Identification of Endonucleolytic Cleavage Sites Involved in Decay of *Escherichia coli* *trx4* mRNA

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The degradation of individual mRNAs in *Escherichia coli* has been studied through the use of a multiple mutant carrying the *pnp-7* (polynucleotide phosphorylase), *rnb-500* (RNase II), and *rne-1* (RNase E) alleles. In this triple mutant, discrete mRNA breakdown products are stabilized in vivo at the nonpermissive temperature (Arraiano, C. M., S. D. Yancey, and S. R. Kushner, J. Bacteriol. 170:4625–4633, 1988). In the case of thioredoxin (*trx4*) mRNA decay, degradation fragments accumulated at early times after a shift to the nonpermissive temperature. Using Northern (RNA) blots, S1 nuclease analysis, and primer extensions, we identified a series of specific endonucleolytic cleavage sites that occur throughout the transcript in both the triple mutant and a wild-type control. The implications of the complex decay patterns observed are discussed.

Efforts to understand the detailed mechanism of mRNA turnover in *Escherichia coli* have been hampered by the rapidity of the decay process. Previous studies have shown that the observed variations in the rate of decay of individual transcripts in *E. coli* are not related to their length (7). In addition, regions within individual transcripts can decay at different rates (42). Recent results suggest that specific structural features of RNA molecules (13) and possibly specific sequences within the RNA determine the susceptibility to enzymatic attack (12). As pointed out by Deutscher (9), many of the enzymes involved in mRNA decay still remain to be identified.

Of the exonucleases already characterized, polynucleotide phosphorylase and RNase II are believed to be the principal degradative enzymes (9, 11). Endonucleolytic cleavages would generate 3' termini that could become substrates for these exonucleases. For example, in phage lambda, cleavage of the major leftward transcript by RNase III renders the upstream *int* mRNA more susceptible to degradation by 3'-to-5' exonucleases such as RNase II (17). Polycistronic mRNA molecules may also be endonucleolytically cleaved to monocistronic units prior to further degradation (8). Evidence for endonucleolytic cleavages in the *lac*, *ompA*, *bla*, and *papA* mRNAs has been obtained (5, 27, 32, 38).

Arraiano et al. (3) demonstrated that in multiple mutants containing the *pnp-7* (polynucleotide phosphorylase), *rnb-500* (RNase II), and *ams-1* (altered message stability) alleles, the breakdown products of specific mRNAs were dramatically stabilized. One of the transcripts analyzed was the thioredoxin (*trx4*) mRNA, a monocistronic message of 493 nucleotides (nt) that was progressively converted into discrete fragments of smaller size. Recently, Mudd et al. (30), Babitzke and Kushner (4), Meleforts and von Gabain (28), and Taraseviciene et al. (39) have shown that *ams* and *rne* (RNase E) are the same gene.

In this report, we demonstrate that the *trx4* mRNA is initially degraded by a series of specific endonucleolytic

cleavages. We have used S1 nuclease protection experiments and primer extension analysis to map the nucleotide sequences associated with a series of cleavage sites throughout the transcript. For the most part, the cleavage patterns of the *trx4* mRNA were indistinguishable in the *pnp rnb rne* multiple mutant and the wild-type control. The complex patterns observed suggested the possible existence of multiple decay pathways.

MATERIALS AND METHODS

Bacterial strains and plasmids. Host cells were *E. coli* K-12 strains MG1693 (*thyA715*) and SK5704 (*rne-1 pnp-7 rnb-500 thyA715*). The *rne-1* allele was formerly called *ams-1* (4). MG1693 was provided by B. Bachmann. The isogenic strain SK5704 (3) was constructed by P1-mediated transduction (44). Mutants deficient either in polynucleotide phosphorylase (*pnp-7*) (36) or RNase II (*rnb-500*) (11) or with the *rne-1* allele (34) have been previously described. To overproduce the *trx4* mRNA, plasmid pBHK10, a pBR322 derivative containing the promoter and the coding region of *trx4* (43), was transformed into MG1693 and SK5704. The resultant strains were called SK5734 and SK5749, respectively. Transformations were carried out as described by Kushner (20). Plasmid pBHK10 was digested with *Hae*III, and the fragment containing the promoter and the coding region of *trx4* was cloned in the *Sma*I site of the M13mp11 polylinker (29). The *Eco*RI-*Hind*III fragment carrying the *trx4* gene was then cloned into the *Eco*RI-*Hind*III sites of pUC19 (46). The resultant plasmid was called pCMAK1. The *trx4* message was transcribed in the direction *Eco*RI-*Hind*III.

RNA isolation. Strains were grown exponentially at 30°C in L broth (19) supplemented with thymine (50 µg/ml). Initiation of transcription was then inhibited by addition of rifampin (500 µg/ml) and nalidixic acid (20 µg/ml) 20 s prior to shifting the culture to 44°C. Samples were removed at the indicated times and harvested as described by Krzyzek and Rogers (18). Total cellular RNA was extracted by the procedure of Williams and Rogers (45). For the S1 analysis and primer extensions, the RNA pellets were resuspended in distilled water. For the Northern (RNA) analysis, the extracted RNA was first treated with RNase-free

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DNase I and then resuspended into 0.1× SSC (saline sodium citrate; 1× SSC is 0.15 M NaCl plus 15 mM sodium citrate).

RNA blotting analysis. Northern blot analysis was performed by fractionation of RNA samples on a 6% polyacrylamide gel containing 7 M urea, followed by electroblotting to a nylon filter according to the method of Fitzwater et al. (15). The RNA samples and molecular size standards (0.16- to 1.77-kb RNA ladder [Bethesda Research Laboratories]) were denatured before electrophoresis by treatment for 5 min at 80°C in deionized formamide containing 0.3% xylene cyanol, 0.3% bromophenol blue, and 0.37% Na₂EDTA (pH 7.0). Hybridization and washing conditions with the oligonucleotide probes were done by the procedure of Grandbastien et al. (16). The other hybridization and washing conditions were carried out as described by Thomas (40). Autoradiography with Kodak XAR-5 X-ray film was performed at -70°C.

The three synthetic oligonucleotides used as probes were 20-mers that were labeled at the 5' ends with [γ -³²P]ATP and T4 polynucleotide kinase (24). One of the 20-mers synthesized (oligo 1; Fig. 1A) was complementary to nt 11 to 30 of the full-length *trxA* mRNA transcript (21). The second 20-mer (oligo 2; Fig. 1A) was complementary to nt 92 to 111 of *trxA* mRNA, hybridizing to the Shine-Dalgarno region (37). The third 20-mer (oligo 3; Fig. 1A) was complementary to nt 260 to 279 of the *trxA* mRNA. The other two probes used in the Northern analysis were obtained by digesting pBHK10 (43) and subsequently gel purifying the desired fragments. Probe A consisted of a 472-bp *HincII* fragment containing most of the *trxA* coding region. Probe B consisted of a 120-bp *HgiAI*-*HincII* fragment complementary to the 3' end of the *trxA* coding sequence. All DNA fragments were radioactively labeled with [α -³²P]dATP as described by Feinberg and Vogelstein (14).

S1 nuclease mapping and DNA sequencing. The method described by Murray (31) was used with the modification of Aldea et al. (1). However, the probes used were ³²P-labeled DNA fragments that were gel purified before hybridization to total cellular RNA.

The 5' probes (*XmnI* and *MspI*) were generated by digestion of pCMAK1 with either *XmnI* or *MspI*, end labeling with [γ -³²P]ATP and T4 polynucleotide kinase (24), and digestion with *EcoI* and *HincII*, respectively. The 3' probes (*HincII* and *RsrII*) were produced by digestion of pCMAK1 with either *HincII* or *RsrII*, end labeling with [α -³²P]dATP and Klenow fragment (24), and digestion with *NlaIV* and *HindIII*, respectively. All probe fragments were gel purified prior to use. The end-labeled fragments used in S1 analysis were sequenced as described by Maxam and Gilbert (26).

Primer extension analysis. For primer extension analysis of *trxA* mRNA cleavage sites, total RNA was isolated from both SK5734 (wild type, pBHK10 [*trxA*⁺]) and SK5749 (*pnp-7 rmb-500 me-1*, pBHK10 [*trxA*⁺]) as described above. Oligonucleotides complementary to the mRNA at positions 249 to 268 (oligo i), 366 to 386 (oligo ii), 445 to 464 (oligo iii), and 471 to 490 (oligo iv) were synthesized on an Applied Biosystems DNA synthesizer and end labeled with [γ -³²P]ATP (6,000 Ci/mmol; New England Nuclear) by using T4 polynucleotide kinase (Boehringer Mannheim) according to the protocol for labeling blunt-ended double-stranded DNA fragments (24). Primer extensions were performed in aqueous solution with 10 μ g of RNA and 5 pmol of end-labeled oligonucleotide (Fig. 1A). After precipitation, hybrids were resuspended in sequencing gel loading dye (Sequenase 2.0 protocol; United States Biochemical) and run on

either 6 or 8% polyacrylamide-7 M urea gels next to sequencing ladders generated from single-stranded M13 DNA containing the noncoding strand of the *trxA* gene and using the same end-labeled oligonucleotide as primer. Gels were run at 60-W constant power for 1.5 to 2 h, dried, and exposed to Kodak X-Omat AR film at -70°C for various lengths of time.

Enzymes and chemicals. Restriction nucleases *HgiAI*, *RsrII*, and *NlaIV* were supplied by New England Biolabs. *HincII* was purchased from Promega. All other restriction nucleases as well as DNase I (RNase free), S1 nuclease, T4 polynucleotide kinase, and the Klenow fragment of DNA polymerase I were obtained from Boehringer Mannheim. The labeled nucleotides were purchased from Dupont. Nylon blotting filters (Biotrans) were obtained from ICN. Formaldehyde (37%) was purchased from Baker. Rifampin and nalidixic acid were obtained from Sigma.

RESULTS

Northern blot analysis of *trxA* mRNA decay. Northern analysis of RNA derived from MG1693 (wild type) and SK5704 (*me-1 pnp-7 rmb-500*) demonstrated that discrete breakdown products were stabilized in the triple mutant compared with the wild-type control (3). To help establish whether the discrete breakdown products observed in SK5704 (*me-1 rmb-500 pnp-7*) were biologically relevant, the sensitivity of the Northern analysis was increased by isolating RNA from derivatives of MG1693 and SK5704 carrying the *trxA* gene on a multicopy plasmid (pBHK10) (SK5734 [wild type] and SK5749 [*me-1 pnp-7 rmb-500*]). Under these conditions, the decay intermediates previously seen in the *me-1 pnp-7 rmb-500* triple mutant (3) were also observed in the wild-type control strain (Fig. 2 and data not shown). In addition, oligonucleotides as small as 80 nt were detected.

To investigate the mechanism of *trxA* mRNA decay, RNA samples extracted from SK5734 (wild type) and SK5749 (*me-1 pnp-7 rmb-500*) were separated by polyacrylamide gel electrophoresis, transferred to a nylon membrane, and hybridized with various segments of the *trxA* gene (Fig. 1A). As shown in Fig. 2, most of the mRNA breakdown products (<380 nt) detected with probe A (full length) were absent when probe B (Fig. 1A) was used, indicating that these fragments had lost their 3' sequences. In contrast, a probe which hybridized specifically to the 5' terminus of the message indicated that the majority of the cleavage products (<380 nt) still retained most of these sequences (data not shown). This result was confirmed with the oligonucleotide probes described in the next paragraph.

To help simplify the interpretation of the Northern results, RNA was hybridized with either oligo 1 (nt 11 to 30; Fig. 1A), oligo 2 (nt 92 to 111; Fig. 1A), or oligo 3 (nt 260 to 279; Fig. 1A). All of the transcripts hybridizing with either oligo 1 or oligo 2 retained the ribosome binding site and the potential for translation. In Fig. 3, the hybridization patterns of oligo 1 and oligo 2 are compared with that of probe A, a probe that hybridized to the full-length transcript (Fig. 1A and 2). With the exception of species 3 and 6 (Fig. 3A), all major breakdown products observed with probe A hybridized with oligo 2 (Fig. 3B). In contrast, only the full-length transcript and breakdown products 4, 7, and 8 hybridized with oligo 1. Oligo 3 hybridized with band 3 and all major breakdown products except species 7 to 9 (data not shown).

The results with the oligo 1 probe indicated that species 4 arose from cleavage of the full-length transcript (Fig. 3A). By 8 min after rifampin treatment, species 4 was the major

FIG. 1. Diagram of *trxA* gene, including hybridization probes, cleavage sites, and model for decay. (A) The rectangular box defines the translated region. The solid bars indicate DNA fragments or oligonucleotides used as probes in the Northern analysis or primer extension. Thin lines underneath indicate DNA probes used in the S1 mapping. Closed circles and restriction sites *MspI*, *HincII*, *RsrII*, and *XmnI* correspond to the ends of the labeled probes. SD, Shine-Dalgarno region (37). The direction of transcription is indicated by the arrow. Restriction site abbreviations: H, *HaeIII*; M, *MspI*; Hi, *HincII*; S, *Sau3AI*; Sc, *SacI*; R, *RsrII*; Hg, *HgiAI*; X, *XmnI*; N, *NlaIV*. (B) Location of cleavage sites in the *trxA* transcript and model for endonucleolytic cleavage. Within the transcribed region, the ribosome binding site (S.D.) and coding sequences are indicated by the rectangular boxes. The capital letters refer to the 5' endpoints mapped by the S1 nuclease analysis (Fig. 5 and 7; Table 1). The roman numerals refer to the 3' endpoints mapped by the S1 nuclease analysis (Fig. 6 and 7; Table 1). Sites marked by 1.Endo, etc., indicate that the 5' and 3' endpoints matched, suggesting the presence of a site-specific endonucleolytic cleavage. The cleavage numbers are assigned according to Table 1. The numbers shown at the left correspond to the fragments identified in Fig. 3. In this model, endonucleolytic decay products would be degraded to mononucleotides (indicated by dashed lines) by either RNase II (*mb*) or polynucleotide phosphorylase (*pnp*). The order of cleavage is based on densitometric scans of Fig. 3A and B as well as analysis of Northern blots with oligo 3 (data not shown).

band, while the smaller breakdown products 7 and 8 had increased in intensity (Fig. 3A). All of these breakdown products retained the 5' end of the transcript, since oligo 1 hybridizes to nt 11 to 30 (Fig. 1A). Breakdown products 7 and 8 may have arisen from species 4, since they accumulated while species 4 was decreasing (Fig. 3A).

From its electrophoretic mobility, fragment 2 appeared to decay initially to fragment 5 by 4 min after rifampin treatment (Fig. 3B). The results obtained with oligo 3 confirmed that breakdown products 4 and 5 most likely arose from species 1 and 2 (data not shown). Densitometric scans of the autoradiograms shown in Fig. 3 indicated that most of the

FIG. 2. Northern analysis of *trxA* mRNA with a 3'-specific probe. RNA was prepared from *E. coli* SK5734 (wild type) and SK5749 (*rne-1 pnp-7 rnb-500*) as described in Materials and Methods; 2 μ g of total RNA was loaded in each lane. The number above each lane indicates the time (minutes) after rifampin addition and temperature shift when RNA was extracted. Duplicate RNA samples of SK5734 and SK5749 were simultaneously separated by electrophoresis and transferred to a nylon membrane. The membrane was divided; one half was hybridized with probe A (Fig. 1A), and the other half was hybridized with probe B (Fig. 1A). Size standards were derived from an *AluI* digest of pBR322 DNA subsequently end labeled with T4 polynucleotide kinase. Sizes are indicated in nucleotides.

radioactivity observed with oligo 1 at time zero in the triple mutant could be accounted for by decay intermediates seen at 8 and 16 min after the shift to the nonpermissive temperature.

Localization of the cleavage sites. The observation of discrete *trxA* mRNA breakdown products (Fig. 2 and 3) could be explained by the occurrence of specific endonucleolytic processing. Alternatively, exonucleolytic degradation in either the 3'→5' or 5'→3' direction could also generate such fragments if there were regions of secondary structure that caused pausing at unique locations. These two possibilities could be distinguished by determining whether adjacent 5' and 3' termini were detected among the breakdown products. Exonucleolytic processing would leave only either a 5' or 3' terminus, depending on the direction of enzyme movement.

Both S1 nuclease protection and primer extension experiments were used to map the termini of the RNA species generated during the decay of the *trxA* mRNA both in an *rne-1 pnp-7 rnb-500* triple mutant and in a wild-type control (Fig. 1B; Table 1). To identify the 5' termini, RNA samples were hybridized with a 5'-end-labeled DNA probe complementary to the 5' region of the *trxA* transcript (*XmnI* probe; Fig. 1A). S1 analyses identified the full-length transcript (the

418-nt species shown in Fig. 4), which corresponded to the 5' terminus of species 1 observed in Fig. 3. In addition, a series of shorter molecules reflecting multiple 5' endpoints throughout the *trxA* transcript was observed (Fig. 4). The patterns were similar for both the wild-type control (SK5734) and the *rne-1 pnp-7 rnb-500* triple mutant (SK5749).

The nucleotide sequences of the 5' termini were determined by using the nucleotide sequence of the *XmnI* probe as a molecular size standard in the same gel with the S1-protected DNA fragments (Fig. 4). The 5' RNA termini corresponding to the major protected DNA fragments C and E (Fig. 4) resided in the 5' noncoding region of the *trxA* transcript (Fig. 1B). DNA fragments L and M as well as several other minor protected DNA fragments demonstrated the presence of RNA transcripts with 5' termini within the *trxA* coding region.

When the *RsrII* probe (Fig. 1A and 5) was used to map 3' termini, the results were strikingly different from those observed with a 5'-end-labeled probe (Fig. 4). The major band (a 278-bp species visible at the time of rifampin addition) corresponded to the full-length transcript which ends at a terminator located very close to the *rho* promoter (25). In the triple mutant, a major new breakdown species began to appear by 6 min after the shift to the nonpermissive temperature (Fig. 5A). This species was still present at 12 to 16 min after rifampin addition and the shift to the nonpermissive temperature. In the wild-type control, this species was present as a smear that was very faintly visible 8 min after rifampin treatment.

When the 3' termini were mapped with a DNA sequence ladder (Fig. 5B), several new 3' termini were detected during the decay process, in contrast to the observation with the 5'-end-labeled probe (Fig. 4). Some of the bands, such as I (3' endpoint of the full-length message), II, III, V, and VI, disappeared with time (Fig. 5B). These species terminated in nontranslated sequences at the 3' end of the *trxA* mRNA (Fig. 1B). Other bands, such as species X, XII, XV, XVI, XVII, and XX, ended within the translated region (Fig. 1B), and these 3' endpoints arose during mRNA decay (Fig. 5B).

Some differences in the pattern of the triple mutant (SK5749) versus the wild-type control (SK5734) were observed. For instance, species XVII was not present in SK5734 (wild type), while species XVIII and XIX were present only in SK5734. The smear that was observed with SK5734 RNA at 8 min (Fig. 5A) was composed of three bands (XVIII, XIX, and XX), but only species XX was present with mRNA isolated from SK5749.

Evidence for specific endonucleolytic cleavage(s). As shown in Fig. 4 and 5, a number of specific 5' and 3' termini were generated during the decay of the *trxA* mRNA. However, to confirm the existence of endonucleolytic cleavages, it was necessary to demonstrate the presence of contiguous 5' and 3' termini.

To span the entire *trxA* transcript, two additional probes for S1 nuclease protection experiments were generated (*HincII* and *MspI*; Fig. 1A). While most of the 3' termini observed with the *HincII* probe (Fig. 6A) corresponded with the species already identified for the *RsrII* probe (Fig. 5B), species XXI to XXV were identified only with the *HincII* probe. Species XXI, XXIII, and XXV accumulated in the triple mutant. Of particular interest was species XXV because its 3' endpoint was contiguous to the 5' end of the RNA fragment L, identified with the *XmnI* probe (Fig. 4B). The results obtained with the second 5'-end-labeled *MspI* probe (Fig. 6B) confirmed the observations shown in Fig. 4B as well as identifying several additional 5' termini.

FIG. 3. Northern analysis of *trxA* mRNA with oligonucleotide probes. RNA was prepared from *E. coli* MG1693 (wild type [WT]) and SK5704 (*me-1 pnp-7 rnb-500*) as described for Fig. 2 except that 6 μ g of total RNA was loaded in each lane. (A) Duplicate samples of RNA from MG1693 (wild type) and SK5704 (triple mutant) were simultaneously separated by electrophoresis and transferred to a nylon membrane. The membrane was divided; one half was hybridized with probe A (Fig. 1A), and the other half was hybridized with 32 P-labeled oligo 1 (Fig. 1A). This oligomer is complementary to nt 11 to 30 of the full-length *trxA* mRNA. (B) The same as panel A except that 32 P-labeled oligo 2 (Fig. 1A) was used. This oligomer is complementary to nt 92 to 111 of the full-length transcript, a segment that contains the Shine-Dalgarno region but not the translation initiation start at nt 115. The RNA species are identified on the right as 1 to 9. The number above each lane indicates the time (minutes) after rifampin addition and temperature shift when the RNA was extracted. Size standards were derived from *Hae*III digests of pBR322 that were end labeled with T4 polynucleotide kinase. Sizes are indicated in nucleotides.

The 5' termini were also mapped by using primer extension analysis with a series of oligonucleotide primers depicted in Fig. 1A. The results of a typical experiment are shown in Fig. 7. In this case, oligo i was used to identify termini D to L. The primer extensions confirmed 16 of the 21 5' termini identified by S1 nuclease protection (Table 1). Species L, N, O, P, Q, R, S, T, U, and V (Fig. 4 and 6B) all had 5' ends contiguous with 3' termini of fragments identified in Fig. 5 and 6A. Table 1 lists the nucleotide sequences associated with the endonucleolytic cleavage sites as well as the sequences associated with all major termini mapped. The locations of the endonucleolytic sites within the *trxA* transcript are shown in Fig. 1B.

DISCUSSION

***trxA* mRNA decay.** In this work, we have studied the degradation of the *E. coli trxA* mRNA, a constitutively expressed monocistronic message. The use of an *me-1 pnp-7 rnb-500* triple mutant has permitted us to identify by Northern analysis discrete breakdown products of the *trxA* mRNA that were not generally stable enough to be detected in the wild-type control strain (3) (Fig. 2 and 3). These *in vivo* experiments showed that even at the time of shift to the nonpermissive temperature, at least three major *trxA* species existed. The overall complex pattern of fragments suggested that there are multiple pathways of decay, with some species being degraded faster than others. Thus, we chose to focus

on identifying cleavage sites rather than delineating the actual order of product formation.

Although endonucleolytic cleavage of *E. coli* mRNAs has been hypothesized for a number of years, supporting evidence has been obtained only recently (5, 8, 23, 27, 32, 33, 37). By comparing the 3' and 5' termini mapped either by S1 nuclease protection or by primer extension experiments (Fig. 4 to 7 and data not shown), it was possible to identify the contiguous 5' and 3' termini shown in Table 1 (1.Endo to 9.Endo), strongly suggesting the occurrence of endonucleolytic cleavages at the sites indicated in Fig. 1B. In addition, a number of other 5' termini that may also have arisen from endonucleolytic cleavages were mapped. However, because of technical problems with the S1 protection experiments, it was not possible to map the complementary 3' ends. These termini could also have arisen from 5'→3' exonucleolytic degradation that was halted by some structural feature of the RNA, but such an enzyme has yet to be found in *E. coli*.

By comparing the fragment sizes observed in Fig. 3 with those predicted from Table 1, a possible series of events can be hypothesized (Fig. 1B). Thus, the full-length *trxA* transcript (Fig. 3, species 1) could be cleaved at the 3' end to produce breakdown product 4. This decay product accumulated until about 8 min after rifampin addition, and it appeared to correspond to species XIX and XX in Fig. 5B. The 3' end of product 4 would be located at either endonucleolytic cleavage site 5.Endo or 6.Endo (Table 1; Fig. 1B).

TABLE 1. Nucleotide sequences associated with observed cleavage sites^a

Sequence	Downstream product (5')	Upstream product (3')
G T C G <u>A</u> T T T C T G G	L ^b -1.Endo-	XXV
C A A A <u>A</u> T G A T C G C	N ^b -2.Endo-	XXIII
T G A A C A T <u>C</u> G A T C	O ^c -3.Endo-	XXII
C C G A A <u>A</u> T A T G G C	Q ^b -4.Endo-	XXI
C T G C T G C T <u>G</u> T T C	R ^c -5.Endo-	XIX
G C T G T T <u>C</u> A A A A A	S ^b -6.Endo-	XVIII
C G G C A <u>A</u> C C A A A G	T ^b -7.Endo-	XV
G T C T A <u>A</u> A G G T C A	U ^c -8.Endo-	XII
A A G A G T T <u>C</u> C T C G	V ^b -9.Endo-	X
T G C T T T <u>A</u> C G A A A	B ^b	
T A A A G T <u>C</u> A A C C T	C ^b	
A C C T T T <u>A</u> G T T G G	D ^b	
C C <u>A</u> G G C T T <u>A</u> T T C	E, F ^b	
A T A T A T <u>G</u> A G C G A	G ^c	
A G C G A T <u>A</u> A A A T T	H ^c	
A A A T T <u>A</u> T T C A C C	I ^b	
A C A G T T <u>T</u> T G A C A	J ^b	
G T A C T <u>C</u> A A A G C G	K ^b	
T G G G <u>C</u> A G A G T G G	M ^b	
C C C G <u>A</u> T T C T G G A	N ^d	
C A G G G <u>C</u> A A A C T G	N nd	
C A T C G <u>A</u> T C A A A A	P ^b	
A A A A C <u>G</u> T G A A G		XVII
G G T G C <u>A</u> C T G T C T		XIV, XIII
T C A G T T <u>G</u> A A A G A		XI
A A C C T G G C <u>G</u> T A A		IX, VIII
A A G G <u>G</u> A A T T T C A		VII
C A T G T T <u>C</u> G G G T G		VI, V
C C C G T C G <u>C</u> T A A		IV, III
A A A <u>A</u> C T G G A A C G		II

^a All 3' termini were determined by S1 protection. Dashes indicate cleavage sites.

^b 5' cleavage sites determined by both S1 protection and primer extension.

^c 5' cleavage sites determined only by S1 protection.

^d 5' cleavage sites determined only by primer extension.

The complementary 5' terminus (species R) was identified with the *MspI* probe in Fig. 6B.

The smaller breakdown products 7 and 8 (Fig. 3A) also have their 3' ends located at the possible endonucleolytic cleavage sites 2.Endo and 1.Endo, respectively (Table 1; Fig. 1B). This observation would explain why these breakdown products were not detected with oligo 3 (data not shown). They especially accumulated at 16 min after rifampin treatment, when the full-length transcript had disappeared and product 4 was considerably reduced (Fig. 3A). Thus, species 7 and 8 could have arisen from endonucleolytic processing of species 4 (Fig. 3A) or from secondary endonucleolytic cleavages of the full-length transcript.

The origin of breakdown product 2 (Fig. 3) is still not clear. Although its 5' terminus corresponds to the location of a putative second promoter (31), our in vitro transcription

FIG. 4. S1 nuclease mapping of the 5' endpoints of *trxA* mRNA with the *XmnI* probe. Total RNA preparations were subjected to S1 mapping analysis with the 5'-end-labeled *XmnI* probe (Fig. 1A) as described in Materials and Methods. S1-resistant species were analyzed by 6% denaturing polyacrylamide gel electrophoresis. The number above each lane indicates the time (minutes) after rifampin addition when RNA was extracted. Numbers on the left indicate the lengths (in nucleotides) of denatured radiolabeled DNA restriction fragments used as markers. The *XmnI* probe, 5' end labeled on its antisense strand, was chemically cleaved by the base-specific reactions of Maxam and Gilbert (26) in order to generate sequence ladders. Lanes: a, b, and l, DNA markers (*AluI*, *HinfI*, and *HaeIII* kinase-labeled digests of pBR322, respectively); c, S1 nuclease reaction without RNA; d and k, G sequencing ladder; e and k, G+A sequencing ladder; f and g, S1 nuclease reaction with RNA extracted from SK5734 (wild type); h and i, S1 nuclease reaction with RNA extracted from SK5749 (*rne-1 pnp-7 rmb-500*). Fifty micrograms of RNA was added to each lane. Species A to M indicate the major S1-resistant DNA fragments that were found with the *XmnI* probe. Their 5' endpoints were mapped in relation to the sequencing ladder and are shown in the *trxA* map of Fig. 1B. Species A represents the 5' end of the full-length transcript. WT, wild type; M, mutant.

experiments (data not shown) suggest that this species most likely arises as a result of an early 5' cleavage of the full-length transcript. Irrespective of its origin, species 2 appears to be degraded at its 3' end into product 5 (Fig. 3B). If this is the case, then its 3' end would be localized to the 5.Endo-6.Endo region (Table 1; Fig. 1B), where the full-length transcript (species 1) also had a major 3' end when degraded to species 4 (Fig. 3A). Additionally, cleavage of

FIG. 5. S1 nuclease mapping of 3' endpoints of *trxA* mRNA with the *RsrII* probe. Total RNA preparations were subjected to S1 mapping analysis with the 3'-end-labeled *RsrII* probe (Fig. 1A) as described in Materials and Methods; 50 μ g of RNA was added to each lane. (A) Lanes are as in Fig. 4. (B) Lanes: a, b, g, and h, C+T, C, G+A, and G sequence ladders, respectively, of the *RsrII* 3'-end-labeled probe; c and d, S1 nuclease reaction with the RNA extracted from SK5734 (wild type); e and f, S1 nuclease reaction with the RNA extracted from SK5749. Species I to XX indicate the major S1-resistant DNA fragments that were found with the *RsrII* probe. Their 3' endpoints have been mapped with reference to the sequencing ladder and are indicated in Fig. 1B. WT, wild type; M, mutant. Sizes are indicated in nucleotides.

product 5 at the 1.Endo and 2.Endo sites would result in RNA fragments the size of products 8 and 9 (Fig. 3B).

Analysis of cleavage sites. In their analysis of the 5' region of the *lacZ* mRNA, Cannistraro et al. (8) found that certain pyrimidine-adenosine bonds, namely, the sequence UUAU, appeared to be particularly vulnerable to endonucleolytic attack. In the *trxA* mRNA, there are two of these sequences in the untranslated 5' region and a third between amino acids 5 and 6. Cleavage was found to occur at only one of these sites (species F; Fig. 4).

The sequences of the nine cleavage sites shown in Table 1 did not suggest any significant similarities at the nucleotide level. This observation would tend to rule out a sequence-specific endonuclease unless it had properties similar to those of the type I restriction enzymes and cleaved at a distance from the recognition sequence. Indeed, at this point it is not clear whether there is a single endonuclease or an array of such enzymes (9). It is of note that the 1.Endo site (Table 1; Fig. 1B) is homologous to the proposed RNase E recognition site ([A/U]AUU[A/U] [2, 12, 22, 41]). It is possible that at early times after the shift to the nonpermissive temperature, there is some residual RNase E activity. The 188-nt fragment seen at time zero with oligo 1 in SK5749 (Fig. 3A) is the correct size to have arisen from an RNase E cleavage at 1.Endo. We have now shown that RNase E will cleave the 1.Endo site in vitro (22a).

Secondary structures could be recognized and be preferred targets for certain endonucleolytic cleavages. When the Zuker and Stiegler (47) folding program was run on each of the possible endonucleolytic cleavage sites shown in

Table 1, including 25 nt on either side of the cleavage, no pattern of stable stem-loop structures was observed (data not shown). Specifically, RNase III recognition sites such as those demonstrated by Guarneros et al. (17) and Portier et al. (35) for the *int* and *pnp* mRNAs, respectively, were not observed.

Models for mRNA decay in *E. coli*. How do these observations fit into the models of mRNA degradation that have been proposed? It has been suggested that many mRNAs are initially degraded by a rate-determining cleavage at the 5' segment of the mRNA molecule (13). This initial cleavage is then followed by a series of endonucleolytic cleavages and subsequent exonucleolytic degradation of the oligonucleotides to mononucleotides (6). Subbarao and Kennell (38) have also proposed that an endonuclease could follow along behind the last ribosome cleaving the mRNA in a 5'→3' direction at specific sites. A prediction of this model is that as full-length transcripts decay, the smaller breakdown products will be missing their 5' sequences.

The results described above suggest that the *trxA* mRNA decays initially through a series of endonucleolytic cleavages, with certain products being preferentially stabilized. Some of the breakdown products retain their original 5' termini and are shortened at the 3' end as a function of time after shift to the nonpermissive temperature, as shown diagrammatically in Fig. 1B. These species still have the potential to be translated into truncated polypeptides.

While the data presented in Fig. 2 and 3 could be interpreted to suggest decay in the 3→5' direction, the S1 protection experiments shown in Fig. 4 and 6B indicate that

FIG. 6. S1 mapping of additional 3' and 5' endpoints. RNA from *E. coli* SK5734 or SK5749 was annealed with the *HincII* or *MspI* probe (Fig. 1A). Hybrids were treated with S1 nuclease as described in Materials and Methods and analyzed by 6% denaturing polyacrylamide gel electrophoresis using radiolabeled digests of pBR322 as markers. Probes were also cleaved by the base-specific reactions of Maxam and Gilbert (26) in order to generate sequence ladders. Numbers on the left of panel A and on the right of panel B indicate the lengths (in nucleotides) of denatured DNA markers. The number above each lane indicates the time (minutes) after rifampin addition when the RNA was extracted. (A) S1 analysis of the 3' endpoints with the *HincII* 3'-end-labeled probe (Fig. 1A). Lanes: a, denatured kinase-treated DNA markers derived from a *HaeIII* digestion of pBR322; b, G+A sequence ladder of the *HincII* probe 3' end labeled on the antisense strand; c and d, S1 nuclease reaction with RNA extracted from SK5734 (wild type [WT]); e and f, S1 nuclease reaction with RNA extracted from SK5749 (*rne-1 pnp-7 mb-500* [M]); g, C+T sequence ladder of the *HincII* probe 3' end labeled on the antisense strand. Species X to XXV indicate the major S1-resistant fragments that were found with the *HincII* probe. Their 3' endpoints were determined with the sequencing ladder, and their localization is shown in the *trxA* map of Fig. 1B. (B) S1 analysis of the complementary 5' endpoints with the 5'-end-labeled *MspI* probe (Fig. 1A). Lanes: a, G+A sequence ladder of the *MspI* probe 5' end labeled on the antisense strand; b and c, S1 nuclease reaction with RNA extracted from SK5734; d and e, S1 nuclease reaction with RNA extracted from SK5749 (*rne-1 pnp-7 mb-500*); f, S1 nuclease

FIG. 7. Primer extension analysis of the 5' endpoints of *trxA* mRNA, using the oligo i primer. The primer extension analysis was carried out as described in Materials and Methods. RNA was isolated from either SK5734 (wild type [WT]) or SK5749 (*rne-1 pnp-7 mb-500*), and 10 μ g was used in each primer extension with 5 pmol of end-labeled oligo i. Numbers above lanes indicate times (minutes) after the shift to the nonpermissive temperature. Lanes C, T, A, and G are sequencing reactions and give the sequence of the sense strand. Primer extension products are indicated on the left and correspond to products D to L (Table 1).

some full-length species remained up to 6 min after the shift to the nonpermissive temperature. It is thus of interest that while primer extension experiments gave good results with oligos i and ii (Fig. 7 and data not shown), few extension products were seen with oligos iii and iv shortly after the shift to the nonpermissive temperature (data not shown). In addition, although a number of new 3' termini were observed in Fig. 5 and 6A, only one new 5' terminus (O; Fig. 6B) was detected during decay. Thus, it is not possible at this time to determine whether in fact there is any directionality in the decay of the *trxA* mRNA or, for that matter, whether there is a single pathway for decay of this mRNA. It seems clear that while the approach described above can be used to identify cleavage sites, additional experiments will be

reaction without RNA but with the *MspI* probe; g and h, denatured DNA markers (*HinfI* and *HaeIII* kinase-treated digestions of pBR322, respectively). Species C to V indicate the major S1-resistant fragments that were found with the *MspI* probe. Their 5' endpoints, which were mapped with the sequencing ladder, are shown in the *trxA* map of Fig. 1B. Sizes are indicated in nucleotides.

needed to determine the actual mechanism(s) of decay for any given mRNA.

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