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Cloning, Sequence Analysis, and Expression of Alteration of the mRNA Stability Gene (*ams*$^+$) of *Escherichia coli*

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The *ams*$^+$ gene, which influences the stability of mRNA in *Escherichia coli* was cloned in pBR322. The product of the gene, which is a 17,000-dalton protein, was expressed in expression vector pRC23, a derivative of pBR322. The molecular weight is consistent with sequencing analysis which shows that the gene contains 595 nucleotides and has an open reading frame of 149 amino acids. We discussed the possible role(s) of the *ams*$^+$ gene product in affecting mRNA stability.

It is known that in *Escherichia coli*, mRNA decays exponentially with a half-life of 0.5 to 2 min at 37°C, and the average mRNA is translated by about 20 to 30 ribosomes before it is destroyed (9). In addition, the overall decay of mRNA chains proceeds in a 5′-to-3′ direction (11) (the same direction that transcription and translation occur on DNA and RNA templates, respectively) or a 3′-to-5′ direction (19). Furthermore, in both whole cells and extracts, decay is inhibited when translation is slowed or blocked (2, 4, 8). A coupling of mRNA decay to translation analogous to the coupling of mRNA transcription and translation (3, 5) was therefore suggested. The mechanism of the decay is unknown, although a mutant with low RNase III activity was found to stabilize mRNA (16). Thus, to study the properties of a gene(s) that may be involved in the turnover of mRNA in growing cells, it is important to have mutants that show a prolonged mRNA half-life. Such a mutant (designated *ams*, alteration of mRNA stability) has been isolated and described by Kuwano et al. and Ono and Kuwano (6, 12). Using this mutant, we cloned the wild-type *E. coli* mRNA stability gene (*ams*$^+$) in pBR322. In this communication we report the cloning, sequencing, and expression of the cloned gene which complements the *ams* mutation. Sequence analysis shows that the gene (595 nucleotide) can code for a protein of 149 amino acids, corresponding to a molecular weight of ≈17,000.

Molecular cloning of mRNA stability gene. To clone the *ams*$^+$ gene, wild-type *E. coli* DNA was cleaved with *Eco*RI and then ligated to *Eco*RI-digested pBR322 which had been treated with calf intestine alkaline phosphatase. The ligated DNA was used to transform *E. coli* HAK117, a temperature-sensitive mutant. This mutant has an mRNA half-life of 10 to 12 min at 42°C compared with 1.5 to 2 min for the wild type (12). Colonies which grew at 42°C were selected, and the mRNA half-lives of several of them were determined by studying the degradation of ^{3}H pulse-labeled mRNA (12). A temperature-resistant clone (A) that had an insert of 8.1 kilobases and showed an mRNA half-life comparable to that of the wild type (2 min) was selected for additional studies. The *ams*$^+$ gene was then subcloned into pBR322 as shown in Fig. 1A. Clone (A) was digested with *Bam*HI and *Eco*RI and two *Eco*RI-*Bam*HI fragments (4.3 and 3.8 kilobases) were purified by gel electrophoresis and ligated separately to *Eco*RI- and *Bam*HI-digested pBR322. This produced clones B and C. Clone B was digested with *Kpn*I, and the large *Kpn*I fragment was isolated and self-ligated at the *Kpn*I site to produce clone D. Clone D was digested with either *Eco*RI and *Kpn*I or *Bam*HI and *Kpn*I. The large fragments in both cases were isolated after gel electrophoresis and treated with T4 DNA polymerase. The 5′-to-3′ polymerizing activity as well as the 3′-to-5′ exonucleolytic activities of T4 DNA polymerase gave rise to clones E and F (Fig. 1B).

mRNA degradation. Measurement of mRNA degradation was done by the method of Ono and Kuwano (12). Exponentially growing cells (2×10^8 to 4×10^8 cells per ml) (*E. coli* HAK117 transformed with different plasmids) were pulse labeled for 2 min with 2 μCi of [^{3}H]uridine per ml at 42°C, and labeling was immediately stopped by the addition of 500 μg of rifampin, 200 μg of uridine, and 20 μg of nalidixic acid per ml. Samples were then withdrawn at different times, and Cl_3CCOOH precipitable counts were measured. The percentage of [^{3}H]RNA remaining was calculated as described previously (12). Figure 2 shows the mRNA degradation of various clones. It can be seen that the half-lives of clones A, B, D, and E are 2, 2.25, 2.5, and 3 min, respectively, whereas the half-life of the mutant is 11.5 min. These values are very similar to that of the wild type, which is 2 to 2.5 min (data not shown). These results show that when the mutant was transformed with a plasmid containing the *ams*$^+$ gene, the half-life of the mRNA population decreased to a value similar to that of the wild type.

Nucleotide sequence of *ams*$^+$ gene. Subclone E was the smallest plasmid of those tested and was therefore used for *ams*$^+$ gene sequencing. The strategy used to determine the complete nucleotide sequence of the *ams*$^+$ gene is shown in Fig. 3. The 595-nucleotide insert as sequenced by the method of Maxam and Gilbert (10) is shown in Fig. 4. DNA sequencing was carried out by using both 5′ single end-labeled fragments as well as filling the restriction sites with Klenow fragment of *E. coli* DNA polymerase. Both strands were sequenced in their entirety.

Expression of *ams*$^+$ gene. There is an open reading frame of 149 amino acids within the *ams*$^+$ gene sequence, and the expression of the gene was achieved by using the expression vector designated pRC23 (R. Crowl, Methods Enzymol., in press). pRC23 is a derivative of pBR322 containing the λ p_L promoter and a synthetic ribosome binding site. Expression of the *ams*$^+$ gene is controlled by the temperature-sensitive λ *c*I repressor produced from the compatible plasmid pRK248

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cIts. The *Eco*RI-*Bam*HI fragment of clone E was inserted into pRC23. Overnight cultures of *E. coli* RR1(pRK248 cIts) containing the expression vector pRC23 with the *ams*$^+$ gene insert, (designated pRC23/*ams*$^+$) were grown in minimal M9 medium at 30°C. The culture was then diluted with minimal M9 medium containing Casamino Acids. During the logarithmic phase of growth (absorbance at 600 nm $\simeq$ 0.4), the culture was shifted from 30 to 42°C, and growth was continued for another 2 h. Bacteria were harvested by centrifugation, and the pellet was dissolved and boiled in sample buffer (0.05 M Tris-hydrochloride [pH 6.8], 1% sodium dodecyl sulfate, 10% glycerol, 0.7 M β-mercaptoethanol) and electrophoresed on a 12.5% polyacrylamide gel by the method of Laemmli (7).

Figure 5 shows the Coomassie brilliant blue stained gel, demonstrating the expression of the *ams*$^+$ gene. From Fig. 5, lane C, it is evident that a polypeptide (band Y) of the expected molecular weight ($\simeq$17,000) is observed when pRC23/*ams*$^+$ is introduced into *E. coli* RR1(pRK248 cIts) and subsequently heat induced. This protein was produced in large quantity 2 h after induction compared with the amount produced in uninduced cells (Fig. 5, lane B). Another protein (band X) with a slightly higher molecular weight ($\simeq$19,000) was also obtained. This protein may be a result of read through. To demonstrate that the protein product might be responsible for the *ams*$^+$ gene function, we changed the reading frame of the coding region of the *ams*$^+$ gene. This was done by digesting pRC23/*ams*$^+$ with *Cla*I and filling in the *Cla*I sites with Klenow fragment of *E. coli* DNA polymerase I. This construction (designated pRC23/*ams**) shifts the reading frame and results in an early termination, giving rise to a protein with a molecular weight of $\simeq$11,000 (data not shown). Next, we transformed *E. coli* HAK117 with pBR322 vector containing the *ams*$^+$ as well as the *ams** gene to determine which one could transform the mutant to the wild-type phenotype. Only *ams*$^+$ complemented *E. coli* HAK117, and *ams** did not, suggesting that the *ams*$^+$ gene product (a protein of $\simeq$17,000 daltons) is responsible for its function. Since the sequenced 595-base-pair fragment does not seem to have a promoter consensus sequence, different cloned fragments isolated from plasmids A, B, D, and E (Fig. 1A) were transcribed in vitro with purified *E. coli* RNA

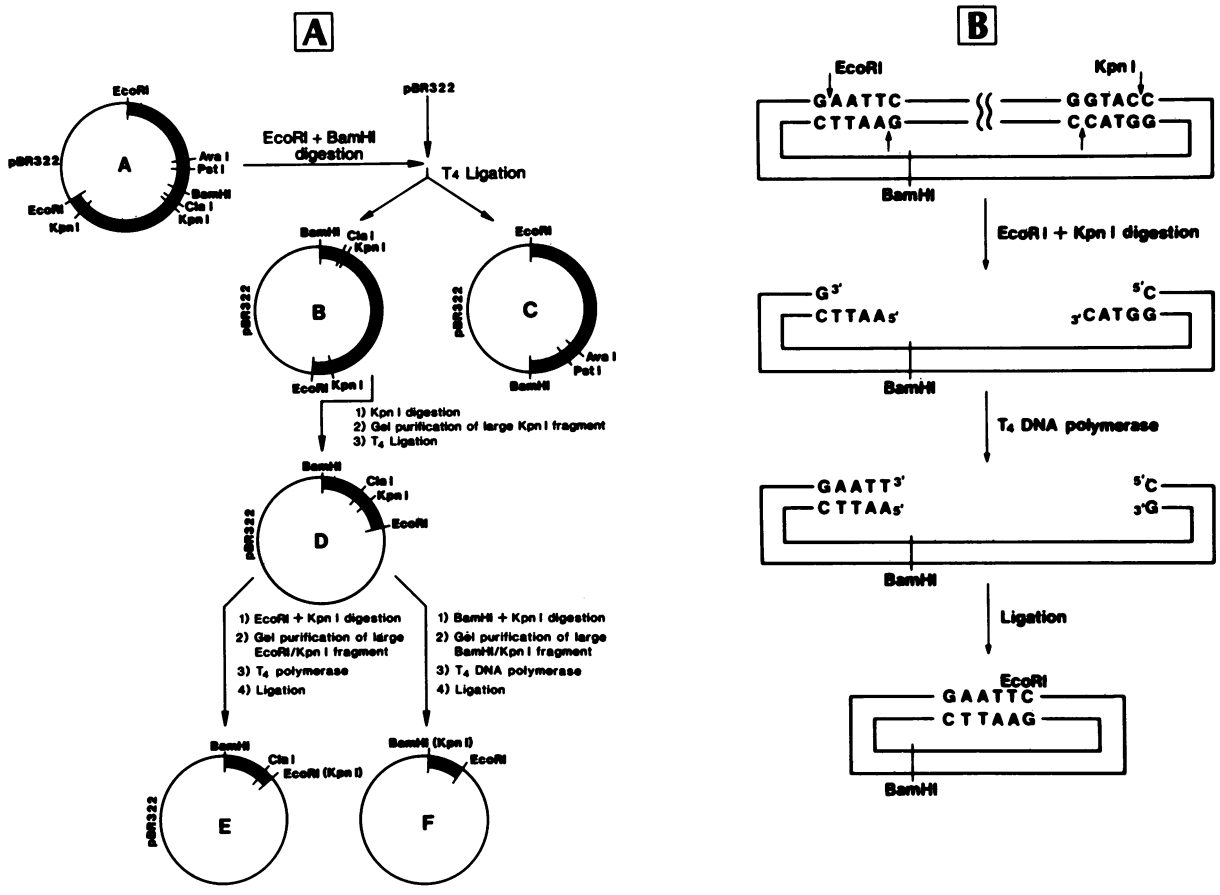

FIG. 1. Construction of *ams*$^+$ gene clones. Clone A containing the 8.1-kilobase insert which was found to complement *E. coli* HAK117 as described in the text was digested to completion with the restriction enzymes *Eco*RI and *Bam*HI. Two *Eco*RI and *Bam*HI fragments (3.8 and 4.3 kilobases) were isolated by agarose gel electrophoresis and then ligated to pBR322 similarly digested with *Eco*RI and *Bam*HI. This gave rise to clones B and C. Clone C was found not to complement *E. coli* HAK117. Clone B, which complemented *E. coli* HAK117, was then digested with *Kpn*I, and the large *Kpn*I fragment was isolated and self-ligated at the *Kpn*I site, producing clone D. Clone D was then digested in one case with *Eco*RI and *Kpn*I, and a large *Eco*RI-*Kpn*I fragment which contains *Bam*HI and *Cla*I sites was isolated from the gel. In another case, clone D was digested with *Bam*HI and *Kpn*I, and a large *Bam*HI-*Kpn*I fragment was similarly isolated. In both cases the DNA fragments were treated with T4 DNA polymerase, followed by blunt-end ligation, producing clones E and F. The latter part of the constructions are shown in more detail in (B). Clone E was found to complement *E. coli* HAK117 but not clone F. All the inserts (black bars) have been drawn to scale with respect to pBR322.

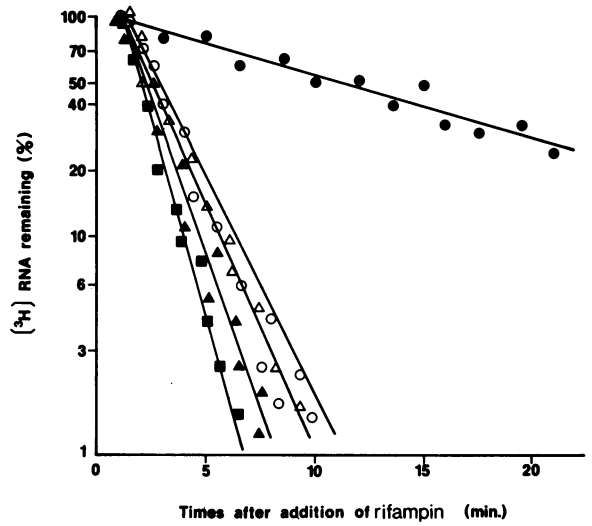

FIG. 2. Degradation of pulse-labeled RNA in clones A, B, D, and E. *E. coli* HAK117 containing the different plasmids (A through E) was grown to exponential phase in minimal A medium [10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.5 g of sodium citrate-$2H_2O$, 2 g of glucose per liter] at 30°C and then incubated at 42°C for 15 min before being pulse-labeled with 2 μCi of [^{3}H]uridine per ml for 2 min at 42°C. The labeling was immediately stopped by the addition of rifampin (500 μg/ml), uridine (200 μg/ml), and nalidixic acid (20 μg/ml). Samples (0.2 ml) were withdrawn at the indicated times and the radioactivity of the acid-insoluble fraction was measured. The percentage of [^{3}H]RNA remaining was then calculated from the radioactivity at a given time minus that for the "stable RNA" present at min 50 after rifampin addition and divided by the maximum radioactivity in the unstable RNA fraction (12). Symbols: ●, HAK117; ○, clone A; △, clone B; ▲, clone D; and □, clone E.

polymerase, and the transcripts were hybridized with the 595-base-pair fragment. The transcripts from the inserts of plasmid A, B, and D did show hybridization, whereas no hybridization was observed in the insert of plasmid E. These results suggested that the promoter of the *ams*$^+$ gene may be located between the *Eco*RI and *Kpn*I fragment of clone D, and the expression of the *ams*$^+$ gene in clone E may be the result of transcription initiating from the pBR322 vector.

The most attractive hypothesis is that the *ams*$^+$ gene codes for an RNase. Since the *ams*$^+$ gene in *E. coli* HAK117 is temperature sensitive, the RNase at the nonpermissive

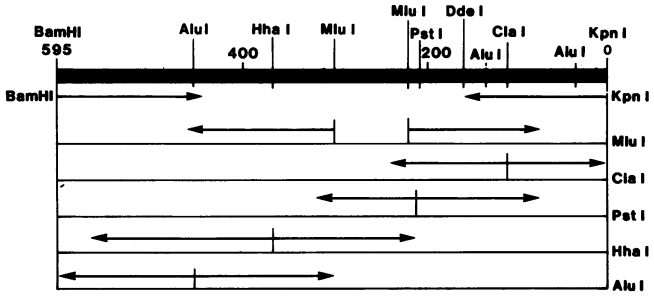

FIG. 3. Restriction map and sequence strategy of *ams*$^+$ gene. The black bar indicates the cloned segment of *ams*$^+$ gene in pBR322. The positions of the restriction endonuclease sites are based on analysis of the size of DNA fragments from single and double digestion of the 595-base-pair DNA fragment. The arrows indicate the direction and extent of individual sequencings.

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KpnI          10          20          30          40          50
GGT ACC GTG ATC TCT GAA GAG ATC GGT ATG GAG CTG GAA AAA GCA ACC CTG GAA
                                    MET Glu Leu Glu Lys Ala Thr Leu Glu

      60          70          80          90          100
GAC CTG GGT CAG GCT AAA CGT GTT GTG ATC AAC AAA GAC ACC ACC ACT ATC ATC
Asp Leu Gly Gln Ala Lys Arg Val Val Ile Asn Lys Asp Thr Thr Thr Ile Ile

 110         120         130         140         150         160
GAT GGC GTG GGT GAA GAA GCT GCA ATC CGA GGC CGT GTT GCT CAG ATC CGT CAG
Asp Gly Val Gly Glu Glu Ala Ala Ile Arg Gly Arg Val Ala Gln Ile Arg Gln

          170         180         190         200         210
CAG ATT GAA GAA GCA ACT TCT GAC TAC GAC CGT GAA AAA CTG CAG GAA CGC GTA
Gln Ile Glu Gly Ala Thr Ser Asp Tyr Asp Arg Glu Lys Leu Gln Glu Arg Val

    220         230         240         250         260             270
GCG AAA CTG GCA GGC GGC GTT GCA GTT ATC AAA GTG GGT GCT GCT ACC GAA GTT
Ala Lys Leu Ala Gly Gly Val Ala Val Ile Lys Val Gly Ala Ala Thr Glu Val

          280         290         300         310         320
GAA ATG AAA GAG AAA AAA GCA CGC GTT GAA GAT GCC CTG CAC GCG ACC CGT GCT
Glu MET Lys Glu Lys Lys Ala Arg Val Glu Asp Ala Leu His Ala Thr Arg Ala

      330         340         350         360         370
GCG GTA GAA GAA GGC GTG GTT GCT GGT GGT GGT GTT GCG CTG ATC CGC GTA GCG
Ala Val Glu Glu Gly Val Val Ala Gly Gly Gly Val Ala Leu Ile Arg Val Ala

 380         390         400         410         420         430
CTA AAC TGG CTG ACC TGC GTG GTC AGA ACG AAG ACC AGA ACG TGG GTA TCA AAG
Leu Asn Trp Leu Thr Cys Val Val Arg Thr Lys Thr Arg Thr Trp Val Ser Lys

          440         450         460         470         480
TTG CAC TGC GTG CAA TGG AAG CTC CGC TGC GTC AGA TCG TAT TGA ACT GCG GCG
Leu His Cys Val Gln Trp Lys Leu Arg Cys Val Arg Ser Tyr  *  Thr Ala Ala

    490         500         510         520         530         540
AAG AAC CGC TTG TTG TTG CTA ACA CCG TTA AAG GCG GCG ACG GCA ACT ACG GTT
Lys Asn Arg Leu Leu Leu Leu Thr Pro Leu Lys Ala Ala Thr Ala Thr Thr Val

          550         560         570         580         590
ACA ACG CAG CAA CCG AAG AAT ACG GCA ACA TGA TCG ACA TGG GTA TCC TGG ATC C
Thr Thr Gln Gln Pro Lys Asn Thr Ala Thr  *  Ser Thr Trp Val Ser Trp Ile

                                                              BamHI
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FIG. 4. Nucleotide sequence of the *ams*$^+$ gene. DNA sequencing was carried out by the method of Maxam and Gilbert (10), and the nucleotide sequence of the coding strand is given from the 5′-to-3′ direction. The predicted amino acid sequence is shown below the DNA sequence. Numbering starts from the first base in the sequence.

temperature (42°C) is nonfunctional, and thus mRNA is stabilized. A mutant with low RNase III has been found to stabilize mRNA. The *ams* gene has been mapped at 23′ on the *E. coli* chromosome (13), but RNase III maps at 55′ (1, 17). In addition to RNase III, other RNases, RNase I and RNase II, map at 14′ and 28′ on the *E. coli* chromosome, respectively (12–15). From the map position of the *ams*$^+$ gene, it is unlikely that the product of the *ams*$^+$ gene is one of these RNases. However, it may be a new RNase yet to be defined. Isolation and characterization of the 17,000-dalton protein will be an important step to answer the question raised above. The degradation process might also operate through the action of ribosomes or other unknown mechanisms such as transcription and antitermination (12). With respect to the latter possibility, a protein, "tof," coded by phage lambda has been shown to be indicated in the stability of mRNA transcribed from phage lambda or tryptophan mRNA transcribed from the phage promoter (18, 20). We are now in the process of cloning and sequencing the *ams* gene from *E. coli* HAK117. Comparison of the *ams*$^+$ and mutant *ams* sequences will allow us to identify the site(s) of mutation and thus give a better understanding of the mechanism(s) by which the half-life of mRNA in the mutant changed. Finally, we should note the formal possibility that we have isolated a second *ams* locus that can function as a suppressor of *ams* when present on a high-copy plasmid. Further biochemical and genetic experiments will be needed to clarify this point.

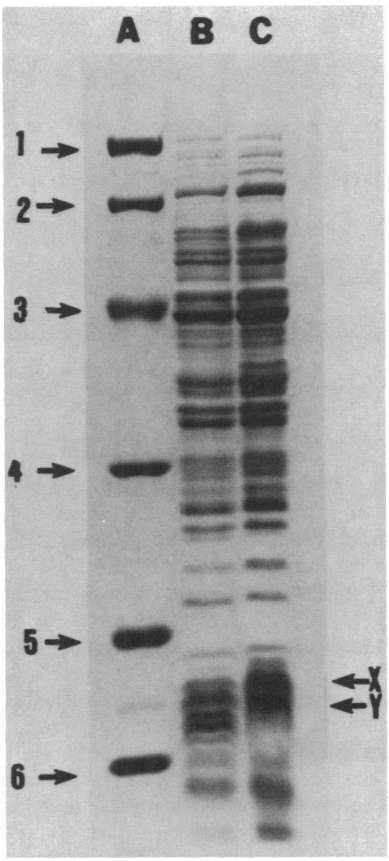

FIG. 5. In vivo expression of the *ams*$^+$ gene. The *ams*$^+$ gene was expressed in *E. coli* by using the expression vector designated as pRC23 as described in the text. *E. coli* RR1 (pRK248*c*Its) containing the expression vector with the *ams*$^+$ gene, designated pRC23/*ams*$^+$, was grown in minimal M9 medium at 30°C overnight. A 1-ml amount of the overnight culture was then diluted to 50 ml with minimal M9 medium. A logarithmic phase (optical density at 600 nm = 0.4), the culture was shifted from 30 to 42°C, and the growth was continued for another 2 h. Bacteria (1 ml) were harvested by centrifugation, and the pellet was dissolved in a loading sample and then electrophoresed on a 12.5% polyacrylamide gel by the method of Laemmli (7). The gel was then stained with Coomassie brilliant blue R250 and then destained with methanol-acetic acid (5%:7%). Lane A, molecular weight standards: 1, phosphorylase *b*, 93,000; 2, bovine serum albumin, 66,000; 3, ovalbumin, 45,000; 4, carbonic anhydrase, 31,000; 5, soybean trypsin inhibitor, 21,500; 6, lysozyme, 14,500. Lane B, cell extracts made before heat induction; lane C, cell extracts made after heat induction.

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