JOURNAL OF BACTERIOLOGY, Oct. 1988, p. 4625–4633
0021-9193/88/104625-09$02.00/0
Copyright © 1988, American Society for Microbiology
Vol. 170, No. 10

Stabilization of Discrete mRNA Breakdown Products in *ams pnp rnb* Multiple Mutants of *Escherichia coli* K-12

CECILIA M. ARRAIANO, STEPHANIE D. YANCEY, AND SIDNEY R. KUSHNER*

Department of Genetics, University of Georgia, Athens, Georgia 30602

Received 20 May 1988/Accepted 11 July 1988

The degradation of mRNA in *Escherichia coli* is thought to occur through a series of endonucleolytic and exonucleolytic steps. By constructing a series of multiple mutants containing the *pnp-7* (polynucleotide phosphorylase), *rnb-500* (RNase II), and *ams-1* (altered message stability) alleles, it was possible to study general mRNA turnover as well as the degradation of specific mRNAs. Of most interest was the *ams-1 pnp-7 rnb-500* triple mutant in which the half-life of total pulse-labeled RNA increased three- to fourfold at the nonpermissive temperature. RNA-DNA hybridization analysis of several specific mRNAs such as *trxA* (thioredoxin), *ssb* (single-stranded-DNA-binding protein), *uvrD* (DNA helicase II), *cat* (chloramphenicol acetyltransferase), *nusA* (N utilization substance), and *pnp* (polynucleotide phosphorylase) demonstrated two- to fourfold increases in their chemical half-lives. A new method for high-resolution Northern (RNA) analysis showed that the *trxA* and *cat* mRNAs are degraded into discrete fragments which are significantly stabilized only in the triple mutant. A model for mRNA turnover is discussed.

Although factors that affect mRNA decay can play an important role in controlling gene expression, analyzing the mechanism of mRNA degradation in procaryotes has been particularly difficult because most mRNAs undergo rapid chemical decay, defined as the degradation to oligonucleotides and mononucleotides. Thus, the average mRNA molecule in *Escherichia coli* has a chemical half-life of between 1.3 to 4.0 min depending on the growth temperature (12, 18).

Despite this problem, previous studies have established some general aspects of mRNA decay. (i) The rate of mRNA turnover has no relation to the length of the gene (4). (ii) The segments of the message that decay most rapidly may be located anywhere on the mRNA (35). (iii) The growth rate affects the stability of some transcripts, but this phenomenon is not general (25). (iv) The nucleases responsible for stable RNA (ribosomal and transfer) processing in general do not seem to be involved in mRNA degradation (1, 8).

Recent work by Donovan and Kushner (12) has shown that two exoribonucleases, polynucleotide phosphorylase (PNPase) and RNase II, are directly involved in mRNA degradation. Both enzymes degrade single-stranded RNA processively in the 3′ to 5′ direction (15, 33). PNPase degrades RNA by a reversible phosphorolytic reaction and generates mononucleoside 5′-diphosphates, while RNase II irreversibly hydrolyzes RNA to 5′-monophosphates.

The product of the altered message stability (*ams*) locus has been implicated in mRNA turnover, since the chemical half-life of total pulse-labeled RNA increased significantly at the nonpermissive temperature (26). However, the functional half-lives of several messages were not altered (26). The location of the *ams* gene at 23 min on the *E. coli* chromosome (27) makes it unlikely that it encodes any of the known RNases. Although a DNA fragment that reportedly complements the *ams-1* mutation has been cloned (6), the function of the Ams protein still has not been established.

As part of our continuing effort to characterize the biochemical pathway(s) by which mRNAs are degraded, we constructed a series of multiple mutants containing the *ams-1*, *pnp-7*, and *rnb-500* alleles. All the multiple mutants analyzed showed increased stability of pulse-labeled mRNA. In addition, the chemical half-lives of specific mRNAs such as *trxA* (thioredoxin), *ssb* (single-stranded-DNA-binding protein), *uvrD* (DNA helicase II), *cat* (chloramphenicol transacetylase), *nusA* (N utilization substance), and *pnp* (PNPase) were also increased in these mutants. Northern (RNA) analysis of the *trxA* and *cat* mRNAs on polyacrylamide gels demonstrated the existence of discrete mRNA breakdown products which were dramatically stabilized in an *ams-1 pnp-7 rnb-500* triple mutant. This work provides conclusive evidence that the *ams*, *pnp*, and *rnb* gene products are required for normal mRNA turnover.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. The relevant genotypes of the bacterial strains used are listed in Table 1. Mutants deficient in either PNPase (*pnp-7*) (31) or RNase II (*rnb-500*) (12) or with the *ams-1* allele (26) have been previously described. Both the *ams-1* and *rnb-500* alleles encode thermolabile proteins, while *pnp-7* appears to be a nonsense mutation (S. R. Kushner and R. Ivarie, unpublished data).

P1-mediated transduction (38) was used to construct isogenic strains of the desired genotypes in either *E. coli* C600 or MG1693. The *ams* gene is 30% cotransducible with *pyrC* (27), *rnb* is 80% linked with *pyrF* (2), and *pnp* is 77% cotransducible with *argG* (30). Transposable drug resistance elements inserted into or adjacent to these markers (specifically, Tn*10* insertions adjacent to *argG* and in *pyrC*, and Tn*5* in *pyrF*) were used for the transductions. Drug-resistant transductants were selected and screened for either arginine or uracil requirements by replica plating to appropriate minimal agar medium. P1 lysates grown on strains containing the *ams-1*, *pnp-7*, or *rnb-500* mutation were used to transduce the appropriate nearby marker to prototrophy. Transductants were screened qualitatively for PNPase and RNase II activities as described below. For the *ams-1* mutation, inability to grow at 44°C was determined.

Plasmid pDK39 (Cm^r *rnb-500*) has been described previously (12). Probes for the RNA-DNA hybridizations were derived from the following plasmids: *trxA*, a 470-base-pair *Hinc*II fragment from an M13mp11 derivative (36); *ssb*, a

TABLE 1. *E. coli* strains used

Strain	*ams*[a]	*pnp*[a]	*rnb*[a]	Other marker(s)	Source or derivation
HAK117	*1*	+	+	*thr leu proA argE rpsL gal*	26
JM2111	+	+	+	*pyrC*::Tn*10*	M. Jones-Mortimer
MG1693	+	+	+	*thyA715*	B. Bachmann
SK107	+	*7*	+	*thr leu rna-19 rpsL lac mal mtl xyl*	16
SK1105	+	+	+	*thr leu trpE pyrF*::Tn*5*	11
SK4900	+	+	+	*thr leu argG6 zgi-203*::Tn*10*	S. Kushner
SK5003	+	*7*	*500*	*thr leu*, pDK39 (Cm[r] *rnb-500*)	12
SK5005	+	+	*500*	*thr leu*, pDK39 (Cm[r] *rnb-500*)	12
SK5006	+	+	+	*thr leu*, pDK39 (Cm[r] *rnb-500*)	12
SK5665	*1*	+	+	*thyA715*	This study
SK5671	*1*	*7*	+	*thyA715*	This study
SK5675	+	+	*500*	*thr leu*	This study
SK5684	*1*	*7*	*500*	*thr leu*	This study
SK5689	+	+	*500*	*thyA715*	This study
SK5691	+	*7*	+	*thyA715*	This study
SK5695	*1*	+	+	*thr leu*	This study
SK5704	*1*	*7*	*500*	*thyA715*	This study
SK5715	*1*	+	*500*	*thyA715*	This study
SK5721	*1*	*7*	*500*	*thr leu*, pDK39 (Cm[r] *rnb-500*)	This study
SK5725	*1*	*7*	*500*	*thyA715*, pDK39 (Cm[r] *rnb-500*)	This study
SK5726	+	*7*	*500*	*thyA715*, pDK39 (Cm[r] *rnb-500*)	This study
SK6605	+	+	+	*thyA715*, pDDK1 (Cm[r] *ssb*$^+$)	This study
SK6606	*1*	*7*	*500*	*thyA715*, pDDK1 (Cm[r] *ssb*$^+$)	This study

[a] +, Wild type for the specified genes. Mutant allele numbers are specified.

675-base-pair *Hae*III fragment from pDDK1 (a pBR325 derivative that contains a functional *cat* gene [D. Dorsett and S. R. Kushner, unpublished data]); *cat*, a 1.2-kilobase (kb) *Hha*II-*Bcl*I fragment from pBR325; *pnp*, a 1.9-kb *Pst*I-*Sac*II fragment from pKAK7 (10); *uvrD*, a 2.9-kb *Pvu*II fragment from pVMK45 (22); and *nusA*, a 1.3-kb *Hae*II-*Bgl*II fragment derived from pKAK6 (K. Armstrong and S. R. Kushner, unpublished data). Transformations were carried out as described by Kushner (20).

Growth conditions. Luria broth (L broth) and K medium were as described by Dykstra et al. (13). Thymine (50 μg/ml) was added to the growth medium of strains carrying the *thyA715* allele. Media were supplemented with appropriate antibiotics as needed. Antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.), and final concentrations were 20 μg/ml for chloramphenicol and tetracycline and 50 μg/ml for kanamycin.

Strains were grown at 30°C in a gyratory water bath. They were shifted to 44°C at 40 Klett units (1×10^8/ml) (green filter no. 54 for L broth and no. 42 for K medium). When cell cultures reached a Klett reading of 100 units, they were diluted 1:10 into fresh prewarmed medium. Generation times were determined in L broth at 30°C. Cell cultures were grown to 40 Klett units and diluted back to 15 Klett units, and then readings were taken every 25 min. To determine the viability of the strains, samples were removed periodically during the growth experiments, diluted in M56/2 buffer (21), plated on L agar plates, and incubated at 30°C for 24 to 36 h.

Enzyme assays. PNPase was assayed in cell lysates by the $P_i \rightleftharpoons$ADP exchange reaction described by Grunberg-Manago (15) and modified by Reiner (30, 31). RNase II activity was assayed qualitatively in vitro as described by Donovan and Kushner (10). Lysates were first incubated for 10 min at 44°C to inactivate the thermolabile RNase II protein (encoded by *rnb-500*) and then assayed for activity at 30°C.

Determination of chemical half-lives. The chemical decay of pulse-labeled RNA was assayed essentially as described by Donovan and Kushner (12). Thus, in these experiments, mRNA decay was measured as the degradation of RNA to oligonucleotides too short to be precipitated with trichloroacetic acid. Exponentially growing cells were pulse-labeled for 1 min with 3 μCi (90 nmol) of [5, 6-^{3}H]uridine (Dupont, NEN Research Products, Boston, Mass.) per ml. The labeling was stopped by the addition of 500 μg of rifampin (Sigma) per ml, 20 μg of nalidixic acid (Sigma) per ml, and 200 μg of uridine per ml.

To determine the decay of specific messages, we added the rifampin and nalidixic acid to stop initiation of new RNA synthesis just prior to shifting to 44°C. Samples of 7 ml were removed at the indicated times and harvested as described by Krzyzek and Rogers (19). Total cellular RNA was extracted by the procedure of Williams and Rogers (39), treated with DNase I (RNase-free; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and hybridized with specific probes (see RNA-DNA hybridization). Only curves having a least-squares measure of fit of ≥0.9 were used in half-life determinations. Errors were estimated from the standard deviation of the slope.

RNA-DNA hybridization. The purified RNA samples were analyzed by using dot blots, slot blots, and agarose or polyacrylamide gel electrophoresis. The dot-blot and slot-blot techniques were a modification of the method of White and Bancroft (37). To an appropriate volume of RNA, 0.4 volume of formaldehyde (37%; J. T. Baker Chemical Co., Phillipsburg, N.J.) and 0.6 volume of 20× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate) were added. The solution was placed in a 65°C water bath for 15 min and subsequently transferred to ice. The denatured RNA was diluted into 15× SSC to the desired concentrations and spotted onto nitrocellulose paper (Schleicher & Schuell, Inc., Keene, N.H.). RNA blots were hybridized with radioactively labeled DNA fragments by the method of Thomas (34) and then autoradiographed at −70°C. All DNA fragments were radioactively labeled with [^{32}P]dATP (Dupont, NEN Research Products) as described by Feinberg and Vogelstein (14). Quantitative determination of hybridization was done by two methods: (i) direct counting in a scintillation counter of the dots cut from the nitrocellulose paper; (ii)

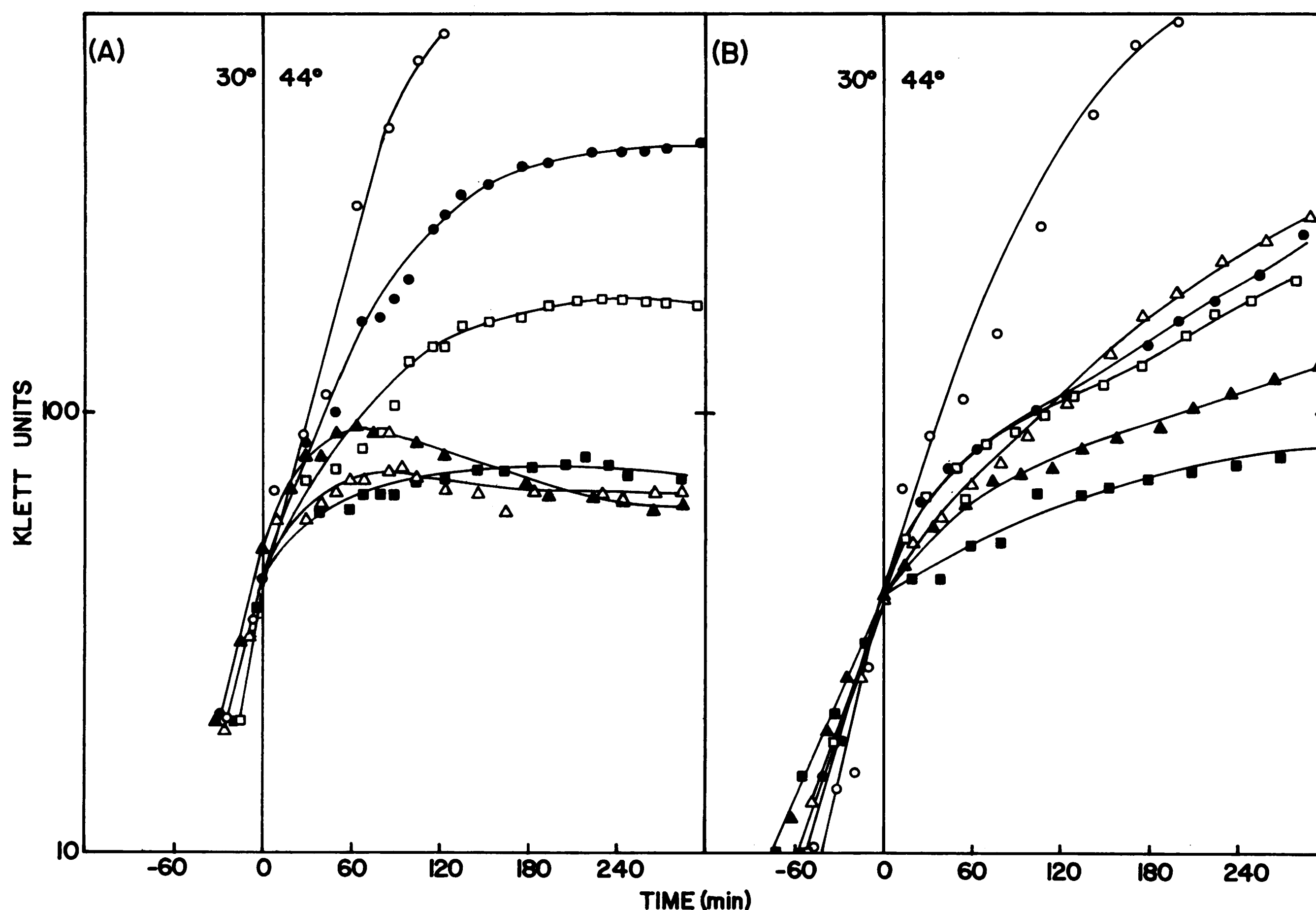

FIG. 1. Growth rates of various strains at 30 and 44°C. Cells were grown in either L broth (A) or K medium (B) as described in Materials and Methods. When cell cultures reached a Klett reading of ≈100 units, they were diluted 1:12 into fresh prewarmed medium. Data were plotted after multiplying Klett units by the appropriate dilution factor. Symbols: ○, MG1693 (*ams*$^+$ *pnp*$^+$ *rnb*$^+$); ●, SK5665 (*ams-1*); □, SK5715 (*ams-1 rnb-500*); ■, SK5704 (*ams-1 pnp-7 rnb-500*); ▲, SK5725 [*ams-1 pnp-7 rnb-500*(pDK39)]; △, SK5726 [*pnp-7 rnb-500*(pDK39)].

densitometric analysis of the autoradiograms. The plateau level of stable RNA was subtracted from all the time points. The percentage of specific mRNA hybridized was calculated from the radioactivity (counts per minute) at a given time divided by the maximum radioactivity in the unstable RNA fraction. The half-life of mRNA was estimated from a plot of the percentage of specific mRNA hybridized versus time after the addition of rifampin.

In the Northern analyses, total mRNA and molecular size standards were denatured in deionized formamide containing 0.3% xylene cyanol, 0.3% bromophenol blue, and 0.37% disodium EDTA. The RNA species were then separated in either 5 or 6% polyacrylamide gels containing 7 M urea unless noted otherwise. Molecular size standards were derived by *Alu*I digestion of pBR322 plasmid DNA and end labeled with polynucleotide kinase (Boehringer Mannheim) or a 0.16- to 1.77-kb RNA ladder obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). After electrophoresis, the RNA was electroblotted onto nylon membranes (Biotrans; ICN Pharmaceuticals Inc., Irvine, Calif.) by a procedure developed by T. Fitzwater and B. Polisky (personal communication). Hybridizations were done as described by Thomas (34). Autoradiography with Kodak XAR-5 X-ray film was performed for various lengths of time at −70°C.

RESULTS

Construction of multiple mutants. A series of isogenic strains containing various combinations of the *ams-1*, *pnp-7*, and *rnb-500* alleles were constructed in both the C600 and MG1693 genetic backgrounds (Table 1). Of particular interest was the fact that the *pnp-7 rnb-500* double mutant could only be constructed if the *rnb-500* mutation was also present on a multicopy plasmid (pDK39). In contrast, the *ams-1 pnp-7 rnb-500* multiple mutant was viable in the absence of pDK39.

Growth properties of multiple mutants. Growth experiments were performed in either L broth or K medium. In L broth, both SK5726 [*pnp-7 rnb-500*, pDK39 (*rnb-500*)] and SK5725 [*ams-1 pnp-7 rnb-500*, pDK39 (*rnb-500*)] ceased growing within 60 to 85 min after the shift to the nonpermissive temperature (Fig. 1A). The profile of SK5704 (*ams-1 pnp-7 rnb-500*) was interesting in that the growth rate dropped immediately after the shift to 44°C, in contrast to SK5725 [*ams-1 pnp-7 rnb-500*, pDK39 (*rnb-500*)]. SK5665 (*ams-1*) and SK5715 (*ams-1 rnb-500*) continued to grow in L broth for several generations, but their growth rates decreased progressively in comparison with that of the wild-type control, MG1693.

TABLE 2. Generation times and chemical half-lives of pulse-labeled RNA in various strains

Strain	ams[a]	pnp[a]	rnb[a]	Generation time (min)[b]	Half-life (min)[c]	SD (σ)
MG1693	+	+	+	35	3.4 (2.7)[d]	0.4 (0.4)[d]
SK5006	+	+	+		3.6	0.5
SK5665	*1*	+	+	35	9.0 (4.2)[d]	1.8 (0.8)[d]
SK5695	*1*	+	+		7.9	1.3
SK5671	*1*	*7*	+	66	7.3	1.3
SK5715	*1*	+	*500*	43	7.5	1.7
SK5726	+	*7*	*500*, pDK39 (*rnb-500*)	67	4.8 (3.8)[d]	0.7 (0.8)[d]
SK5003	+	*7*	*500*, pDK39 (*rnb-500*)		5.3	0.7
SK5704	*1*	*7*	*500*	63	8.9 (2.7)[d]	1.5 (0.4)[d]
SK5684	*1*	*7*	*500*		10.8	2.7
SK5721	*1*	*7*	*500*, pDK39 (*rnb-500*)		15.8	4.1
SK5725	*1*	*7*	*500*, pDK39 (*rnb-500*)	62	11.4	2.4

[a] +, Wild type for the specified genes. Mutant allele numbers are specified.
[b] Generation times were determined in L broth at 30°C as described in Materials and Methods.
[c] The half-life of total pulse-labeled RNA was determined at 44°C as described in Materials and Methods. Half-lives were estimated from a plot of percentage of [^{3}H]RNA remaining versus time after the addition of rifampin. Only curves having a least-squares measure of fit of >0.9 were used in the mRNA half-life estimations. The standard deviations (σ) for the half-lives were calculated from the σ of the slopes determined by the standard statistical methods.
[d] Half-lives determined at 30°C.

In K medium (Fig. 1B), SK5725 [*ams-1 pnp-7 rnb-500*(pDK39) (*rnb-500*)] and SK5726 [*pnp-7 rnb-500*, pDK39 (*rnb-500*)] continued growing after the temperature shift, in contrast with the L broth results. Similarly, SK5715 (*ams-1 rnb-500*) and SK5704 (*ams-1 pnp-7 rnb-500*) continued growing slowly in K medium at 44°C. The other strains did not show any significant difference in the K medium relative to their growth in L broth. Interestingly, SK5715 (*ams-1 rnb-500*) grew more slowly in L broth than SK5665 (*ams-1*) but matched its growth in K medium.

Generation times were determined in L broth at 30°C (Table 2). SK5671 (*ams-1 pnp-7*) grew considerably slower (66 min) than SK5715 (*ams-1 rnb-500*) (43 min). The triple mutant SK5704 (*ams-1 pnp-7 rnb-500*) had a generation time slightly faster (63 min) than that of SK5671 (*ams-1 pnp-7*). The presence of multiple copies of the *rnb-500* allele did not seem to affect growth at 30°C, since the generation times were similar for SK5704 (*ams-1 pnp-7 rnb-500*) and SK5725 [*ams-1 pnp-7 rnb-500*, pDK39 (*rnb-500*)].

The viability of single and double mutant strains deficient in PNPase or RNase II or both at 44°C has previously been reported (12). When the viability of SK5704 (*ams-1 pnp-7 rnb-500*) was determined after a shift to the nonpermissive temperature, a 10-fold drop in viability was observed (Fig. 2). It took only 28 min at 44°C to reduce the number of CFU at 30°C by 50%.

Chemical half-life of total pulse-labeled RNA. The chemical half-life of total pulse-labeled RNA was measured at 44°C in strains carrying various combinations of the *ams-1*, *pnp-7*, and *rnb-500* alleles (Table 2). Significant differences were noted between the wild type (3.4 to 3.6 min) and the *ams-1 pnp-7 rnb-500* triple mutant (11.4 to 15.8 min). The presence of the *ams-1* allele had the most striking effect on the measured mRNA half-life (Table 2). There was no obvious correlation between generation time at 30°C and the half-lives obtained after the shift to 44°C (Table 2). Of interest was that the *ams-1* single mutant had a slower rate of decay (9 min) than either the *ams-1 pnp-7* (7.3 min) and or the *ams-1 rnb-500* (7.5 min) double mutant. In the same genetic background, the *pnp-7 rnb-500* double mutant had a half-life of 4.8 min. The results were comparable in both the C600 and MG1693 genetic backgrounds. In addition, the presence of pDK39 (*rnb-500*) in *ams-1 pnp-7 rnb-500* genetic backgrounds led to an increase in chemical half-life (SK5704 [*ams-1 pnp-7 rnb-500*], 8.9 min; SK5725 [*ams-1 pnp-7 rnb-500*, pDK39 (*rnb-500*)], 11.4 min) (Table 2).

Chemical decay of specific mRNAs. After growth of the cells in L broth to 40 Klett units at 30°C, rifampin and

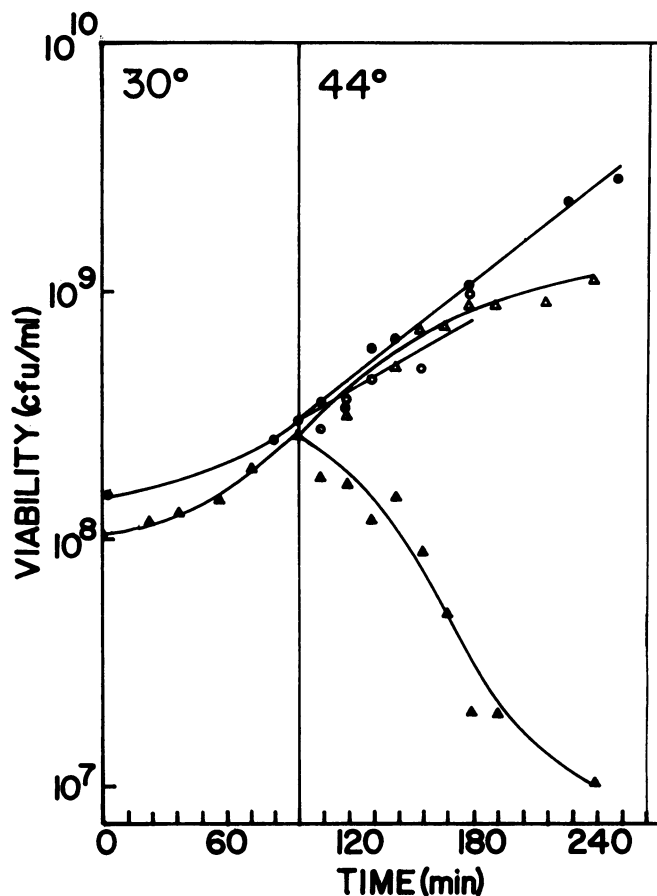

FIG. 2. Viability of MG1693 and SK5704 at 30 and 44°C. The strains were grown in L broth as described in Materials and Methods. Symbols: ○, MG1693 (wild type) at 30°C; ●, MG1693 (wild type) at 44°C; △, SK5704 (*ams-1 pnp-7 rnb-500*) at 30°C; ▲, SK5704 (*ams-1 pnp-7 rnb-500*) at 44°C.

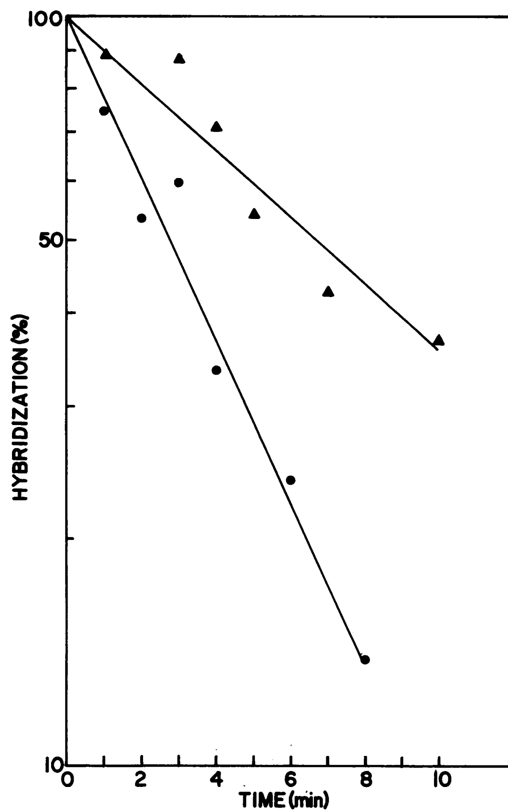

FIG. 3. Chemical decay of *trxA* mRNA at 44°C. SK5704 and MG1693 were grown in L broth. The half-life determination was done as described in Materials and Methods. Symbols: ▲, MG1693 (ams^+ pnp^+ rnb^+); ●, SK5704 (*ams-1 pnp-7 rnb-500*).

nalidixic acid were added as described in Materials and Methods and the cultures were immediately shifted to 44°C. The chemical decay of specific mRNAs was analyzed by hybridization of the isolated RNAs with DNA fragments containing the coding sequences of the genes indicated (Materials and Methods).

The decay of *trxA* mRNA at 44°C was significantly slower in the triple mutant SK5704 (Fig. 3). Analysis of these data yielded a chemical half-life for *trxA* mRNA of 6.8 min in SK5704 (*ams-1 pnp-7 rnb-500*) versus 2.6 min in the wild-type control (Table 3). The half-lives observed in *ams-1* and *pnp-7 rnb-500* control strains were shorter than that of the triple mutant (Table 3). At 30°C, the chemical half-lives were similar: 5 min for SK5704 (*ams-1 pnp-7 rnb-500*) and 4.5 min for MG1693 (wild type). When the chemical half-lives were determined for the *ssb*, *uvrD*, *pnp*, *nusA*, and *cat* genes, the results were consistent: the transcripts were always degraded two- to fourfold more slowly in the triple mutant (Table 3).

Northern analysis of *trxA* mRNA. Northern analysis employing RNA denaturation with formaldehyde and agarose gel electrophoresis produced a single broad band at zero time in the range of 450 to 500 nucleotides (nt) (Fig. 4A). A second, smaller species appeared in the triple mutant approximately 4 min after the shift (Fig. 4A). Although this result suggested some stabilization of the mRNA breakdown products upon a shift to 44°C, it was clear that agarose gel electrophoresis would not provide adequate resolution. Accordingly, a different method based on DNA sequencing technology was used. RNA samples were denatured in 99% formamide and electrophoresed in 6% polyacrylamide gels containing 7 M urea.

The results of a typical Northern analysis by this technique are shown in Fig. 4B. At the time of shift to 44°C, four major mRNA species ranging in size from 493 to 369 nt were observed. In the wild-type strain (MG1693), only the 387-nt RNA molecule was still present after 7 min at the nonpermissive temperature (Fig. 4B). In the *ams-1 pnp-7 rnb-500* triple mutant (SK5704), the same four species were observed at the time of shift. However, by 4 min after the shift, a series of discrete breakdown products began to appear (Fig. 4B). Further processing occurred at longer times at 44°C with RNA products as small as 185 nt appearing (Fig. 4B). By 60 min after the shift, all the RNA species had been degraded in both strains.

Since the chemical half-life of total pulse-labeled RNA also increased in the *ams-1* single mutant and the *pnp-7 rnb-500* double mutant (Table 2), the degradation of *trxA* mRNA in strain SK5665 (*ams-1*) and strain SK5726 (*pnp-7 rnb-500*) was also examined. While the kinetics of *trxA* mRNA decay changed in SK5665 (*ams-1*) compared with that of the wild-type control, no discrete breakdown products appeared (Fig. 5A). The pattern in the *pnp-7 rnb-500* double mutant (SK5726) initially also appeared identical to that of the wild-type control (Fig. 5B). However, upon significantly longer exposure, it became apparent that breakdown products similar to those observed in the triple mutant were present (Fig. 5C). Longer exposure of the autoradiograms of the RNA isolated from the *ams-1* strain did not show this pattern (data not shown). No hybridization was observed when total RNA was isolated from a *trxA* deletion mutant (data not shown).

Northern analysis of *cat* mRNA. To determine the generality of the above-described observations, we tested addi-

TABLE 3. Chemical half-lives of specific mRNAs

Gene	Half-life (min)[a]			
	Wild type	*ams-1*	*pnp-7 rnb-500*	*ams-1 pnp-7 rnb-500*
trxA (thioredoxin)	2.6[b]	5.1[b]	4.2[b]	6.8[b]
ssb (single-stranded-DNA-binding protein)	1.6[c]			4.1[c]
uvrD (DNA helicase II)	1.7[c]			4.3[c]
pnp (PNPase)	0.43[c]			1.4[c]
nusA (N utilization substance)	1.0[c]			3.9[c]
cat (chloramphenicol transacetylase)	2.2[c]			5.6[c]

[a] Chemical half-lives were determined in L broth at 44°C as described in Materials and Methods. Half-lives were estimated from a plot of the percentage of RNA hybridized versus time after the addition of rifampin.
[b] Determined in MG1693 background.
[c] Determined in C600 background.

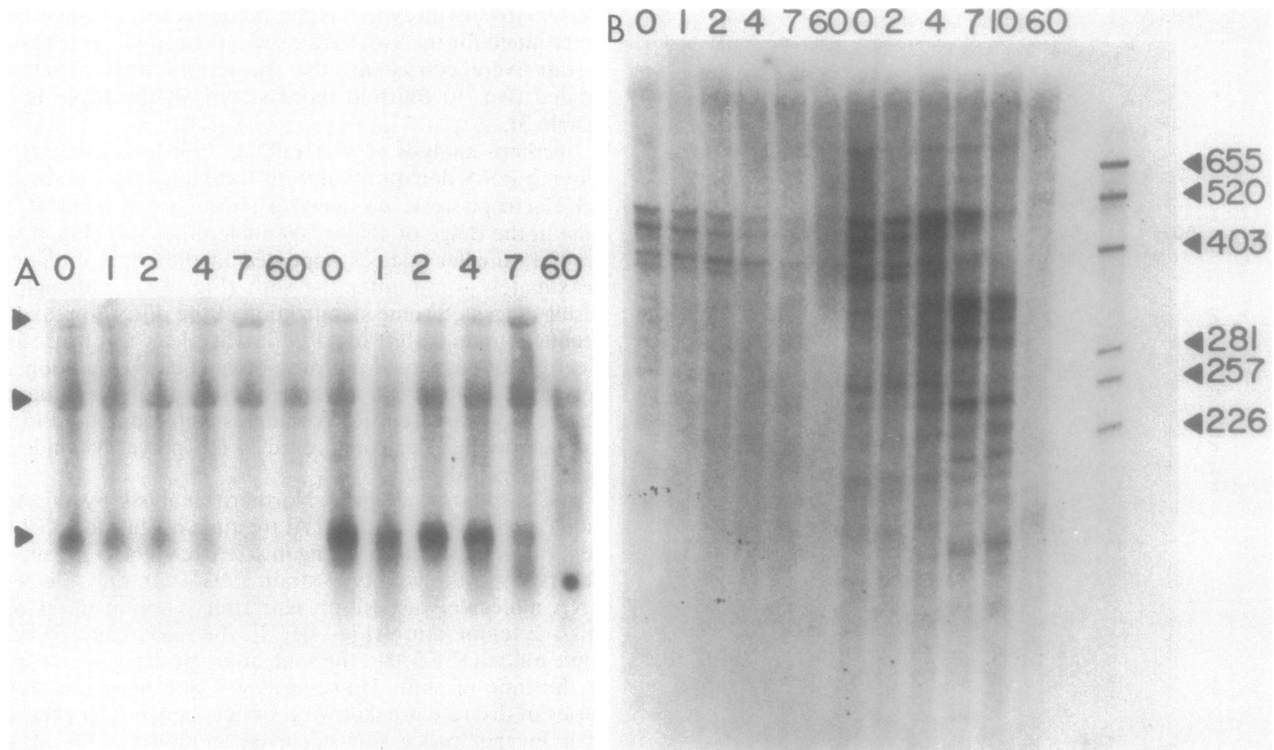

FIG. 4. Northern analysis of *trxA* mRNA in the *ams-1 pnp-7 rnb-500* triple mutant. Strains were grown at 30°C in L broth. At 40 Klett units, cell cultures were shifted to 44°C with the concomitant addition of rifampin. At various times, cell culture samples were removed and total RNA was extracted from MG1693 (*ams*$^+$ *pnp*$^+$ *rnb*$^+$) and SK5704 (*ams-1 pnp-7 rnb-500*) as described in Materials and Methods and electrophoresed through a 1.5% agarose–2.2 M formaldehyde gel (A) or a 6% polyacrylamide–7 M urea gel (B). The same amount of RNA (3.5 μg) was loaded in each lane. After electrophoresis, the RNA was transferred to a nitrocellulose filter by wicking (A) or by electroblotting to Biotrans (B), and the filters were hybridized with ^{32}P-labeled *trxA* DNA. The first six lanes show RNA extracted from the wild-type MG1693, and the last six lanes show RNA extracted from the triple mutant SK5704 (A and B). Numbers above each lane indicate the time (minutes) after the temperature shift at which the RNA was extracted. Arrows on the left indicate the positions of 23S rRNA (2.9 kb), 16S rRNA (1.5 kb), and *trxA* mRNA (≈0.5 kb) (A). *Alu*I-derived pBR322 DNA fragments labeled with ^{32}P are indicated in panel B (nucleotides).

tional mRNAs in the *ams-1 pnp-7 rnb-500* genetic background. For the *cat* mRNA, four discrete large transcripts of 1,020 to 1,200 nt were observed after electrophoresis in a 5% acrylamide gel (Fig. 6). By 10 min after the shift to 44°C, all these transcripts had been degraded in the wild-type strain without any evidence of smaller discrete breakdown products. In contrast, in the triple mutant, the full-length transcripts appeared to be sequentially processed into a series of smaller mRNA species, some of which were still visible 30 min after the shift to 44°C. The degradation pattern of the *cat* mRNA was clearly distinct from that observed with *trxA* (Fig. 4). Similar results have been obtained with additional mRNAs (data not shown).

DISCUSSION

The results described above demonstrate that RNase II, PNPase, and the Ams protein are required for the terminal steps of mRNA breakdown. In their absence, the appearance of discrete breakdown products of the *trxA* and *cat* mRNAs can be visualized on Northern blots (Fig. 4 to 6). It is particularly important to note that these breakdown products were only significantly stabilized in the triple mutant. While the absence of the Ams protein alone increased the chemical half-life of the *trxA* mRNA (Table 3), the degradation pattern observed on Northern blots closely resembled that of the wild-type control (Fig. 5A). In contrast, in the PNPase-RNase II double mutant, products similar to those seen in the triple mutant could be seen after very long exposure of the autoradiograms (Fig. 5C), even though the chemical half-life of the *trxA* mRNA was closer to that observed in the wild-type control strain.

Several important questions regarding the nature of the breakdown products need to be answered. Perhaps most important are the oligonucleotides observed in the triple mutant normal mRNA breakdown products that are being stabilized by the absence of the Ams protein, PNPase and RNase II? Analysis of a wild-type strain overproducing thioredoxin mRNA showed the transient appearance of fragments identical in size to those observed at longer times in the triple mutant (data not shown). This result implies that the patterns shown in Fig. 4 are an accurate representation of *trxA* mRNA degradation.

A second important issue relates to how the initial breakdown products are generated. Although it remains to be proven, the simplest explanation would be endonucleolytic cleavage. Recently, Portier et al. (28) have suggested that RNase III plays a role in the functional inactivation of the *pnp* mRNA by cleaving the transcript 5′ to the coding sequence. It has been previously shown that RNase III-processing sites exist in some intercistronic regions (3, 29). To determine whether RNase III is an important enzyme in mRNA turnover, we are currently constructing *ams pnp rnb rnc* multiple mutants.

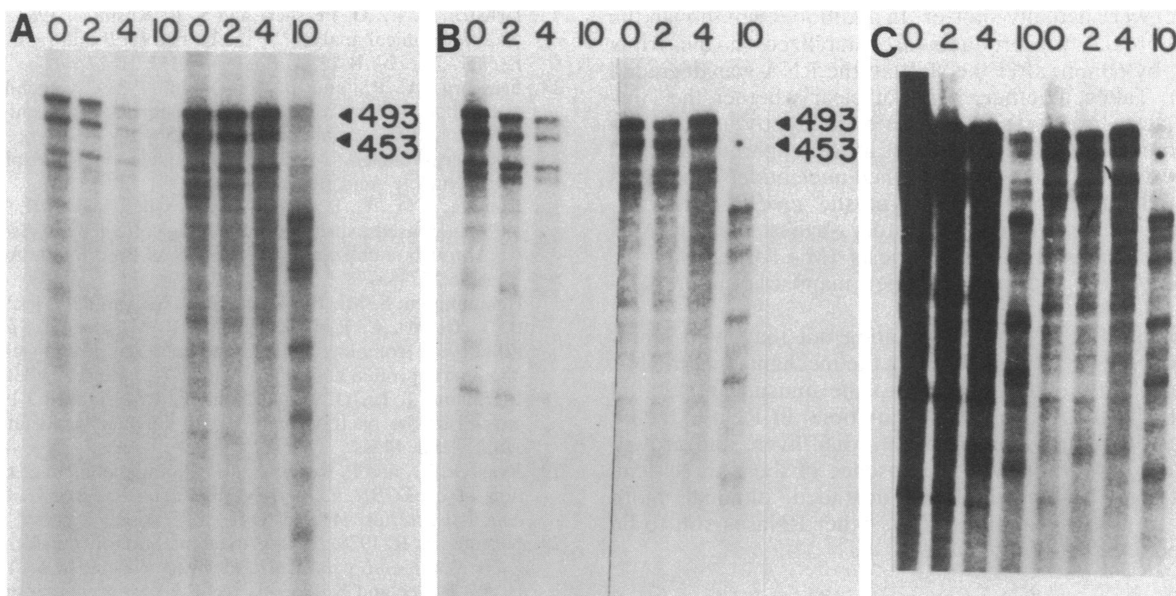

FIG. 5. Northern analysis of *trxA* mRNA in *ams-1* or *pnp-7 rnb-500* genetic backgrounds. Strains were grown at 30°C in L broth. At 40 Klett units, cell cultures were shifted to 44°C with the concomitant addition of rifampin. Cell culture samples were removed at the indicated times, and total RNA was extracted from SK5665 (*ams-1*), SK5626 (*pnp-7 rnb-500*), or SK5704 (*ams-1 pnp-7 rnb-500*) as described in Materials and Methods and electrophoresed through a 6% polyacrylamide–7 M urea gel. In panel A, the first four lanes were from SK5665 (*ams-1*) and the last four lanes were from SK5704 (*ams-1 pnp-7 rnb-500*). In panel B, the first four lanes were from SK5626 [*pnp-7 rnb-500*, pDK39 (*rnb-500*)] and the last four lanes were from SK5704 (*ams-1 pnp-7 rnb-500*). Panel C is a longer exposure of panel B. Numbers above each lane indicate the time (minutes) after the temperature shift at which the RNA was extracted. The sizes (nucleotides) of the two largest transcripts are indicated.

Other RNases are known to exist in the cytoplasm, but most of them have not been characterized in relation to mRNA turnover (9). It is possible that the discrete RNA fragments observed in Fig. 4 to 6 could have arisen from an exonucleolytic activity which is inhibited by specific secondary structures. Such secondary structures have been demonstrated to influence the rate at which nucleases degrade a given species of mRNA (7, 23, 24, 32).

Determination of the nucleotide sequences where cleavages occur will help answer the question of whether unique sequences or special secondary structures are recognized. The work of Cannistraro et al. (5) suggested that the *lacz* mRNA was preferentially cleaved at UUAU sequences. There are three of these sequences in the *trxA* mRNA. Two of them occur in the untranslated 5′ region and the third occurs between amino acids 5 and 6. It is thus not possible for all the fragments to have arisen from cleavage only at these sites.

Taken together, these data indicate a complex mechanism for the breakdown of mRNA molecules. We propose that full-length transcripts are initially processed by endonucleolytic cleavages, with each mRNA having a unique series of sites. Complete degradation of the initially cleaved transcripts occurs through possible additional endonucleolytic steps, followed by exonucleolytic degradation by RNase II, PNPase, and possibly the Ams gene product.

The results presented above indicate a complex role for the Ams protein in mRNA decay. While the absence of the protein alone leads to increased chemical half-lives of mRNAs (Table 2), discrete breakdown products were not visualized (Fig. 5). Furthermore, while the viability of *ams-1 rnb-500* (Fig. 1) and *ams-1 pnp-7* (data not shown) mutants was reduced at 44°C compared with that of the *ams-1* single mutant, the chemical half-lives measured in these strains

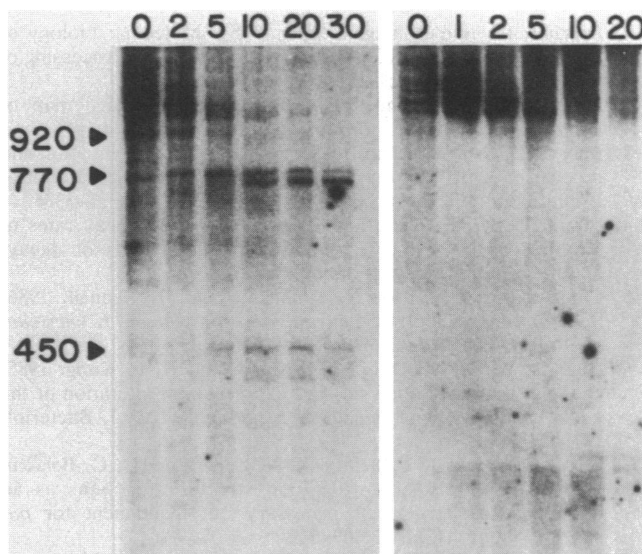

FIG. 6. Northern analysis of *cat* mRNA in the *ams-1 pnp-7 rnb-500* triple mutant. Strains SK6605 (wild type containing pDDK1) and SK6606 (*ams-1 pnp-7 rnb-500* containing pDDK1) were grown and treated as described in the legend to Fig. 4. At the times indicated, total RNA was extracted from the cells as described in Materials and Methods and electrophoresed through a 5% polyacrylamide–7 M urea gel. The same amount of RNA (4 μg) was loaded in each lane. After electrophoresis, the RNA was transferred by electroblotting to Biotrans and the filters were hybridized with a 1.2-kb DNA fragment containing the *cat* gene. The first six lanes show RNA extracted from SK6606 (*ams-1 pnp-7 rnb-500*), and the last six lanes show RNA extracted from SK6605 (wild type). The sizes (nucleotides) of representative bands are indicated.

(Table 2) were actually shorter. In addition, even though the mRNA breakdown products are stabilized in the triple mutant, by 60 min after the shift all the RNA was degraded (Fig. 4). Taken together, it is not clear whether the Ams protein acts directly as an RNase or indirectly as a regulatory protein. Although Chanda et al. (6) apparently cloned the *ams* structural gene, its published nucleotide sequence is in fact identical to a segment of the *groEL* gene (17). Preliminary results in our laboratory suggest that the Ams protein regulates its own synthesis (M. Torres-Diaz, F. Claverie-Martin, and S. R. Kushner, manuscript in preparation).

In conclusion, it is worth pointing out that many unresolved questions remain regarding the mechanism of mRNA turnover. Some of these relate to the unusual interaction between the *ams-1* allele and mutations in RNase II and PNPase regarding viability, mRNA half-lives, and growth rates. In addition, even in the absence of these three gene products, mRNA is still being degraded, although more slowly, to mononucleotides. Thus other RNases still to be identified are clearly involved.

ACKNOWLEDGMENTS

We thank Jan Smith for excellent technical assistance, Richard Gourse for helpful discussions, and T. Fitzwater and B. Polisky for describing to us their Northern procedure prior to publication.

This work was supported in part by Public Health Service grant GM28760 to S.R.K. from the National Institutes of Health and ACS grant PF-2749 to S.D.Y.

LITERATURE CITED

1. **Apirion, D., and P. Gegenheimer.** 1984. Molecular biology of RNA processing in prokaryotic cells, p. 35–62. *In* Processing of RNA. CRC Press, Inc., Boca Raton, Fla.
2. **Bachmann, B. J., and K. B. Low.** 1980. Linkage map of *Escherichia coli* K-12, edition 6. Microbiol. Rev. **44**:1–56.
3. **Barry, G., C. Squires, and C. L. Squires.** 1980. Attenuation and processing of RNA from the *rplJL-rpoBC* transcription unit of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77**:3331–3335.
4. **Blundell, M., E. Craig, and D. Kennell.** 1972. Decay rates of different mRNAs in *Escherichia coli* and models of decay. Nature (London) New Biol. **238**:46–49.
5. **Cannistraro, V. J., M. N. Subbarao, and D. Kennell.** 1986. Specific endonucleolytic cleavage sites for decay of *Escherichia coli* mRNA. J. Mol. Biol. **192**:257–274.
6. **Chanda, P. K., M. Ono, M. Kuwano, and H. F. Kung.** 1985. Cloning, sequence analysis, and expression of alteration of the mRNA stability gene (ams^+) of *Escherichia coli*. J. Bacteriol. **161**:446–449.
7. **Chen, C.-Y. A., J. T. Beatty, S. N. Cohen, and J. G. Belasco.** 1988. An intercistronic stem-loop structure functions as an mRNA decay terminator necessary but insufficient for *puf* mRNA stability. Cell **52**:609–619.
8. **Deutscher, M. P.** 1985. *Escherichia coli* RNases: making sense of the alphabet soup. Cell **40**:731–732.
9. **Deutscher, M. P.** 1988. The metabolic role of RNases. Trends Biochem. Sci. **13**:136–139.
10. **Donovan, W. P., and S. R. Kushner.** 1983. Amplification of ribonuclease II (*rnb*) activity in *Escherichia coli* K-12. Nucleic Acids Res. **11**:265–275.
11. **Donovan, W. P., and S. R. Kushner.** 1983. Cloning and physical analysis of the *pyrF* gene (coding for orotidine-5′phosphate decarboxylase) from *Escherichia coli* K-12. Gene **25**:39–48.
12. **Donovan, W. P., and S. R. Kushner.** 1986. Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **83**:120–124.
13. **Dykstra, C. C., D. Prasher, and S. R. Kushner.** 1984. Physical and biochemical analysis of the cloned *recB* and *recC* genes of *Escherichia coli* K-12. J. Bacteriol. **157**:21–27.
14. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
15. **Grunberg-Manago, M.** 1963. Polynucleotide phosphorylase. Prog. Nucleic Acid Res. **1**:93–133.
16. **Hautala, J. A., C. L. Bassett, N. H. Giles, and S. R. Kushner.** 1979. Increased expression of a eukaryotic gene in *Escherichia coli* through stabilization of its mRNA. Proc. Natl. Acad. Sci. USA **76**:5774–5778.
17. **Hemmingsen, S. M., C. Woolford, S. M. van der Vies, K. Tilly, D. T. Dennis, C. P. Georgopoulos, R. W. Hendrix, and R. J. Ellis.** 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. Nature (London) **333**:330–334.
18. **Ingraham, J. L., O. Maaløe, and F. C. Neidhardt.** 1983. The growth of the bacterial cell, p. 12. Sinauer Associates, Inc., Sunderland, Mass.
19. **Krzyzek, R., and P. Rogers.** 1972. Arginine control of transcription of *argECBH* messenger ribonucleic acid in *Escherichia coli*. J. Bacteriol. **110**:945–954.
20. **Kushner, S. R.** 1978. An improved method for transformation of *Escherichia coli* with ColE1-derived plasmids, p. 17–23. *In* H. W. Boyer and S. Nicosia (ed.), Genetic engineering. Elsevier/North-Holland Biomedical Press, Amsterdam.
21. **Low, B.** 1973. Rapid mapping of conditional and auxotrophic mutations in *Escherichia coli* K-12. J. Bacteriol. **113**:798–812.
22. **Maples, V. F., and S. R. Kushner.** 1982. DNA repair in *Escherichia coli*: identification of the *uvrD* gene product. Proc. Natl. Acad. Sci. USA **79**:5616–5620.
23. **Mott, J. E., J. L. Galloway, and T. Platt.** 1985. Maturation of *Escherichia coli* tryptophan operon: evidence for 3′ exonucleolytic processing after *rho*-dependent termination. EMBO J. **4**:1887–1891.
24. **Newbury, S. F., N. H. Smith, E. C. Robinson, I. D. Hiles, and C. F. Higgins.** 1987. Stabilization of translationally active mRNA by prokaroytic REP sequences. Cell **48**:297–310.
25. **Nilsson, G., J. G. Belasco, S. N. Cohen, and A. Von Gabain.** 1984. Growth-rate dependent regulation of mRNA stability in *Escherichia coli*. Nature (London) **312**:75–77.
26. **Ono, M., and M. Kuwano.** 1979. A conditional lethal mutation in an *Escherichia coli* strain with a longer chemical lifetime of mRNA. J. Mol. Biol. **129**:343–357.
27. **Ono, M., and M. Kuwano.** 1980. Chromosomal location of a gene for chemical longevity of messenger ribonucleic acid in a temperature-sensitive mutant of *Escherichia coli*. J. Bacteriol. **142**:325–326.
28. **Portier, C., L. Dondon, M. Grunberg-Manago, and P. Regnier.** 1987. The first step in the functional inactivation of the *Escherichia coli* polynucleotide phosphorylase messenger is a ribonuclease III processing at the 5′ end. EMBO J. **6**:2165–2170.
29. **Portier, C., and P. Regnier.** 1984. Expression of the *rpsO* and *pnp* genes: structural analysis of a DNA fragment carrying their control regions. Nucleic Acids Res. **12**:6091–6102.
30. **Reiner, A. M.** 1969. Isolation and mapping of polynucleotide phosphorylase mutants of *Escherichia coli*. J. Bacteriol. **97**:1431–1436.
31. **Reiner, A. M.** 1969. Characterization of polynucleotide phosphorylase mutants of *Escherichia coli*. J. Bacteriol. **97**:1437–1443.
32. **Schmeissner, U., K. McKenney, M. Rosenberg, and D. Court.** 1984. Removal of a terminator structure by RNA processing regulates *int* gene expression. J. Mol. Biol. **176**:39–53.
33. **Spahr, P. F.** 1964. Purification and properties of ribonuclease II from *Escherichia coli*. J. Biol. Chem. **239**:3716–3726.
34. **Thomas, P.** 1983. Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. Methods Enzymol. **100**:255–266.
35. **Von Gabain, A., J. G. Belasco, J. L. Schottel, A. C. Y. Chang, and S. N. Cohen.** 1983. Decay of mRNA in *Escherichia coli*: investigation of the fate of specific segments of transcripts. Proc. Natl. Acad. Sci. USA **80**:653–657.

36. **Wallace, B. J., and S. R. Kushner.** 1984. Genetic and physical analysis of the thioredoxin (*trxA*) gene of *Escherichia coli* K-12. Gene **32:**399–408.
37. **White, B. A., and F. C. Bancroft.** 1982. Cytoplasmic dot hybridization. J. Biol. Chem. **257:**8569–8572.
38. **Willetts, N. S., and D. W. Mount.** 1969. Genetic analysis of recombination-deficient mutants of *Escherichia coli* K-12 carrying *rec* mutations cotransducing with *thyA*. J. Bacteriol. **100:** 923–934.
39. **Williams, M. G., and P. Rogers.** 1987. Expression of *arg* genes in *Escherichia coli* during arginine limitation dependent upon stringent control of translation. J. Bacteriol. **169:**1644–1650.