce thioredoxin reductase in about the ratio that would be expected from the consideration of gene copy number alone. Thioredoxin reductase is a dimer and has been thought to consist of identical subunits (26). This is consistent with the observation that thioredoxin reductase activity is provided by a phage whose 1.7-kb insert can code for only a single 32,000-dalton protein.

Expression of thioredoxin reductase from plasmids in purified minicells was higher when transcription from the lipoprotein promoter in the vector was oriented in the same direction as the transcription and translation of the *trxB* gene insert (as in pPMR14 and pPMR16). Orientation-specific expression was not observed in plasmid-containing whole cells in the absence or presence of isopropyl-β-D-thiogalactopyranoside (data not shown). This somewhat puzzling observation can be rationalized by assuming that there is a factor (presumably Rho)-dependent transcription termination signal located upstream of the thioredoxin reductase promotor and that the concentration of the termination factor is low in minicells.

The availability of the *trxB* clone permits the isolation of substantial amounts of thioredoxin reductase by a greatly simplified purification scheme. This should facilitate a number of in vitro studies, particularly in filamentous phage assembly, which has hitherto not been reproducibly achieved. With the cloned gene it should be possible to introduce genetically defined *trxB* null mutants into the chromosome so as to better assess the role of thioredoxin reductase in filamentous phage assembly in vivo.

ACKNOWLEDGMENTS

We thank Kathy Bowdish and Sally Thurston for excellent technical assistance and Christian Gillespie for help in preparing the manuscript. We also thank Norton D. Zinder for helpful critical reading of the manuscript.

This work was supported by grants from the National Science Foundation and the National Institutes of Health.

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