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Specific Endonucleolytic Cleavage of the mRNA for Ribosomal Protein S20 of *Escherichia coli* Requires the Product of the *ams* Gene In Vivo and In Vitro

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Endonucleolytic cleavage is believed to initiate the degradation of most bacterial mRNAs, but with several exceptions, the enzymes responsible have yet to be identified. Crude (S-30) or partially fractionated extracts of *Escherichia coli* strains with reduced exonuclease activities catalyze the cleavage of a 372-residue RNA substrate containing the sequences coding for ribosomal protein S20 to yield a number of discrete products. The major product of 147 residues is obtained in 60 to 70% yield, is coterminal with the 3′ end of the substrate, and is identical to an mRNA fragment previously characterized in vivo (G. A. Mackie, J. Bacteriol. *171*:4112–4120, 1989). A number of other products of 150 to 340 residues are also formed, and the cleavage sites, typically N ↓ AU sequences, have been identified in the S20 mRNA substrate by Northern (RNA) blotting and primer extension. All cleavages required a native rather than a denatured RNA substrate. The rate of cutting of the S20 mRNA substrate at the site yielding the prominent 147-residue product appears to be independent of cleavages at other sites. In addition, the activity of the putative endonuclease(s) depends strongly, both in vivo and in vitro, on the product of the *ams* gene, which is known to influence mRNA lifetimes in vivo. Taken together, the data show that the fractionated extract described here reproduces steps in the degradation of some mRNAs which occur in living cells.

Since the discovery that bacterial mRNAs are metabolically unstable, a number of models have been proposed to explain how these macromolecules are degraded (1, 4). Currently, degradation is believed to be the consequence of the concerted action of endo- and exoribonucleases. The only endonucleases known to date to act on mRNAs in *Escherichia coli* are ribonuclease III (3, 10, 28) and ribonuclease E (22, 23, 24), the latter in T4-infected cells. The major role of these two enzymes, however, is thought to be the processing of rRNA precursors (12). It is not yet clear, therefore, whether these and other known enzymes (5, 8) can fulfill the essential mRNA endonuclease activities of *E. coli* or whether additional endoribonucleases remain to be identified. The importance of such activities is clear from the characterization of intermediates in the decay of several mRNAs in vivo as products of endonucleolytic attack (6, 17, 25). An essential criterion for the involvement of any ribonuclease in mRNA decay is a demonstration that the enzyme postulated to catalyze a step in decay in vivo does so faithfully in a reconstructed in vitro system. Site-specific cleavage of the *ompA* and *bla* mRNAs in vivo and in vitro has been demonstrated by Nilsson et al. (25). Several sites of cleavage of the *ompA* substrate RNA have now been determined, and the responsible activity, termed RNase K, has been partially purified (13). The relationship of this activity to known endonucleases of *E. coli* remains to be established.

The two nested mRNAs encoding ribosomal protein S20 of *E. coli* serve as simple prototypes for determining steps which occur during mRNA decay since they are small (447 and 356 residues) and relatively abundant (14–16). In addition, their times of synthesis are short relative to their half-lives, and several structural or functional features of these mRNAs which control their lifetimes in vivo have been characterized (15, 16, 27). In particular, examination of the metabolism of these mRNAs in strains displaying reduced 3′ exonuclease activity has revealed two potential pathways for their decay. The first and major pathway requires polynucleotide phosphorylase, while the second involves at least one site-specific endonucleolytic cleavage (17). I have focused on the latter in view of its potential relevance to the decay of many bacterial mRNAs. As an initial step in reconstructing the decay process with purified components, I have sought to prepare and subsequently fractionate extracts of *E. coli* capable of accurately reproducing endonucleolytic cleavages of the S20 mRNAs identical to those observed in vivo. The activity or activities responsible have been identified, characterized, and shown to depend on the product of the *ams* gene (2, 7, 26).

MATERIALS AND METHODS

Bacterial strains and media. The following strains were obtained from Sidney Kushner (Department of Genetics, University of Georgia, Athens): SK5003 (*thr leu pnp-7 rnb-500* pDK39 [Cm^r *rnb-500*]), SK5005 (*thr leu rnb-500* pDK39 [Cm^r, *rnb-500*]), SK5684 (*thr leu pnp-7 rnb-500 ams-1*), SK5665 (*ams-1*), SK5671 (*ams-1 pnp-7*), SK5704 (*ams-1 pnp-7 rnb-500*), and SK5714 (*ams-1 rnb-500*) (2, 9). Plasmid pSP65 (20) was obtained from Promega Biotec, Inc. Plasmid pGM75 has been described previously (17), while the construction of pGM79 from pSP65 is discussed in the text. All strains were grown in Luria-Bertani medium at 29°C or as indicated (described in reference 17).

Oligodeoxyribonucleotides. Oligodeoxyribonucleotides used in this study are listed in Table 1. They were synthesized on an Applied Biosystems model 380A DNA synthesizer and purified by preparative polyacrylamide gel electrophoresis or by using oligonucleotide purification cartridges obtained from Applied Biosystems Inc. Where required, oligonucleotides were phosphorylated as previously described and purified from free ATP and salts by gel filtration (17).

Preparation of extracts. Cultures (1 to 2 liters) were grown

TABLE 1. Oligodeoxyribonucleotides used in this work

Oligodeoxyribonucleotide[a]	Sequence[b]	S20 sequence coordinates[c]
7	5′-CCTTTTCAGACTGAATGG	Complementary to 164–181
14	5′-TGTGGATCAGACCTTTAGCAGC	Complementary to 316–337
15	5′-ACGTTCAGCAAATTGGCG	Complementary to 398–415
16	5′-AGCTTTGTGAAAAAGCCCGCGCAAGCGGGTTTTTTTAaag	412–448
17	5′-gatccttTAAAAAAACCCGCTTGCGCGGGCTTTTTCACAA	Complementary to 416–448
21	5′-CACGATCGGTTGCATTTC	Complementary to 289–306

[a] Oligodeoxyribonucleotides were synthesized and purified as described in Materials and Methods.
[b] Lowercase letters indicate residues not in the S20 sequence which have been included to facilitate molecular cloning.
[c] Sequence coordinates are renumbered from those in reference 14; thus, the +1 residue of the P1 promoter (coordinate 141 in the original sequence) is residue 1 (17).

to mid-exponential phase in Luria-Bertani medium supplemented with $MgSO_4$ to 1 mM and antibiotics as required to 20 μg/ml. Cultures were chilled thoroughly in a slurry of water and ice and harvested by centrifugation at 4°C in a Beckman JA-14 rotor for 20 min at 6,000 × *g*. Cell pellets were suspended in 100 ml of cold buffer I (50 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, 60 mM NH_4Cl, 0.5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride) supplemented with 6 mM 2-mercaptoethanol. The cells were harvested by centrifugation as before (but at 8,000 × *g*). The cell pellet was drained and frozen at −70°C. The frozen pellet was suspended in cold buffer II (buffer I supplemented with 0.1 mM dithiothreitol and glycerol to 5% [vol/vol]) at 4 ml/g of wet cell pellet. Cells were disrupted by two passages through an Aminco French pressure cell at 8,000 lb/in^2. DNase (3 U/ml of lysate) and dithiothreitol (0.1 μmol/ml of lysate) were added to the lysate, which was incubated on ice for 5 to 10 min prior to centrifugation for 10 min at 8,000 × *g* to remove unbroken cells and debris. The resultant supernatant was clarified by centrifugation for 40 min at 30,000 × *g* in a Beckman JA-21 rotor. The supernatant (S-30) was frozen in liquid N_2 in small aliquots or was diluted with 4 volumes of buffer II (to about 3 mg/ml). Sufficient powdered $(NH_4)_2SO_4$ was added slowly with stirring to make the S-30 26% (wt/vol) in $(NH_4)_2SO_4$. The precipitated protein was recovered by centrifugation for 20 min at 12,000 rpm in a Beckman JA-20 rotor. The pellet was suspended with 2 to 3 ml of buffer II and dialyzed against 3 changes (200 volumes each) of buffer IV (10 mM Tris-HCl [pH 8.0], 1 mM NH_4Cl, 0.1 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 5% [wt/vol] glycerol). The dialyzed material (referred to below as the AS-26 fraction) was frozen in small portions in liquid N_2 and stored at −70°C.

Protein concentrations in the various fractions were measured by a dye-binding assay with a kit from Bio-Rad, Inc., and crystallized bovine serum albumin as the standard.

Assay of ribonuclease activity. Assays were performed at 30°C in 25 mM Tris-HCl (pH 8.0)–5 mM $MgCl_2$–60 mM KCl–100 mM NH_4Cl–0.1 mM dithiothreitol–5% (wt/vol) glycerol–50 μg of yeast RNA per ml. Normally, the substrate RNA (prepared as described below) was heated in this buffer for 2 min at 50°C and 10 min at 37°C and then chilled. Alternatively, the RNA was heated for 5 min at 94°C and chilled, after which appropriately concentrated assay buffer was added. Where needed, complementary oligodeoxynucleotides were present at four- to tenfold molar excess over substrate. The final concentration of substrate was 20 nM. Incubations were initiated by the addition of protein (typically to 0.05 to 1.0 μg/μl). Samples were diluted at various times into a stop mixture. When the source of activity was S-30, the stop consisted of 9 volumes of 0.2 M sodium acetate (pH 6) containing 50 μg of yeast RNA carrier per ml, 5 mM EDTA, and 0.1% sodium dodecyl sulfate. The diluted sample was extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and the aqueous phase was precipitated with ethanol. The precipitated nucleic acids were recovered by centrifugation and dissolved in a small volume of H_2O. The dissolved material was diluted with 4 volumes of loading buffer (90% deionized formamide containing 0.1% bromphenol blue and 0.1% xylene cyanole FF in half-strength Tris-borate-EDTA buffer [19]). When the source of activity was the fractionated S-30 (i.e., the AS-26 fraction), samples were removed from incubation and diluted directly into 4 volumes of loading buffer without prior extraction.

Substrates were prepared by transcription of the appropriate linear template (36 pmol) with 100 U of SP6 RNA polymerase in 200 μl of the buffer described by Melton et al. (20). Unlabeled ribonucleotides were present at 500 μM; the α-^{32}P-labeled ribonucleotide (when used) was present at 100 μM. After incubation at 37°C for 60 min, reaction mixtures were diluted with EDTA to 5 mM and with ammonium acetate to 2 M, extracted twice with phenol-chloroform-isoamyl alcohol, and precipitated with ethanol. The RNA product was recovered by centrifugation, reprecipitated once more from 2 M ammonium acetate with ethanol, washed with 80% ethanol, and finally dissolved at 0.2 pmol/μl in a buffer containing 5 mM Tricine (pH 8.0), 50 mM sodium acetate, and 0.5 mM EDTA. The RNA was stored at −20°C and could be frozen and thawed several times with little apparent damage. Yields of product were determined by measuring its acid-precipitable radioactivity.

Analysis of RNA products. Labeled RNA samples were heated for 2 min at 90°C, quick cooled on ice, and analyzed by electrophoresis on a 0.75-mm-thick 6% polyacrylamide gel (29:1 acrylamide:bisacrylamide) containing 8 M urea in Tris-borate-EDTA buffer (19). Subsequently, the gel was fixed, dried, and exposed to Kodak XAR-2 X-ray film. Unlabeled RNAs derived from 0.06 pmol of substrate were separated by electrophoresis as described above and electrophoretically transferred to Zeta-Probe charge-modified nylon (from Bio-Rad, Inc.) in a Bio-Rad miniblot apparatus following the manufacturer's instructions (60 min at 80 V in half-strength Tris-borate-EDTA buffer). RNA was fixed to the support by exposure to a UV germicidal lamp for 3 min (11) and drying in vacuo for 60 min. Conditions for hybridization were essentially those of Zoller and Smith (31). Removal of excess probe was performed as previously described (17). Primer extension was performed as previously described (17), using as template either 5.0 μg of total cellular RNA extracted by method II of reference 17 or in vitro RNAs derived from 0.06 pmol of substrate.

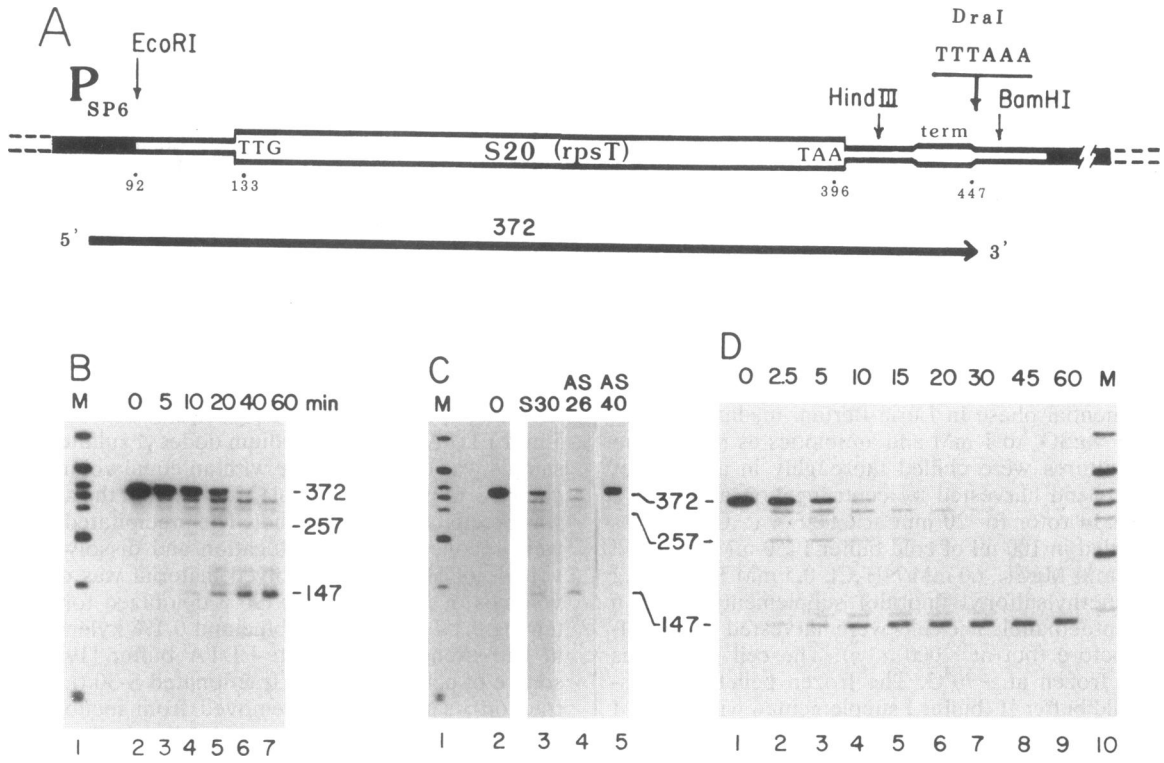

FIG. 1. Activities of different enzyme fractions against a synthetic S20 mRNA substrate. (A) Structure of pGM79. An *Eco*RI-*Hin*dIII fragment spanning residues 92 to 411 of the S20 sequence (numbered as in reference 17) was mixed with 5'-phosphorylated oligonucleotides 16 and 17 (see text and Table 1) and ligated into pSP65 (20) previously digested with *Eco*RI and *Bam*HI. The open box denotes the S20 coding sequence, and the horizontal arrow denotes the 372-residue in vitro transcript obtained from a *Dra*I-linearized template. P_{SP6}, Promoter for SP6 RNA polymerase. (B) Time course of digestion of S20 mRNA by an S-30 fraction. The ^{32}P-labeled 372-residue substrate was digested at 30°C with an S-30 extract (0.48 μg/μl from strain SK5003 for the time indicated above each lane). Aliquots containing 10 μl of the digest were deproteinized and analyzed electrophoretically (see Materials and Methods). Lane 1 (M) contains end-labeled markers derived from *Hin*fI-digested pBR322. The inferred sizes (numbers of residues) of major products are noted in the right margin. (C) Fractionation of the S-30 extract with ammonium sulfate. The ^{32}P-labeled 372-residue substrate was undigested (lane 2) or digested for 20 min at 30°C with 0.48 μg of S-30 extract per μl (lane 3), 0.078 μg of AS-26 per μl (lane 4), or 0.5 μg of AS-40 per μl (lane 5). Aliquots containing 10 μl of digest were deproteinized and analyzed as for panel B. (D) Time course of digestion of the S20 mRNA substrate with the AS-26 fraction. The ^{32}P-labeled 372-residue substrate was digested with AS-26 from strain SK5003 (0.10 μg/μl) for the time (in minutes) indicated above each lane. Aliquots containing 4 μl of digest were denatured and analyzed electrophoretically (see Materials and Methods).

RESULTS

Preparation of substrates. Previous work has shown that the stability of the S20 mRNAs in vivo and their susceptibility to specific internal cleavages depend on the presence of the natural rho-independent terminator distal to the coding sequence in the S20 mRNAs (16, 17). It seemed important, therefore, to design a template which would result in the product RNA containing the same 3' terminus that the S20 mRNAs possess in vivo. Two partially complementary oligodeoxyribonucleotides (16 and 17 in Table 1), each 40 residues in length, were synthesized, phosphorylated on their 5' termini, and annealed. The duplex so created contains a 5'-AGCT overhang on one strand and a 5'-GATC overhang on the other, permitting its ligation between *Hin*dIII and *Bam*HI sites. In addition, the synthetic duplex contains residues 412 to 448 of the S20 sequence encompassing the rho-independent terminator and three additional A residues to the 3' side of the terminator. This creates a new *Dra*I recognition sequence, TTT ↓ AAA, at the end of a run of seven T residues in the template where transcriptional termination normally occurs (15). The 40-residue duplex and a 320-bp fragment encompassing residues 92 to 411 of the S20 sequence were ligated between the *Eco*RI and *Bam*HI sites of pSP65 (20) to generate pGM79, as illustrated in Fig. 1A. Transcription of *Dra*I-linearized pGM79 by SP6 RNA polymerase yielded a single transcript of 372 residues, as expected (Fig. 1B, lane 2). This RNA is identical to the shorter of the two nested S20 mRNA transcripts in vivo (the P2 mRNA), except that the 5' end of the in vitro transcript contains 16 additional residues encoded by the vector and an *Eco*RI linker.

Cleavage of the S20 mRNA in vitro. A 372-residue RNA encompassing the S20 P2 mRNA was labeled with [α-^{32}P]CTP by transcription of pGM79 linearized with *Dra*I (Fig. 1A). A time course of incubation of this substrate with an S-30 extract prepared from strain SK5003 (see Materials and Methods) is shown in Fig. 1B. The substrate RNA disappears within 60 min of incubation, and three major products of approximately 340, 260, and 150 residues accumulate during the incubation, the latter to the highest yields (see below). The 150-residue product of digestion is similar in size to the 147-residue Po RNA which is coterminal with the 3' end of the S20 mRNAs and which is observed in vivo (17). Primer extension experiments confirmed that the 5' end of the 150-residue product is identical to that of the Po RNA (18) (see below) and that its actual size is 147 residues.

The S-30 extract was fractionated directly by precipitation with ammonium sulfate (see Materials and Methods). Trials showed that most of the activity precipitated at 10 to 20% (wt/vol) ammonium sulfate, with the balance recovered in the 20 to 30% (wt/vol) fraction. Subsequent precipitation of S-30 extracts with 26% (wt/vol) ammonium sulfate yielded complete recovery of activity in the precipitate (the AS-26 fraction) with at least sevenfold purification (Fig. 1C; compare lanes 3 and 4). Proteins precipitated from the S-30 fraction by a further addition of ammonium sulfate to 40% (wt/vol) to yield the "AS-40" fraction appeared to display only slight activity toward the 372-residue substrate (Fig. 1C, lane 5) and produced the 147-residue product at a low rate. The spectrum of products obtained with the AS-26 fraction was identical to that obtained with the crude S-30 extract (compare Fig. 1B and D). More quantitative results were obtained by scanning autoradiograms obtained from time course experiments similar to those illustrated in Fig. 1D. The major 147-residue product accumulated linearly as long as intact substrate remained. Thereafter, it increased gradually to reach a yield of 0.66 mol/mol of substrate (average of three experiments).

The activity of the AS-26 fraction in producing the 147-residue product was assayed as a function of monovalent and divalent cation concentrations in the incubation. At least 1 mM $MgCl_2$ was required for activity, with the optimum occurring over a broad range between 2 and 20 mM (18). Accumulation of the 147-residue product was maximal at about 150 mM KCl or NH_4Cl; the products appeared to be cleaner with NH_4Cl (18).

Characterization of the products of digestion. Three methods were applied in combination to identify the products of digestion of the 372-residue substrate: sizing (cf. Fig. 1D), primer extension, and Northern (RNA) blot hybridization with specific oligodeoxyribonucleotide probes. Detailed information about the 5′ termini of the various products of digestion of the 372-residue substrate was obtained from primer extension experiments with primers 15 and 21 (see Materials and Methods and Table 1). Unlabeled RNA transcribed from *Dra*I-cut pGM79 was digested for increasing times with the AS-26 fraction, after which the RNA products were purified and analyzed by primer extension. In one case, the analysis was performed in parallel with RNAs extracted from growing cells so that a comparison of the termini of cleavage products in vitro with those of minor S20 RNAs detectable in vivo could be made. Primer extension with oligodeoxyribonucleotide 21 demonstrated the time-dependent formation of a major terminus at residue 191 (A) (Fig. 2A, lanes 1 through 5). Additional termini form during incubation at residues 117, 147, 152, 194, and 211, as indicated in the margin to Fig. 2A, as well as at residues 125 and 237 (18). Other bands are visible in all lanes, including lane 1 (zero time), and presumably represent strong stops to reverse transcription.

The major 5′ end detected by the extension of oligodeoxyribonucleotide 15 annealed to digested substrate RNA occurs as a doublet at residues 301 (A) and 302 (U) (Fig. 2B, lanes 1 through 11). A parallel analysis of RNAs extracted from cultures of strains containing pGM75 demonstrates clearly that this new end is identical to the 5′ end of the Po RNA detected in strains of *E. coli* carrying the *pnp-7* allele of polynucleotide phosphorylase (Fig. 2B, compare lanes 11 and 13; see also reference 17). RNA from an otherwise isogenic strain containing the wild-type allele for polynucleotide phosphorylase does display the novel 5′ ends at residues 301 and 302 but in much lower abundance, which is in agreement with earlier work (Fig. 2B, lane 12) (17). Minor termini also occur at residues 282 (A), 292 (A), and 341 (A). Extension of primer 15 also detects 5′ ends uncovered by oligodeoxyribonucleotide 21 (e.g., at residue 191; compare Fig. 2A and B).

In the third method, unlabeled substrate was digested for increasing times with the AS-26 fraction and the products were separated on a denaturing polyacrylamide gel prior to electrophoretic transfer to a charge-modified nylon membrane (see Materials and Methods). Replicate membranes were probed with one of four end-labeled oligodeoxyribonucleotides to permit a determination of whether a particular product of digestion originated in the 5′, central, or 3′ portion of the substrate. Figure 3A illustrates a typical result, with probe 7 complementary to residues 164 to 181 in the 5′ portion of the substrate (Fig. 3E and Table 1). The major products detected are the undigested substrate, a slightly shortened 340-residue molecule, and a 110- to 120-residue product most prominent after 10 min of digestion. Almost no products retaining sequences complementary to the 5′ probe are detectable after 45 min of digestion (Fig. 3A, lane 4). In contrast, probe 15, complementary to residues 398 to 415 in the 3′ region of the substrate (Fig. 3E and Table 1), detects undigested substrate, three products of 260 to 340 residues, and four closely spaced products of 147 to 175 residues, of which the 147-residue product accumulates to the highest levels (Fig. 3B, lanes 1 through 4). The prominent 147-residue "limit product" seen in Fig. 1D must therefore originate in the 3′ portion of the substrate. Probes 14 and 21 complementary to the central portion of the substrate (Fig. 3E and Table 1) confirm the location of the 147-residue product. Probe 14 (complementary to residues 316 to 337) detects the same spectrum of products as probe 15, whereas probe 21 (complementary to residues 289 to 306) fails to detect the abundant 147-residue limit product. Thus, the 5′ end of the 147-residue product must lie between the extremities of the termini of probes 14 and 21 (i.e., between residues 289 and 337), confirming the results obtained with primer extension (Fig. 2B). Since probes 14, 15, and 21 all anneal to the same digestion products of between 160 and 340 residues in length, it follows that digestion of the substrate results in its successive shortening from the 5′ end rather than from the 3′ end (see Discussion). Taken collectively, the set of four probes identifies all fragments detected by cleavage of the uniformly labeled substrate (compare Fig. 3 and 1D).

Figure 3E summarizes the data obtained by the combined methodologies. The major endpoints determined by primer extension predict fragments of the size and provenance observed in the Northern blotting assay shown in Fig. 3A through D. Figure 7A (see below) summarizes the sequences which appear to be most susceptible to cleavage by the activity or activities in the AS-26 fraction.

Effect of substrate structure on its cleavage in vitro. The data summarized in Fig. 3E show that the cleavage site between residues 300 and 301 of the 372-residue substrate is the most 3′ of all efficiently recognized sites. This raises the question of whether the recognition of this site requires the prior cleavage of sites more 5′ on the substrate RNA. This issue was addressed by annealing oligodeoxyribonucleotide 7 to the 372-residue substrate and cleaving the heteroduplex with RNase H prior to incubation with the AS-26 fraction. Figure 4A illustrates data from one experiment, while the kinetics of formation of the 147-residue product averaged in two experiments are plotted in Fig. 4B. Incubation of the 372-residue substrate with RNase H in the absence of

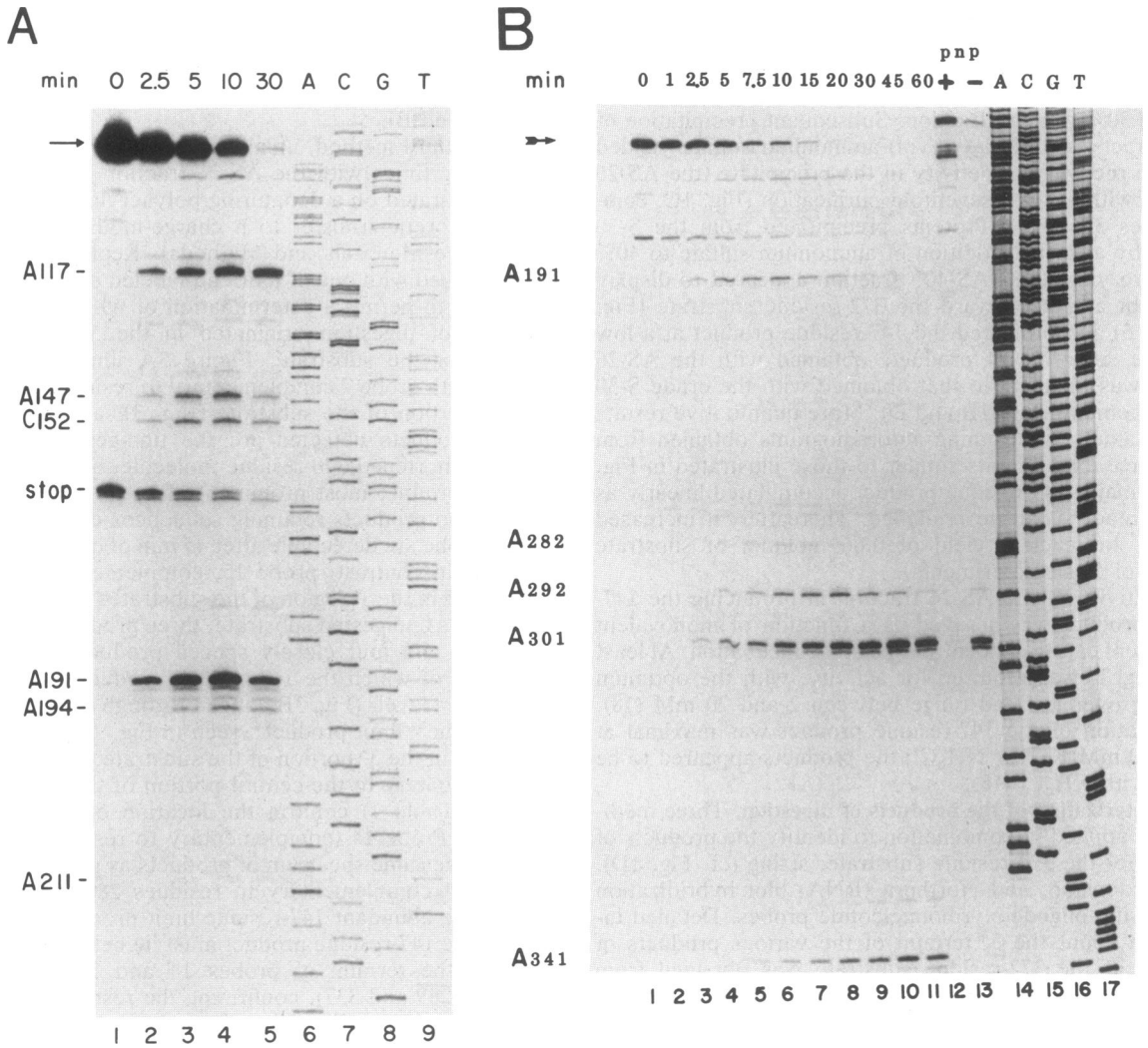

FIG. 2. Time course of new 5′ end formation in the S20 mRNA substrate. Unlabeled 372-residue RNA transcribed from *Dra*I-cut pGM79 (Fig. 1A) was digested with AS-26 from strain SK5003 (0.1 μg/μl) at 30°C for increasing times. The digestion products were deproteinized and precipitated with ethanol. Portions were analyzed by primer extension (see Materials and Methods). The DNA products were analyzed on an 8% polyacrylamide sequencing gel and visualized by autoradiography. Sequence markers were obtained by using the same (phosphorylated) primer and a single-stranded DNA template containing S20 sequences from −140 to +411. (A) Primer extension with oligonucleotide 21 (Table 1 and Fig. 3E). Aliquots of the 372-residue RNA substrate digested for the times (in minutes) indicated above the lanes served as templates for primer extension (lanes 1 through 5). Residues corresponding to new 5′ ends are indicated in the left-hand margin. The arrow denotes the position of the extension product corresponding to undigested template. Sequencing markers are shown in lanes 6 through 9. (B) Primer extension with oligonucleotide 15 (Table 1 and Fig. 3E). Lanes 1 through 11 contain the extension products obtained from aliquots of the 372-residue substrate digested for the times (in minutes) indicated above lanes 1 through 11. In lanes 12 and 13, the template RNA was 5.0 μg of total RNA extracted from strain SK5005/pGM75 (*rnb-500*) and strain SK5003/pGM75 (*rnb-500 pnp-7*), respectively. Sequencing markers are in lanes 14 through 17.

oligodeoxyribonucleotide had no effect, as expected (Fig. 4A, lane 0). The heteroduplex substrate was cleaved efficiently by RNase H (about 80% yield in this experiment) to produce the anticipated products of about 265 and 100 residues (Fig. 4A, lane 10). Addition of the AS-26 fraction to the RNase H-treated substrate produced the 147-residue product with kinetics indistinguishable from that of the control (Fig. 4B). Thus, prior shortening of the 372-residue substrate at the site chosen does not change the rate of cleavage between residues 300 and 301.

To address the question of whether primary structure is the major determinant of the specificity of the cleavages produced by the AS-26 fraction, the 372-residue RNA substrate was denatured by heating prior to assay. Surprisingly, the denatured substrate RNA was initially inert to digestion, as products appeared only after a 15-min lag (Fig. 4D, lane 5). Substrate RNA which was renatured prior to incubation displayed normal kinetics of accumulation of 257- and 147-residue products (Fig. 4C). This experiment was repeated with independent preparations of both substrate and AS-26 with identical results. These data suggest strongly that the RNA must be folded into an appropriate secondary or tertiary structure in order to serve as a substrate for the enzyme(s) in the AS-26 fraction.

Requirement for the product of the *ams* gene. Strains carrying mutations reducing the activities of several known

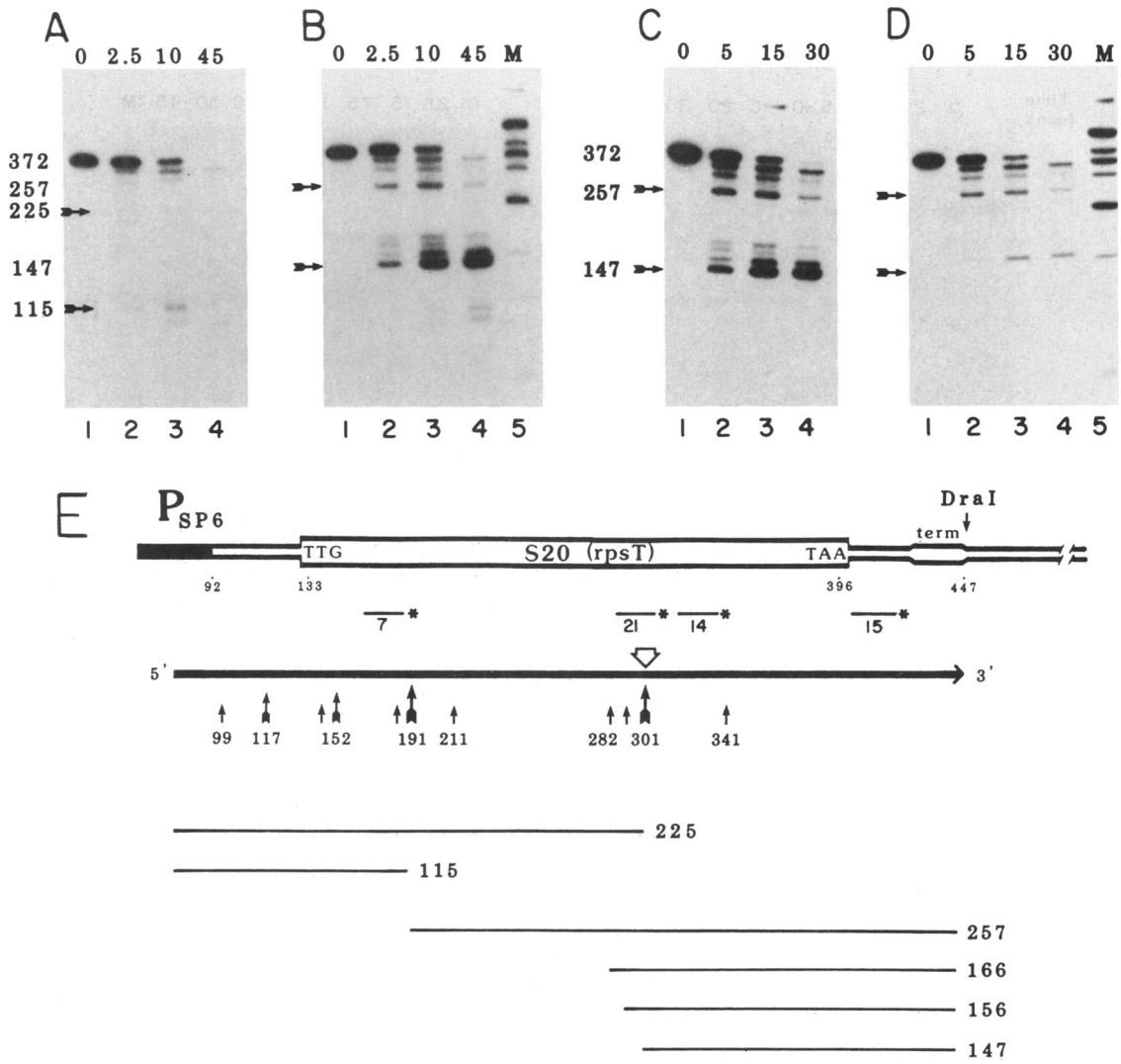

FIG. 3. Northern blot analysis of the products of digestion of an S20 mRNA substrate. (A through D) Unlabeled 372-residue substrate was digested as described in the legend to Fig. 2 for the time (in minutes) indicated above each lane, and blots were prepared as outlined in Materials and Methods. RNAs in the blots were detected by hybridization to ^{32}P-end-labeled oligonucleotides 7 (A), 15 (B), 21 (C), and 14 (D) (see Table 1 and panel E). Lanes 5 contain markers. (E) Schematic showing the major 5′ ends and products of digestion obtained with the AS-26 fraction. The S20 mRNA substrate is denoted by the thick arrow; P_{SP6} indicates the promoter for SP6 RNA polymerase. The four oligonucleotides employed as probes or primers are shown above the mRNA. The open vertical arrow denotes the position of the 5′ end of the Po RNA seen in polynucleotide phosphorylase-deficient strains in vivo (17). The closed vertical arrows below the substrate show the positions of cleavage sites in vitro, with each number indicating the new 5′ end. The height of the arrow is roughly proportional to the apparent frequency of cleavage. The horizontal lines show the extent of some of the products inferred from the results obtained in Fig. 2 and 3A through D. The 156- and 166-residue products are not marked in the margins to panels A through D; they are visible immediately above the prominent 147-residue product.

ribonucleases have been examined previously for alterations in the degradation of the S20 mRNAs (17). The effect of an *ams* mutation (2, 26) on the steady-state sizes of the S20 mRNAs in vivo is shown in Fig. 5. The 147-residue Po RNA and several other less than full-length RNAs can be detected in a *pnp-7* strain (Fig. 5, lane 2) as well as in strains with *ams-1 pnp-7* mutations grown at the permissive temperature (Fig. 5, lanes 4 and 5). One band denoted by the arrow in the margin to Fig. 5 comigrates with the 257-residue product produced in vitro (18). The relative abundance of the Po RNA is lower in strains SK5665 (*ams-1*) and SK5671 (*ams-1 pnp-7*), however, than in strain SK5003 (*pnp-7 rnb-500*) (Fig. 5; compare lane 2 with lanes 4 and 5). This may be a function of the growth rates of the different strains (cf. reference 16). At higher temperatures, strains carrying the *ams-1* mutation do not accumulate the Po RNA, even in a *pnp-7* background (Fig. 5, lanes 8 and 9). Formation of the Po RNA in vivo, therefore, requires a functional product of the *ams* gene.

The analysis of S20 mRNAs extracted from whole cells was extended by assaying S-30 extracts or derived AS-26 fractions from strain SK5684 (*pnp-7 rnb-500 ams-1*) for their ability to catalyze endonucleolytic cleavages of an S20 mRNA substrate. Initially, the S-30 extract from the mutant strain was assayed to avoid uncertainty about how the mutant activity might behave during fractionation. An S-30 extract from strain SK5684 is able to convert the 372-residue

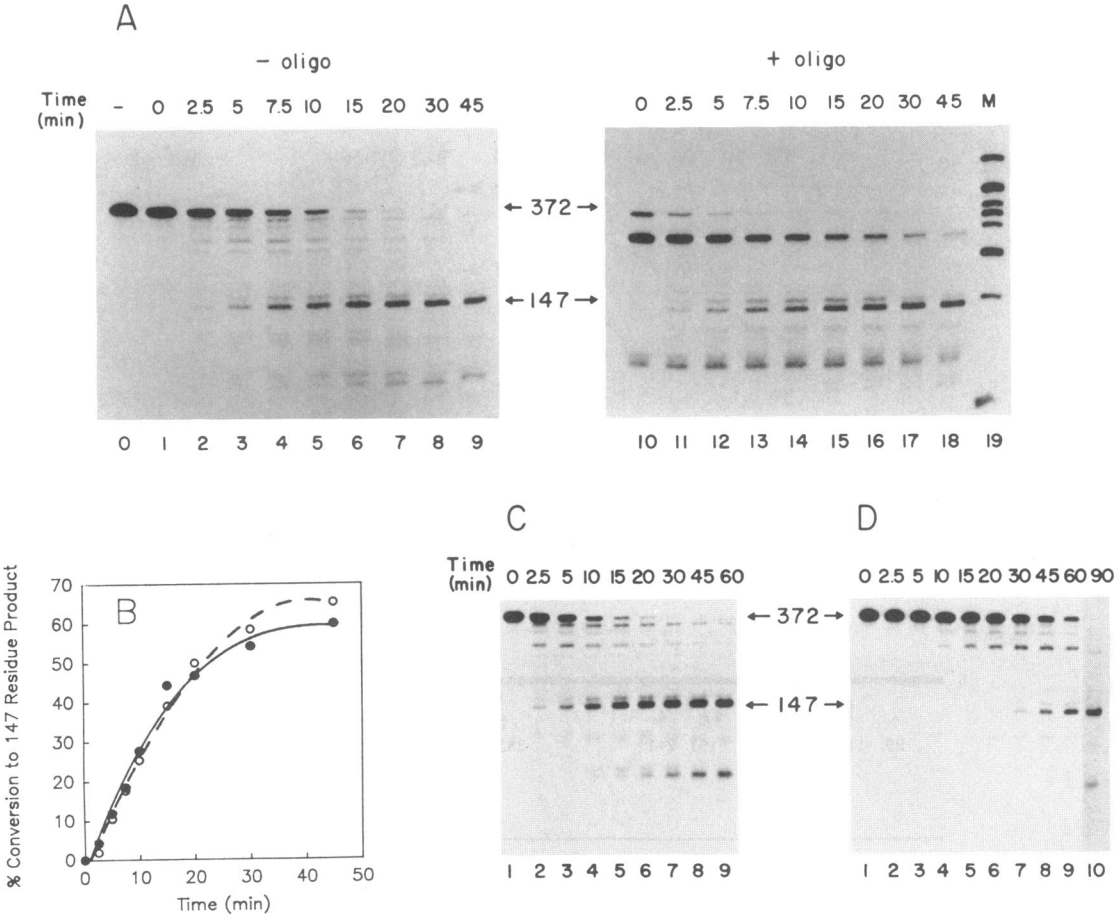

FIG. 4. Time course of digestion of S20 mRNA previously treated with ribonuclease H or denatured prior to assay. (A) The ^{32}P-labeled 372-residue substrate transcribed from *Dra*I-linearized pGM79 (1.2 pmol in 30 μl) was digested with 1 U of RNase H (obtained from Bethesda Research Laboratories, Inc.) for 15 min at 30°C in the absence (lanes 1 through 9) or presence (lanes 10 through 18) of 6 pmol of oligonucleotide 7 (Fig. 3E and Table 2). These mixtures were then incubated with AS-26 (0.08 μg/μl) from strain SK5003 at 30°C for the times indicated above each lane. The RNA products were denatured and analyzed by electrophoresis (see Materials and Methods). The RNA in lane 0 is untreated substrate. The markers in lane 19 are the same as for Fig. 1B. (B) Kinetics of accumulation of the 147-residue product. The experiment illustrated in panel A was repeated with a different preparation of substrate and 15 pmol of oligonucleotide 7. The yields of the 147-residue product from both experiments were determined by densitometry, and the results were averaged. The open circles represent yields obtained with substrate treated in the presence of oligonucleotide 7, while the closed circles denote yields obtained in its absence. (C and D) Effect of prior denaturation of the substrate on its digestion. The ^{32}P-labeled 372-residue substrate was assayed either after annealing in the assay buffer as described in Materials and Methods (C) or after heating in H_2O to 94°C for 5 min, followed by cooling on ice (D). Otherwise, conditions were similar to those used in Fig. 1 D. Samples were analyzed electrophoretically at the times (in minutes) indicated over each lane. Lane 10 in panel D was obtained from a separate experiment.

RNA transcribed from pGM79 into a number of smaller products, including the 147-residue limit product, at either 30 (18) or 42°C (Fig. 6, lanes 6 and 7), albeit inefficiently. Inspection of Fig. 6 shows that the rate of cleavage of substrate by the S-30 extract from strain SK5684 is much reduced compared with the rate of cleavage of substrate by the AS-26 fraction (or S-30 extract) (18) from the parental strain. A mixing experiment was performed to test for the presence of diffusible inhibitors in strain SK5684. An S-30 extract from strain SK5684 was assayed alone (Fig. 6, lanes 6 and 7) or with an AS-26 fraction prepared from strain SK5003 (Fig. 6, lanes 4 and 5). The extent of formation of the 147-residue product in the mixed assay is proportional to the total activity added from the wild-type AS-26 (Fig. 6, lanes 2 and 3) and the mutant S-30 (Fig. 6, lanes 6 and 7).

A quantitative comparison of the activities of the AS-26 fractions from parental and mutant strains is summarized in Table 2. At 29°C, the rate of formation of the 147-residue product in the mutant extract from strain SK5684 (*ams-1 pnp-7 rnb-500*) is only 20% the rate of an AS-26 fraction prepared from the parental strain SK5003 (*pnp-7 rnb-500*). In addition, the rate of disappearance of substrate was reduced fivefold in the mutant extract. At 42°C, the rate at which an S-30 or AS-26 extract from strain SK5684 catalyzes the disappearance of the substrate RNA and the formation of the 147-residue product is markedly reduced. In contrast, the AS-26 fraction from strain SK5003 is almost twofold more active at the higher temperature than at 29°C and is at least 80-fold more active than the AS-26 fraction from strain SK5684 at the permissive temperature (Table 2). While the data presented in Table 2 show only the rate of appearance of the 147-residue product, other products were similarly affected (18).

The stabilities of AS-26 fractions from strains SK5003 and

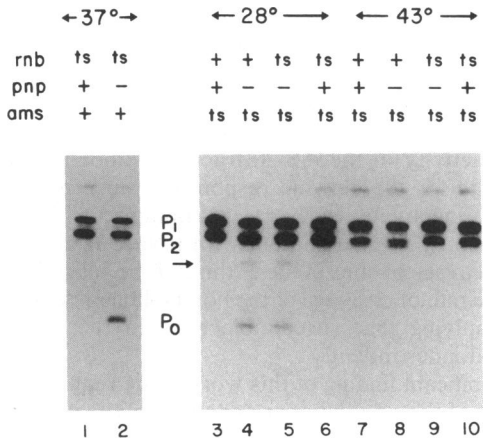

FIG. 5. Accumulation of Po RNA in different strains. Cultures of the strains listed below were grown at permissive temperature (29°C) and shifted to the temperature indicated at the top of the figure for 60 min prior to extraction of RNA by method II of reference 17. Samples containing 2 μg of total RNA were denatured by heating, separated on a 6% polyacrylamide gel, electroblotted to nylon, and detected with a ^{32}P-labeled complementary RNA probe (15) (see Materials and Methods). RNAs were extracted from the following strains: lane 1, SK5005 (*rnb-500*); lane 2, SK5003 (*rnb-500 pnp-7*); lanes 3 and 7, SK5665 (*ams-1*); lanes 4 and 8, SK5671 (*ams-1 pnp-7*); lanes 5 and 9, SK5704 (*ams-1 pnp-7 rnb-500*); lanes 6 and 10, SK5715 (*ams-1 rnb-500*). The positions of the P1, P2, and Po RNAs are noted in the central margin. The arrow points to a faint RNA whose mobility is identical to that of the 257-residue species noted in Fig. 3B through E.

SK5684 at 42°C are also summarized in Table 2. The endonuclease activity obtained from strain SK5684 (*ams-1 pnp-7 rnb-500*) is markedly thermosensitive, with an apparent half-life of less than 5 min at 42°C. The comparable activity from the *ams*$^+$ strain SK5003 is substantially more stable in the AS-26 fraction, with an apparent half-life of 60

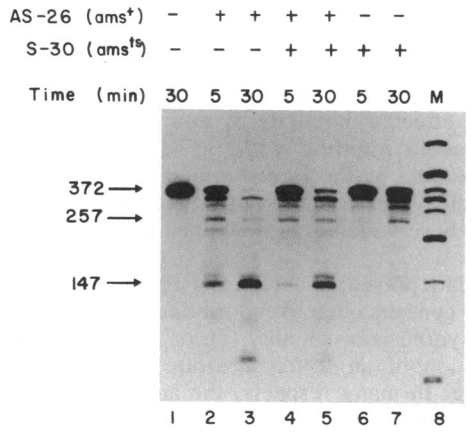

FIG. 6. Activity of extracts from an *ams-1* mutant. The standard cleavage assay with ^{32}P-labeled 372-residue RNA was performed at 42°C with an AS-26 fraction (0.1 μg/μl) from strain SK5003 (*pnp-7 rnb-500*) (lanes 2 and 3), with both AS-26 (0.1 μg/μl) from SK5003 and S-30 (3.0 μg/μl) from strain SK5684 (*ams-1 pnp-7 rnb-500*) (lanes 4 and 5), or with S-30 (3.0 μg/μl) from strain SK5684 alone (lanes 6 and 7). Samples were taken at the times indicated, deproteinized, and analyzed electrophoretically (see Materials and Methods). Lane 1 contains undigested substrate, and lane 8 contains denatured end-labeled DNA markers (cf. Fig. 1B).

TABLE 2. Thermal lability of endoribonuclease activity in extracts prepared from an *ams-1* mutant[a]

Source of AS-26	Sp act[b]		Half-life[c]
	29°C	42°C	
SK5003 (*pnp rnb*)	428	813	>60
SK5684 (*pnp rnb ams*)	57	<10	<5

[a] Separate AS-26 fractions were prepared from strains SK5003 (*pnp-7 rnb-500*) and SK5684 (*pnp-7 rnb-500 ams-1*). Each was assayed at 29 or 42°C on a ^{32}P-labeled 372-residue RNA substrate transcribed from pGM79 linearized with *Dra*I (see Materials and Methods) to yield the specific activities given in the table.

[b] Specific activity is the number of picomoles of 147-residue product formed per hour per milligram of protein. The numbers given are the averages of duplicate experiments with independent preparations of substrate.

[c] To determine the half-life of the endoribonuclease activity, the appropriate AS-26 fraction (0.3 mg/ml in buffer II) was incubated at 42°C. Samples were withdrawn at intervals and assayed by the standard method at 29°C. Each result is given as the time in minutes at 42°C required to inactivate half the activity compared with that of an untreated sample.

min. By two criteria, therefore, the thermosensitive mutation in the *ams* gene in strain SK5684 results in inactivation of the activity or activities capable of producing specific cleavages of the S20 mRNA in vitro.

DISCUSSION

The data presented here demonstrate that crude or partially fractionated extracts of *E. coli* contain one or more endoribonuclease activities capable of cleaving the mRNA for ribosomal protein S20 into discrete products. This system reproduces several features of the decay of the S20 mRNAs in vivo. Most notably, the cleavage produced in vitro between residues 300 and 301 in the S20 coding sequence is identical to the major internal 5′ end observed in vivo in strains with reduced 3′ exonuclease activity (17). At least one other cleavage observed in vitro, that between residues 190 and 191, can also be detected in vivo. A second important feature of the in vitro system is its dependence on the product of the *ams* gene, a feature reflective of mRNA decay in vivo (2, 26). Thus, the specificity obtained in vitro is a relatively faithful reproduction of at least some events in whole cells.

The *ams*-dependent cleavages manifested in the in vitro system exhibit specificity for both primary sequence and structure in the substrate. The bond preferentially hydrolyzed is usually NpA, in the context 5′-PupNpApU-3′ (Fig. 7A). Moreover, two of the sites attacked to yield discrete products (bonds between residues 190 and 191 and between residues 300 and 301) are preceded by imperfect or weak stem-loop structures, as illustrated in Fig. 7B and C. Reversible inactivation of the substrate by prior denaturation supports a significant role for secondary and tertiary structures in the selection of sites of cleavage in this system. To this extent, the cleavage of the S20 substrate resembles a processing event. There are several important differences, however. First, susceptible sites occur very frequently, every 20 to 30 residues, but the distribution of these sites is not random. Most are clustered in the 5′ third of the substrate, whereas the 3′ third is relatively resistant to attack, as is the case in vivo (17). The relative insensitivity of this portion of the substrate to endonuclease cleavage may be due to the extensive secondary structure which can form between residues 380 and 447 (17). Second, in several instances minor cleavage sites occur within several residues of major cleav-

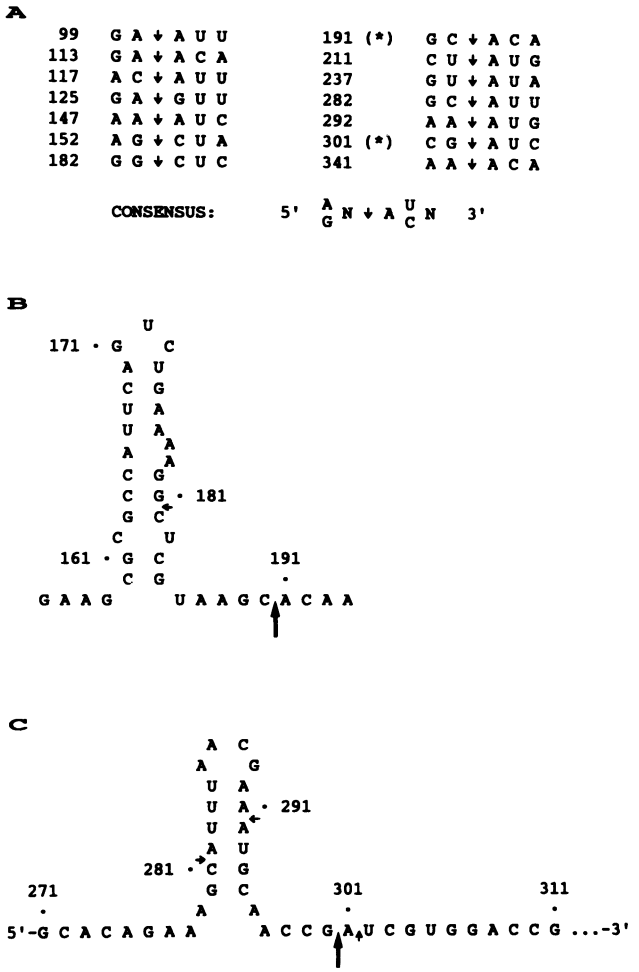

FIG. 7. Primary and secondary structures of *ams*-dependent cleavage sites in the S20 mRNA. (A) Pentanucleotides containing cleavage sites identified by primer extension are numbered according to the coordinates of the new 5′ end in the S20 mRNA sequence. The two cleavages yielding the most prominent products are marked with asterisks. (B) Potential secondary structure of residues 166 to 195 adjacent to the cleavage site between residues 190 and 191. (C) Potential secondary structure of residues 271 to 311 adjacent to the cleavage site between residues 300 and 301.

ages (e.g., a minor 5′ end occurs at residue 302 adjacent to the major 5′ end at residue 301; several minor sites occur 3′ to the abundant 5′ end at residue 191). Thus, while many NpApUp trinucleotides may form potential cleavage sites, certain ones will be attacked much more rapidly than others because they are situated in a highly favorable context created by adjacent secondary and/or tertiary structures. This hypothesis can be tested by modifying the potential for forming secondary structure in the S20 mRNA and by determining *ams*-dependent cleavage sites in other RNA substrates.

Cleavage of the S20 mRNA substrate in the in vitro system does not appear to be processive. First, the cleavage of the phosphodiester bond between residues 300 and 301 proceeds without a lag even though many other sites are situated upstream. The experiments illustrated in Fig. 3 show that most possible combinations of sites in the substrate are attacked. The disappearance of the 5′ portion of the substrate before the 3′ end should not be taken as evidence for 5′-to-3′ movement of an endonuclease along the substrate. It seems more likely that this observation is a reflection of the relative paucity of susceptible sites in the 3′ third of the substrate. Low recoveries of 5′ fragments of the substrate (Fig. 3A) probably also reflect significant levels of 3′ exonuclease activity in the AS-26 fraction. Ribonuclease II is a candidate for the enzyme responsible since no steps were taken to inactivate it. The results obtained with a substrate shortened by prior treatment with RNase H also show that prior cleavage of sites in the 5′ third of the substrate does not alter the rate of cleavage of the bond joining residues 300 and 301, implying that susceptible sites in the substrate are attacked independently.

A significant finding of this work is the requirement of the product of the *ams* gene for endonucleolytic cleavage of the S20 mRNA in vitro and in vivo. Under nonpermissive conditions in vivo, the *ams* mutation causes an increase in the chemical lifetime of total mRNA but does not alter the functional half-lives of either the *lac* or *trp* mRNAs (2, 26). These observations can be interpreted to imply that the *ams* gene product is required at a step in mRNA turnover which follows the initial step(s) committing an mRNA to decay. The properties of the in vitro system described here suggest that the product of the *ams* gene could serve as more than just a scavenging endoribonuclease, however. First, the S20 substrate is cleaved at preferred sites, not randomly, resulting in some relatively large stable products. Second, the substrate does not require previous cleavage to become activated. Third, cleavage at any one of the sites shown in Fig. 7A could potentially inactivate the template activity of the S20 mRNA, particularly if coupled to 3′ exonucleolytic attack. *ams*-dependent activities could, therefore, serve to initiate mRNA decay on any substrate which contains a strong cleavage site. The disappearance of sequences 5′ to the major cleavage between residues 300 and 301 in vivo (17) is consistent with the *ams*-dependent labilization of upstream sequences seen in vitro. Whether this actually occurs in vivo depends on several variables. Those which could influence an *ams*-dependent endonuclease directly include the rate at which it binds RNA and hydrolyzes susceptible bonds, the location of cleavage sites, and the position of secondary structures capable of inhibiting exoribonuclease activity. The activity of an *ams*-dependent endoribonuclease may be affected indirectly by competition from ribosomes which could interfere with the enzyme's search for its substrate and by the rate at which other potentially inactivating cleavages occur on the same mRNA substrate. The role of these variables is now open to systematic investigation.

The data presented here are consistent with the view that the *ams* gene encodes an endoribonuclease, a factor required for its synthesis or stability (7), or a factor required for the recognition of an RNA substrate by a known or novel nuclease. In many respects, the activity documented here resembles that of RNase K (13) and RNase E (22–24). A significant difference is that the *ams*-dependent activity displays a very high molecular weight ($>1.5 \times 10^6$) (18), unlike that reported for RNase K (13) or RNase E (22). Further purification of the *ams*-dependent activity should establish its specificity unambiguously and permit its direct comparison with the known endonucleases of *E. coli*, of which seven, RNases I (30), III (3, 10, 28), IV (29), E (23, 24), K (13), M (5), and N (21), have been implicated directly or indirectly in mRNA turnover.

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ADDENDUM IN PROOF

There is now convincing evidence that *ams* and *rne* (encoding RNase E) are the same genetic locus (P. Babitzke and S. R. Kushner, Proc. Natl. Acad. Sci. USA **88**:1–5, 1991; E. A. Mudd, H. M. Kritsch, and C. F. Higgins, Mol. Microbiol. **4**:2127–2135, 1990), implying that the *ams*-dependent endonuclease activity is RNase E or requires it as a component.

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