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Cloning and Functional Analysis of the Arginyl-tRNA-protein Transferase Gene *ATE1* of *Saccharomyces cerevisiae**

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Elisabetta Balzi‡, Mordechai Choder§¶, Weining Chen‡, Alexander Varshavsky§, and Andre Goffeau‡

From the ‡Laboratoire d'Enzymologie, Unité de Biochimie Physiologique, Université Catholique de Louvain, Place Croix du Sud 1, Louvain-La-Neuve 1348, Belgium and the §Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Aminoacyl-tRNA-protein transferases (Arg-transferases) catalyze post-translational conjugation of specific amino acids to the amino termini of acceptor proteins. A function of these enzymes in eukaryotes has been shown to involve the conjugation of *destabilizing* amino acids to the amino termini of short-lived proteins, these reactions being a part of the N-end rule pathway of protein degradation (Gonda, D. K., Bachmair, A., Wünning, I., Tobias, J. W., Lane, W. S., and Varshavsky, A. (1989) *J. Biol. Chem.* 264, 16700–16712). We have cloned the *ATE1* gene of the yeast *Saccharomyces cerevisiae* which encodes arginyl-tRNA-protein transferase. *ATE1* gives rise to a ~1.6-kilobase mRNA and codes for a 503-residue protein. Expression of the yeast *ATE1* gene in *Escherichia coli*, which lacks Arg-transferases, was used to show that the ATE1 protein possesses the Arg-transferase activity. Null *ate1* mutants are viable but lack the Arg-transferase activity and are unable to degrade those substrates of the N-end rule pathway that start with residues recognized by the Arg-transferase.

It has been known for many years that both bacteria and eukaryotes contain aminoacyl-tRNA-protein transferases, enzymes that catalyze post-translational conjugation of specific amino acids to amino termini of acceptor proteins (Kaji *et al.* 1963, 1965; Hird *et al.*, 1964; reviewed by Soffer, 1974, 1980; Deutch, 1984). This amino acid-protein conjugation is greatly enhanced in stressed or regenerating animal tissues, for instance, in physically injured neuronal axons (Ingoglia *et al.*, 1983; Zanakis *et al.*, 1984; Shyne-Athwal *et al.*, 1986; Chakraborty *et al.*, 1986). Furthermore, in studies initially unconnected to the problem of amino acid-protein conjugation, it has been found that ubiquitin-dependent degradation of some proteins in reticulocyte extracts requires the presence of tRNA (Ferber and Ciechanover, 1986). Discovery of the N-end rule of protein degradation (Bachmair *et al.*, 1986) provided an explanation for these observations and suggested a function for aminoacyl-tRNA-protein transferases. Specifically, it has been shown that in both yeast and mammalian cells, an important component of the degradation signal in a short-lived protein is the protein's amino-terminal residue (Bachmair *et al.*, 1986; Varshavsky *et al.*, 1988; Reiss *et al.*, 1988; Gonda *et al.*, 1989; Chau *et al.*, 1989; Bachmair and Varshavsky, 1989). The resulting code or rule that relates the protein's metabolic stability and the nature of its amino-terminal residue has been called the N-end rule. It has also been suggested (Bachmair *et al.*, 1986) and confirmed (Ferber and Ciechanover, 1987; Gonda *et al.*, 1989) that some of the destabilizing amino-terminal residues in proteins are not directly destabilizing as such but become so only through their ability to be conjugated, via an aminoacyl-tRNA-protein transferase, to other, *primary* destabilizing residues. Amino-terminal glutamic acid and aspartic acid (and also cysteine in mammalian reticulocytes) have been shown to be the *secondary* destabilizing residues in that they are destabilizing through their ability to be conjugated, via an arginyl-tRNA-protein-transferase, to arginine, a *primary* destabilizing residue (Ciechanover *et al.*, 1988; Gonda *et al.*, 1989). Amino-terminal glutamine and asparagine have been shown, in both yeast and mammalian cells, to be the *tertiary* destabilizing residues in that they are converted, via selective deamidation, into the *secondary* destabilizing residues glutamic acid and aspartic acid (Gonda *et al.*, 1989).

Although the existence of several distinct aminoacyl-tRNA-protein-transferase activities has been inferred previously from a variety of evidence in both eukaryotic and prokaryotic cells (see "Discussion"), the only transferases that have actually been purified and characterized in some detail are the arginyl-tRNA-protein-transferase in eukaryotes and the leucyl, phenylalanyl-tRNA-protein-transferase in bacteria (reviewed by Soffer, 1980; Deutch, 1984; see also Kato and Nozawa, 1984).

Savage *et al.* (1983) have isolated a yeast (*Saccharomyces cerevisiae*) mutant that had no obvious phenotype other than the virtual absence of the Arg-transferase[1] activity in cell-free extracts. A wild-type version of the mutant gene has been named *ATE1* and genetically mapped to a locus on chromosome VII, near the *LEU1* gene (Savage *et al.*, 1983).

We now report the cloning of the *S. cerevisiae ATE1* gene, show that it encodes Arg-tRNA-protein transferase, and describe the results of an *ate1* null phenotype analysis. We also

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¶ Supported by a postdoctoral fellowship from the Weizmann Foundation. Present address: Dept. of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138.

[1] The abbreviations used are: Arg-transferase, arginyl-tRNA-protein transferase; Leu,Phe-transferase, leucyl,phenylalanyl-tRNA-protein transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase(s); SDS, sodium dodecyl sulfate; Ub-*X*-β*gal*, ubiquitin-*X*-β-galactosidase.

consider the potential diversity of amino acid conjugation reactions *in vivo* and functional implications of such diversity.

EXPERIMENTAL PROCEDURES

Yeast and Bacterial Strains—The *S. cerevisiae ate1* mutant (strain M522: *MATα ate1 his7* [KIL-1] (Savage *et al.*, 1983)) was kindly provided by Dr. M. J. Leibowitz (Rutgers University, Piscataway, NJ). The *ate1* strain U288-12A (MAT*α ate1 ura3 his7*), used for transformation according to Dieckman and Tsagoloff (1983), has been obtained by a cross between M522 and 22295-C (*MAT***a** *ura3*) and was a gift from Dr. S. Ulaszewski (University of Wroclaw, Poland). The strains 22295-C (*Mat***a** *ura3*) and US86 (*MATα/MAT***a** *ura3/ura3*) were used for *ATE1* gene disruption. The strains IL125-2B (*MATα his 1* ω^+) and Σ1278b (*MATα*) were used as additional controls for wild-type levels of the Arg-transferase activity. YPD medium contained 2% glucose, 1% Bacto-yeast extract, and 2% Bacto-peptone. SD medium contained 2% glucose, 0.67% Difco yeast nitrogen base without amino acids, and nutrients essential for auxotrophic strains (Sherman *et al.*, 1986).

Transformation of *Escherichia coli* was carried out with either the HB101 strain, using the RbCl procedure (Maniatis *et al.*, 1982) or with the strains BJH3 (for selection of *URA3* plasmids), *71-18* (for selection of *lacZ* plasmids), and K38 (Tabor and Richardson, 1985) (for expression of *ATE1* in *E. coli*). Transformation of the latter strains was carried out using the $CaCl_2$ procedure (Maniatis *et al.*, 1982).

In Vitro Assay for Arg-transferase—The Arg-transferase activity was determined in crude extracts (Savage *et al.*, 1983; Deutch, 1984). A stationary culture of *S. cerevisiae* grown at 30 °C in SD medium was centrifuged at 5,000 × *g* for 5 min. The pelleted cells (450 mg, wet weight) were resuspended in 0.5 ml of 0.25 M sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (freshly added from a 0.1 M stock in isopropyl alcohol), 1 mM dithiothreitol, 0.2 M Tris-HCl (pH 8.0). The suspension (0.9 ml) was degassed and thereafter shaken with 0.9 g of 0.45-mm glass beads in a Braun homogenizer (2 min total, with 3-s CO_2 cooling cycles between 20-s homogenizations). The suspension was adjusted to pH 7.5 with 1 N NaOH and centrifuged at 80,000 × *g* for 50 min at 4 °C. The supernatant was adjusted to pH 9.0 with 1 N NaOH and used immediately for the Arg-transferase assay. A reagent for this assay, [^{14}C]arginine-tRNA, was prepared in advance by incubating a supernatant from the extract of strain Σ1278b (obtained as above except for the resuspension of pelleted cells in 0.25 M glucose, 0.1 M Tris-HCl, pH 7.5) with 0.6 nmol of L-[^{14}C]arginine (Amersham Corp., 342 mCi/mmol) and total yeast tRNA (Boehringer Mannheim) at 25 mg/ml for 45 min at 30 °C. The incubation mixture (0.2 ml) also contained 9 mM $MgCl_2$, 2 mM ATP, 0.1 mM dithiothreitol, 0.1 M NaHEPES (pH 7.4), and 4.4 μM unlabeled arginine. After incubation, the sample was extracted with phenol, the RNA precipitated with ethanol, and redissolved in 50 mM Tris-HCl (pH 9.0) to ~2,000 cpm/μl (~0.8 nM in Arg-tRNA). Under these conditions, ~40% of the added [^{14}C]arginine was converted into [^{14}C]Arg-tRNA. To measure the Arg-transferase activity, 0.17 ml of the yeast extract was incubated at 37 °C with [^{14}C]Arg-tRNA (~40,000 cpm) and 84 mg/ml bovine serum albumin (the unlabeled arginine acceptor whose amino-terminal residue is aspartic acid) in a total volume of 0.5 ml containing also 0.15 M KCl, 50 mM 2-mercaptoethanol, 50 mM Tris-HCl (pH 9.0). Yeast extract and the rest of the incubation mixture were preincubated separately for 3 min at 37 °C. Samples (0.1 ml) were withdrawn at different incubation times, spotted on Whatman 3 MM filters, and assayed for hot trichloroacetic acid-insoluble radioactivity (Deutch, 1984). In some experiments, the Arg-transferase assay was carried out as described below for *E. coli* except that *S. cerevisiae* cells were used.

Cloning and Disruption of the ATE1 Gene—The cosmid cosA and its subclones pA-X28, pA-X14, pA-B6.5, pA-B8, and pA-B9 (Balzi *et al.*, 1987) encompass the ~35-kb region from *LEU1* to *TRP5* on chromosome VII of *S. cerevisiae* (strain IL125-2B). For subcloning of the *ATE1* gene, two additional plasmids were constructed: (i) pA-H4, produced by inserting the ~4-kb, *Hin*dIII-produced H4 fragment of pA-X14 into the *Hin*dIII site of pHCG3 (Gerbaud *et al.*, 1981); (ii) pA-XS, produced by *Sal*I digestion of pA-X14 (Balzi *et al.*, 1987) and religation.

The gene disruption method of Rothstein (1983) was used to generate null *ate1* mutants. The H4 fragment of pA-X14 was inserted at the *Hin*dIII site of a pBR322 derivative in which the *Bam*HI site has been eliminated by digesting pBR322 with *Bam*HI and *Eco*RV, blunting the ends, and religating (Maniatis *et al.*, 1982; Ausubel *et al.*, 1987). The *ATE1* gene, contained within the H4 fragment (see "Results"), was then disrupted *in vitro* by the insertion, at its single *Bam*HI site, of the *URA3*-containing 1.17-kb *Bgl*II fragment excised from pFL44 (a gift from Dr. F. Lacroute, Centre de Biologie Moleculaire, Gif-sur-Yvette, France). The resulting plasmid, pA-H4::URA3, was digested with *Hin*dIII, and the 5.17-kb *Hin*dIII fragment was transformed into strains 22295-C and US86. Ura$^+$ transformants were isolated and tested for the Arg-transferase activity as described below for the expression of *ATE1* in *E. coli*.

Sequencing the ATE1 Gene—The *ATE1*-containing H4 fragment of pA-H4 (Fig. 1) was inserted into the plasmids pTZ18R and pTZ19U (United States Biochemical Corp., Cleveland, OH) to yield pTZ18R-H4 and pTZ19U-H4, respectively. The plasmid pTZ18R-H4 was digested with *Bam*HI and *Hin*dIII and religated to yield pTZ-HB1.2, which contained the ~1.2-kb, 5′-proximal portion of *ATE1* (the HB1.2 fragment). Synthetic 15-nucleotide primers complementary to regions of the HB1.2 fragment were produced by Eurogentec (Liege, Belgium). The ~4.5-kb *Sal*I/*Bam*HI fragment containing the 3′-proximal portion of the ATE1 was inserted into pTZ18U, to yield pTZ-SB4.5, and was then progressively deleted as described by Barnes *et al.* (1983). The inserts of pTZ19U-H4, pTZ-HB1.2, and the deletion derivatives of pTZ-SB4.5 were sequenced on both strands using the chain termination method (Ausubel *et al.*, 1987). Computer analysis of the *ATE1* sequence and its comparisons with other sequenced genes were carried out using either the PC/Gene or DNA-STAR programs, and the SWISS-PROT or NBRF-PIR data bases.

Expression of ATE1 in E. coli—The *ATE1*-containing H4 fragment of pA-H4 was inserted, in either orientation, into the *Hin*dIII site of pT712 (obtained from GIBCO-Bethesda Research Laboratories) to yield the plasmids pTH4(+) and pTH4(−), in which the *ATE1*-coding strand was in either the correct or the opposite orientation, respectively, in relation to the T7 promoter of pT712. *E. coli* K38(pGP1-2) cells (Tabor and Richardson, 1985) were transformed with either pTH4(+) or pTH4(−) and grown at 30 °C in Luria broth containing 30 μg/ml kanamycin and 100 μg/ml ampicillin (selective conditions for pGP1-2 and pT712, respectively). To induce the expression of *ATE1*, a 10-ml *E. coli* culture was grown to A_{600} of ~0.35, heated at 42 °C for 15 min to derepress the T7 promoter (Tabor and Richardson, 1985), and incubated for 3 min at 30 °C. Cells were pelleted by centrifugation, washed once with H_2O, and resuspended in 0.3 ml of the assay buffer containing 0.12 M KCl, 10 mM $MgCl_2$, 4 mM dithiothreitol, 0.1 M Tris-HCl (pH 9.0), and 20 μg/ml each of antipain, leupeptin, aprotinin, chymostatin, and pepstatin (Sigma). Glass beads (0.15 ml) were added, and the suspension was vortexed at top speed for 50 s followed by centrifugation at 12,000 × *g* for 5 min at 4 °C. The supernatant (39 μl) was immediately added to 11 μl of premixed regents, to yield the following final concentrations: 1 × assay buffer; 1 mg/ml total yeast tRNA (Sigma); 3 mM ATP; 0.2 mM puromycin; 180 units/ml crude *E. coli* aminoacyl-tRNA synthetase (Sigma); 1 mg/ml bovine serum albumin (the arginine acceptor); 1.5 μCi/ml L-[^{14}C]arginine (Amersham, 342 mCi/mmol). Reactions were carried out at 30°C. Samples (18 μl) taken at 0, 10, and 25 min of incubation were spotted onto Whatman 3MM filters and assayed for hot trichloroacetic acid-insoluble radioactivity (Deutch, 1984). The 25-min samples were also fractionated by electrophoresis in an 18% polyacrylamide-SDS gel, followed by fluorography, to monitor the arginylation of the added bovine serum albumin.

Determination of in Vivo Levels of X-βgal Test Proteins in S. cerevisiae—Unlike the parental strain 22295-C (*Mat***a** *ATE1 ura3*), its derivatives, the ate1-Δ1, ate1-Δ2, and ate1-Δ3 strains, were Ura$^+$ because *URA3* was used to disrupt the *ATE1* gene in these strains (see "Results"). To make possible the transformation with *URA3*-based plasmids, the ate1-Δ1 strain was transferred onto YPD plates containing 5-fluoroorotic acid (Boeke *et al.*, 1987), and Ura$^-$ mutants were isolated. Southern hybridization using either *URA3* or *ATE1* as probes showed that mutations to the Ura$^-$ phenotype in these derivatives of the ate1-Δ1 stain were not accompanied by excision of *URA3* from the disrupted *ATE1* locus (data not shown). A Ura$^-$ derivative of the ate1-Δ1 strain and the parental strain 22295-C (*Mat***a** *ATE1 ura3*) were transformed with yeast expression vectors encoding ubiquitin-*X*-β-galactosidase (Ub-*X*-βgal) proteins (Bachmair *et al.*, 1986), where *X* was either arginine, aspartic acid, glutamic acid, asparagine, or glutamine. Two Ura$^+$ isolates from each transformation were tested for the level of βgal enzymatic activity present, essentially as described by Miller (1972), with the following modifications. An exponential culture of cells growing in SD medium containing 3% galactose instead of glucose was centrifuged, washed, and resuspended in H_2O to 2 × 10^7 cells/ml. A 0.1-ml sample of the suspension was

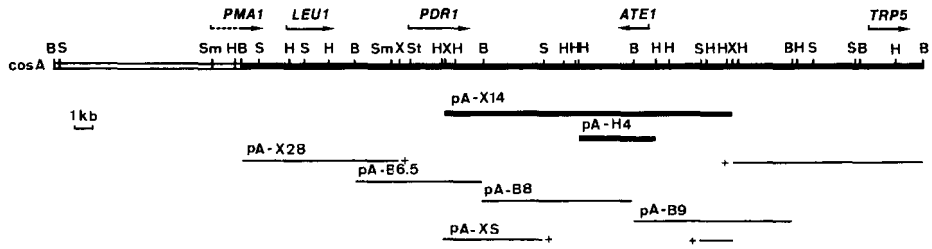

FIG. 1. **Plasmids used in cloning the *ATE1* gene.** The cosmid cosA carries a ~35-kb insert encompassing the region between *LEU1* and *TRP5* on the chromosome VII of *S. cerevisiae*. The positions and orientations of *PMA1*, *LEU1*, *PDR1*, *TRP5* (Balzi *et al.*, 1987) and *ATE1* (this work) are shown. The restriction map of cosA is as described previously (Balzi *et al.*, 1987). Also shown are the inserts of subclones used in the present work. Inserts that complemented the *ate1* mutation are indicated by *thick lines*. + indicates the junctions between two fragments present together on the same plasmid.

added to 0.4 ml of the buffer (10 mM KCl, 50 mM 2-mercaptoethanol, 1 mM $MgSO_4$, 0.1 M sodium phosphate, pH 7.0) in an Eppendorf tube. Two drops of chloroform and 30 μl of 0.1% SDS were added followed by vortexing for 10 s and the addition of 0.1 ml of *o*-nitrophenyl-β-D-galactoside (4 mg/ml) in H_2O. After incubation at 36 °C for 1 h, the reaction was terminated by the addition of 0.5 ml of 1 M Na_2CO_3. The sample was centrifuged at 12,000 × *g* for 30 s, and the absorbance of the supernatant at 420 nm was determined.

Southern Hybridization and Other Procedures—Plasmids were isolated from *E. coli* as described by Birnboim and Doly (1979). Radioactive DNA probes were prepared by nick translation (Rigby *et al.*, 1977) with [γ-^{32}P]dCTP. The *S. cerevisiae* genomic DNA was isolated as described by Davis *et al.* (1980), digested with either *Hin*dIII or *Eco*RI, and processed for low stringency Southern hybridization as described by Özkaynak *et al.* (1987). Filters containing blotted DNA were incubated with labeled DNA probes overnight at 42 °C in 30% formamide, 0.2% SDS, 5 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 5 × Denhardt's solution (Maniatis *et al.*, 1982), 50 mM Na_2HPO_4 (pH 7.0) containing 0.5 mg/ml denatured salmon sperm DNA. The filters were washed four times at 65 °C, for 30 min each, in 0.2% SDS, 1 × SSC. For Northern analyses, total RNA was isolated from a stationary culture of the Y55-1163 strain of *S. cerevisiae* (*HO, ade1-1 ura3-1*) as described by Maccecchini *et al.* (1979). The *ATE1*-specific radioactive probe was prepared by annealing the single-stranded plasmid pTZ-HB1.2 to a synthetic 15-nucleotide primer complementary to the *ATE1* sequences between nucleotides 482 and 468 followed by the addition of the Klenow Pol I in the presence of [γ-^{32}P]dCTP and the three unlabeled dNTPs. The probe was heat denatured before hybridization, which was carried as described above, except that the washes were performed at 60 °C.

RESULTS

Isolation of the ATE1 Gene—By screening mutagenized *S. cerevisiae* for strains that show decreased activity of Arg-transferase in an *in vitro* assay, Savage *et al.* (1983) succeeded in isolating a mutant, which they called *ate1*, that lacked the bulk of Arg-transferase activity in crude extracts. Aside from lacking most of the Arg-transferase activity, this mutant showed no obvious deviations from the parental phenotype. Genetic mapping of the *ate1* mutation placed it near *LEU1* on chromosome VII (Savage *et al.*, 1983).

We have used a series of overlapping genomic DNA fragments encompassing the *LEU1* gene and the previously cloned (Balzi *et al.*, 1987) ~35-kb region centromere-distal to *LEU1* (Fig. 1) to transform the strain US88-12A that carried the original *ate1* mutation of Savage *et al.* (1983). Several independent Ura$^+$ transformants derived from each of the DNA subclones used (Fig. 1) were tested for the Arg-transferase activity *in vitro* (Fig. 2). The cosmid cosA, whose insert encompasses the entire ~35-kb region (Fig. 1), conferred an increase of the Arg-transferase activity in the *ate1* mutant to approximately wild-type levels (Fig. 2). Among the subclones of the cosA, the plasmid pA-X14 also complemented the Arg-transferase deficiency in the *ate1* mutant, whereas pA-X28, pA-B6.5, pA-B8, and pA-XS did not (Fig. 2). Further subcloning of pA-X14 yielded the plasmid pA-H4, whose ~4-kb insert was the smallest among the tested subclones that could restore high levels of the Arg-transferase activity in the *ate1* mutant (Fig. 2). Northern analysis showed that the corresponding region of chromosome VII in an *ATE1* strain is transcribed *in vivo* to yield a ~1.6-kb RNA (Fig. 3*A*).

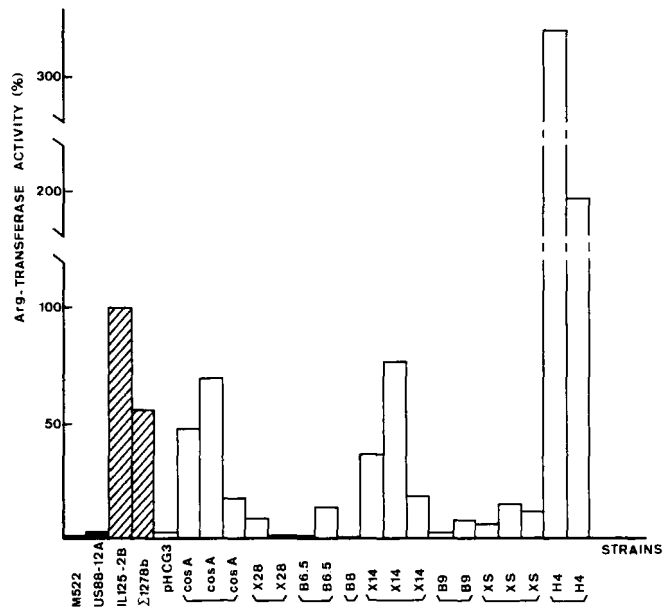

FIG. 2. **Arg-transferase activity in different transformants of *S. cerevisiae*.** cosA and its subclones shown in Fig. 1 were transformed into an *ate1* mutant of *S. cerevisiae* (the US288-12A strain (*MAT*α *ate1 ura3 his7*)), and the Arg-transferase activity in crude extracts was determined as described under "Experimental Procedures." For each DNA subclone, the several values shown were obtained with independent transformants and are expressed as percentages of the value (determined in the same experiment) for an *ATE1* strain IL125-2B (*striped bar*). The value for another *ATE1* strain (Σ1278b) is also shown. *Filled bars* on the *left* indicate the relative Arg-transferase activities in extracts from strains carrying the original *ate1* mutation (M522 and US288-12A).

There are apparently no close homologs of *ATE1* in the *S. cerevisiae* genome because a low stringency Southern hybridization, using the full-length *ATE1* as a probe, detected only *ATE1* in the *S. cerevisiae* genomic DNA (Fig. 3*B*). This result is consistent with the absence of Arg-transferase activity in extracts from *ate1*-Δ mutants and also with the metabolic stabilization in these mutants of *X*-βgal proteins bearing secondary or tertiary destabilizing amino-terminal residues (see below).

The ATE1 Gene Encodes a 503-Residue Protein—The nucleotide sequence of a ~2.1-kb region that contains *ATE1* was

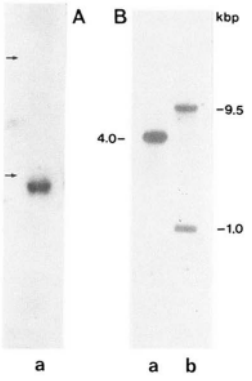

FIG. 3. **The *ATE1* RNA and DNA.** *A*, total RNA from an *ATE1* strain was fractionated by electrophoresis in a 1.6% agarose gel containing 6% formaldehyde (Maniatis *et al.*, 1982), transferred to a nitrocellulose filter, and hybridized to an *ATE1*-specific probe (see "Experimental Procedures"). *Arrowheads* indicate the positions of yeast rRNAs (1789 and 3392 nucleotides, respectively). Using these markers, the *ATE1* transcript (indicated by an *arrow*) was estimated to be ~1.6 kb long. *B*, Southern hybridization analysis. Purified *S. cerevisiae* DNA was digested with either *Hin*dIII (*lane a*) or *Eco*RI (*lane b*), fractionated by electrophoresis in an agarose gel, transferred to a filter, and hybridized to an *ATE1*-specific probe under low stringency conditions (see "Experimental Procedures").

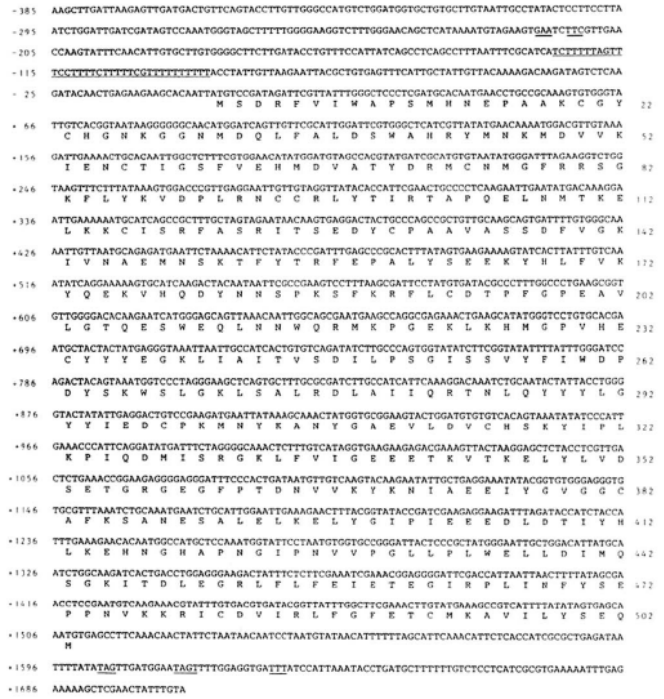

FIG. 4. **Nucleotide sequence of the *ATE1* locus and deduced amino acid sequence of the ATE1 protein.** The 2091-bp nucleotide sequence shown starts at the proximal *Hin*dIII site of the yeast DNA insert in pA-H4 (see Fig. 1). The initiator methionine codon (*position 1* in the amino acid sequence) was assigned at this position because it yielded the longest uninterrupted reading frame. No sequence motifs characteristic of yeast introns (Ballance, 1986) have been found in the ~400-bp stretch of DNA upstream of the (inferred) initiator methionine codon of *ATE1*. The sequence motifs characteristic of *S. cerevisiae* promoters and terminators are *underlined*, respectively, upstream and downstream of the *ATE1* reading frame. The putative heat shock element, GAAtcTTC (Sorger and Nelson, 1989; Perisic *et al.*, 1989) is present near position −220. Note also the presence of another GAA sequence 3 nucleotides downstream from TTC.

determined as described under "Experimental Procedures." The *ATE1* gene appears to encode a 503-residue protein (Fig. 4), with a calculated molecular mass of 57,866 daltons and a calculated isoelectric point of 5.86. The exact location of the start ATG codon in *ATE1* is uncertain; the choice shown in Fig. 4 yielded the longest uninterrupted reading frame. The inferred size of the *S. cerevisiae* Arg-transferase (~58 kDa) is larger than those reported for the mammalian Arg-transferases from rabbit liver (~50 kDa) (Soffer, 1980) and hog kidney (~35 kDa) (Kato and Nozawa, 1984). No sequences characteristic of membrane-spanning helical segments were detectable in ATE1 using the algorithm of either Eisenberg *et al.* (1982) or Rao and Argos (1986). The ATE1 protein also lacks motifs characteristic of either an endoplasmic reticulum-targeting signal sequence (von Heijne, 1986) or nuclear translocation signals (Dingwall and Laskey, 1986; Guiochon-Mantel *et al.*, 1989), suggesting that the *ATE1* gene product is a cytoplasmic protein. This assignment is also consistent with the predicted function of Arg-transferase in the N-end rule pathway of protein degradation which operates in the cytosol and is apparently absent from membrane-enclosed compartments such as Golgi, lysosomes, and endoplasmic reticulum (Gonda *et al.*, 1989).

The codon bias index (Bennetzen and Hall, 1982) for *ATE1* is low (0.04), suggesting that *ATE1* is a weakly expressed gene. No statistically significant homologies were found between the amino acid sequence of ATE1 and those of known proteins using several data bases and the algorithm of Lipman and Pearson (1985). Similarities of marginal statistical significance were observed between ATE1 and several aminoacyl-tRNA synthetases, in particular the *E. coli* Arg-, Ala-, and Thr-tRNA synthetases and the Trp-tRNA synthetase from *Bacillus stearothermophilus* (data not shown). These similarities were not unexpected because aminoacyl-tRNA, the product of an aminoacyl-tRNA synthetase, is the substrate of the ATE1 enzyme.

Flanking nucleotide sequences upstream and downstream of the *ATE1* gene contain motifs characteristic of yeast promoter and terminator regions, respectively (Fig. 4). In particular, a CT-rich tract (92% CT in a 36-nucleotide sequence) precedes by 90 base pairs the putative start codon of ATE1 (Fig. 4). Similar tracts are present in many yeast promoter regions (Dobson *et al.*, 1982; Oberto and Davison, 1985), where they appear to increase the fidelity of transcriptional initiation (McNeil, 1988). The same CT-rich region is also extremely T-rich, with stretches of uninterrupted T-tracts up to 10 nucleotides longs (see Fig. 6). These sequence motifs may play a role in the regulation of constitutive transcription in *S. cerevisiae* (Struhl, 1985; Winter and Varshavsky, 1989; Lue *et al.*, 1989). No TATA element, as defined by Chen and Struhl (1988), is evident in the (sequenced) region upstream of *ATE1*. A purine, adenine, is present at position −3, as observed with most eukaryotic genes (Dobson *et al.*, 1982; Kozak, 1984). A potential heat shock element, a highly conserved DNA sequence that is apparently both necessary and sufficient for heat-inducible transcriptional activation (Perisic *et al.*, 1989; Sorger and Nelson, 1989) is present near position −220. Whether the *ATE1* transcription is in fact regulated by temperature shifts or other stresses remains to be determined. In the 3′-noncoding region, the tripartite motif TAG . . . TAGT . . . TTT, a potential signal for transcription termination and polyadenylation (Zaret and Sherman, 1982), is present 91 base pairs downstream from the TGA stop codon of *ATE1*.

Construction and Analysis of ate1-Δ Mutants—To generate a null allele of the *ATE1* gene and also to verify that the plasmid pA-H4 (Figs. 1, 2, and 4) actually carried *ATE1*, we have disrupted the *ATE1*-coding sequence *in vitro* and re-

placed the wild-type *ATE1* allele in the yeast genome with the disrupted allele by homologous recombination (Rothstein, 1983). The *URA3* marker was inserted at the unique *Bam*HI site of the ~4-kb H4 fragment of pA-H4 which is located in the middle of the *ATE1* reading frame (Fig. 5*A*). The ~5.2-kb H4::URA3 fragment thus obtained was used to transform US86 and 22295-C, the diploid and haploid *ATE1 ura3* strains, respectively. Stable Ura$^+$ transformants were obtained with both of these strains, indicating that the disruption of *ATE1* is compatible with cell viability. The haploid Ura$^+$ transformants of the strain 22295-C were screened by Southern hybridization for the presence of restriction endonuclease sites diagnostic of the *ATE1* gene disruption (Fig. 5, *A* and *B*). For three of the four Ura$^+$ transformants tested (strains ate1-Δ1, ate1-Δ2, and ate1-Δ3), the analysis confirmed that the ~5.2-kb H4::URA3 fragment had integrated at the *ATE1* locus on chromosome VII, where it replaced the resident *ATE1* gene (Fig. 5, *A* and *B*).

The 22295-C (*ATE1*) strain and its *ate1*-Δ derivatives were also tested for the Arg-transferase activity in crude extracts. The level of Arg-transferase activity in the *ate1*-Δ mutants (Fig. 5*C*) was indistinguishable from the background levels of [^{3}H]arginine incorporation in extracts of *E. coli* which apparently lacks Arg-transferase (Soffer, 1980).

Expression of ATE1 in E. coli—To verify directly that the cloned *S. cerevisiae ATE1* gene encodes an Arg-transferase, the gene was expressed in *E. coli*. The ~4-kb *Hin*dIII fragment of pA-H4 which encompasses the *ATE1* gene was inserted into the expression vector pT712 (Tabor and Richardson, 1985) downstream of the T7 RNA polymerase promoter. The *ATE1*-containing fragment was inserted in both orientations, yielding the plasmids pTH4(+) and pTH4(−), with the former plasmid having *ATE1* oriented correctly in relation to the T7 promoter (see "Experimental Procedures"). These plasmids were transformed into the *E. coli* K38 host, which carries pGP1-2, a plasmid that encodes the phage T7 RNA polymerase under the control of the inducible λP_L promoter (Tabor and Richardson, 1985). As shown in Fig. 6, extracts prepared from *E. coli* bearing the plasmids pGP1-2 and pTH4(+) contained the Arg-transferase activity. Much lower levels of the Arg-transferase activity were present in extracts from *E. coli* bearing the plasmids pGP1-2 and pTH4(−) (Fig. 6). That these levels were still greater than the background of the Arg-transferase assay (in extracts from cells lacking *ATE1* plasmids) (Fig. 6) could be explained by a "leaky" expression of *ATE1* from a cryptic promoter in the pTH4(−) plasmid.

We conclude that the *ATE1* gene encodes Arg-transferase rather than an activator of the Arg-transferase expression or activity.

Arg-transferase and the N-end Rule Pathway—S. cerevisiae mutants lacking the *ATE1*-encoded Arg-transferase are viable and apparently normal phenotypically. They lack the Arg-transferase activity in crude extracts (Fig. 5*C*), suggesting, in agreement with the Southern hybridization data (Fig. 3*B*), that *ATE1* is the only gene that encodes Arg-transferase in *S. cerevisiae*.

Previous *in vitro* studies of the N-end rule pathway (Gonda *et al.*, 1989; Ciechanover *et al.*, 1988; Ferber and Ciechanover, 1987; see the Introduction) have shown that amino-terminal aspartic acid and glutamic acid (and also cysteine in reticulocytes) are *secondary* destabilizing residues in that they function by virtue of their ability to be conjugated to a primary destabilizing residue, arginine. To determine whether *ATE1* is essential for this aspect of the N-end rule pathway in yeast, we transformed a Ura$^-$ derivative of the ate1-Δ1 strain (see "Experimental Procedures") and its parental counterpart with

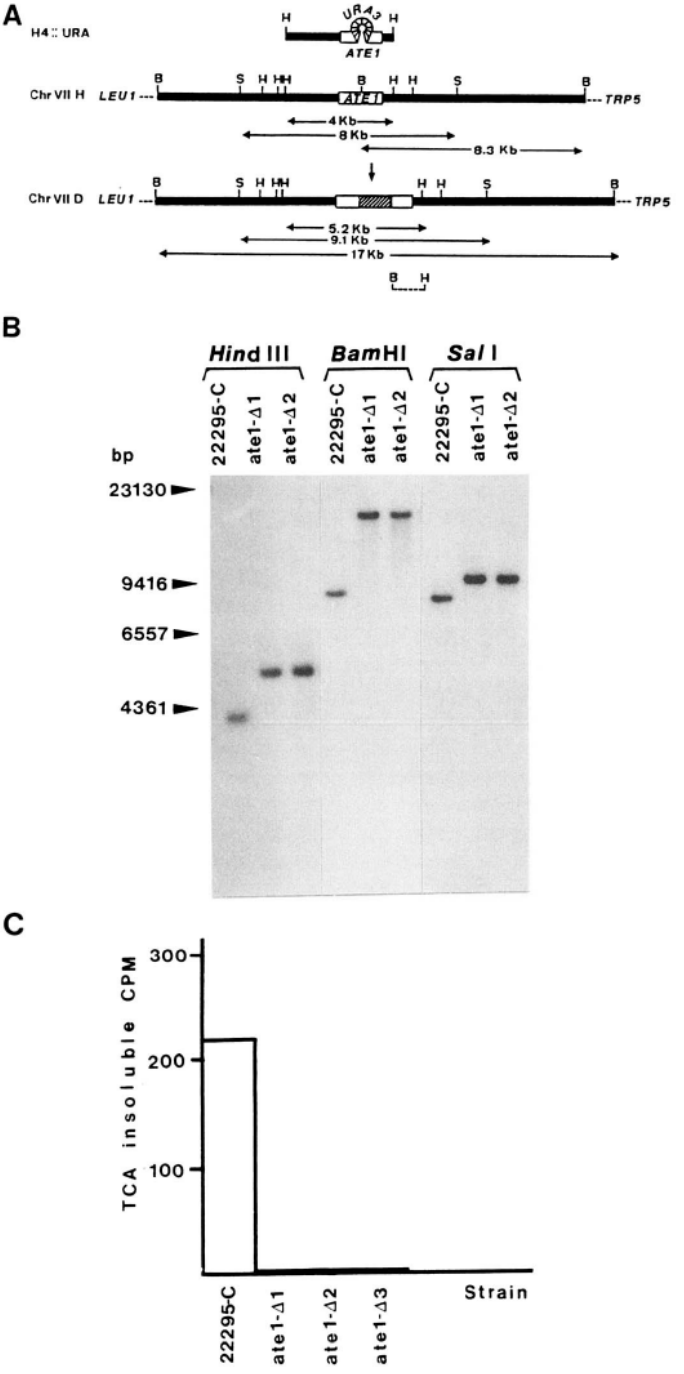

FIG. 5. **Disruption of *Ate 1*.** *A*, diagram of expected recombination events in the ATE1 disruption. The linear ~5.2-kb *Hin*dIII fragment (H4::URA3) which was used to transform *S. cerevisiae*, contained the *ATE1* gene (*open bar*) disrupted by the insertion of *URA3* (*striped box*). Replacement of the wild-type *ATE1* on chromosome VII (*Chr VII H*) with the disrupted *ate1* allele (*Chr VII D*) was carried out as described under "Experimental Procedures." As shown in the diagram, this replacement generates a new arrangement of the recognition sites for *Hin*dIII (*H*), *Bam*HI (*B*), and *Sal*I (*S*). *B*, Southern hybridization analysis of the structure of the integrated *ate1*::*URA3* allele. Purified genomic DNA from the parental (haploid) strain 22295-C and from Ura$^+$ derivatives of this strain (ate1-Δ1 and ate1-Δ2) obtained by transformation with pH4::URA3 (see "Experimental Procedures") was digested with either *Hin*dIII, *Bam*HI, or *Sal*I, fractionated by electrophoresis in a 0.9% agarose gel, and analyzed by Southern hybridization using the ~1.2-kb *Bam*HI/*Hin*dIII fragment (a *dashed segment* in *A*) as a probe. *Arrows* mark the positions of diagnostic DNA fragments shown in *A*. *C*, Arg-transferase activity in extracts of the *ATE1* strain 22295-C and the *ate1*-Δ strains (see "Experimental Procedures").

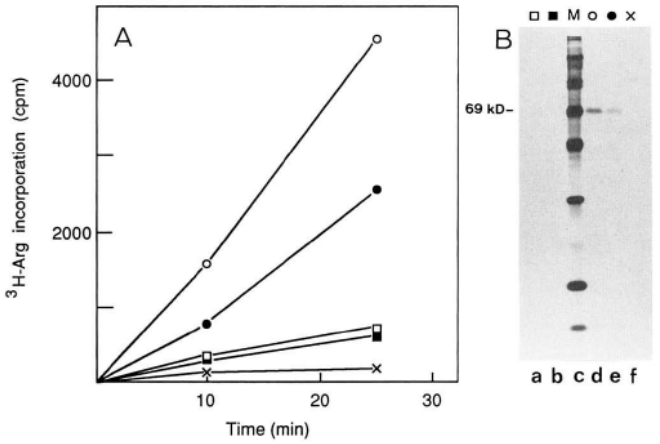

FIG. 6. **Expression of the *S. cerevisiae ATE1* gene in *E. coli*.** *A*, incorporation of [^{14}C]arginine into hot trichloroacetic acid-insoluble material by extracts of *E. coli* K38 bearing either pGP1-2 plus pTH(4+) (○ and ●) or pGP1-2 plus pTH4(−) (□ and ■), with the *open* and *closed symbols* indicating extracts prepared from heat-treated and untreated *E. coli*, respectively. ×, pGP1-2 alone (with either heat-treated or untreated *E. coli*). See "Experimental Procedures" for the assay used. *B*, proteins in equal volume, 25-min samples of *panel A* were fractionated by SDS-polyacrylamide gel electrophoresis followed by fluorography. An *arrow* indicates the 69-kDa band of bovine serum albumin, the added acceptor of [^{14}C]arginine in the *E. coli* extracts (see "Experimental Procedures"). *Lane* designations on *top* correspond to the *curve* designations in *panel A*. *Lane M* contained ^{14}C-labeled molecular mass markers (Amersham).

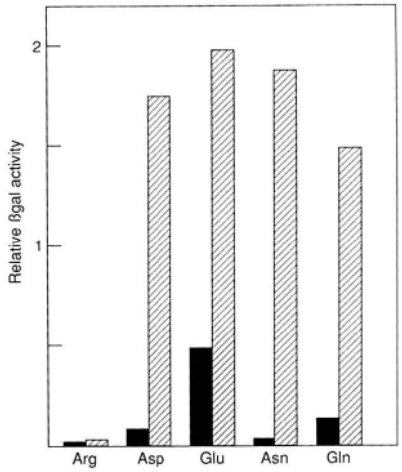

FIG. 7. **Null *ate1* mutant does not degrade *X*-βgal test proteins that bear either *secondary* or *tertiary* destabilizing amino-terminal residues.** The *ATE1* (22295-C, *filled bars*) and null *ate1* (ate1-Δ1, *striped bars*) strains of *S. cerevisiae* were transformed with expression vectors encoding different Ub-*X*-βgal proteins, and the enzymatic activity of βgal was determined in extracts from the transformants (see "Experimental Procedures"). Measurements were carried out with samples in duplicate; the values shown are each an average of two measurements that differed from each other by less than 7%. Note the increase of βgal activity in *ate1*-Δ cells for Asn-βgal, Gln-βgal, Asp-βgal, and Glu-βGal but not for Arg-βgal (see also "Results"). The *in vivo* half-lives of Asn-, Gln-, Asp-, Glu-, and Arg-βgal in wild-type (*ATE1*) *S. cerevisiae* at 30 °C are ~3, ~10, ~3, ~30, and ~2 min, respectively (Bachmair *et al.*, 1986; Bachmair and Varshavsky, 1989).

yeast expression vectors encoding ubiquitin-*X*-β-galactosidase (Ub-X-βgal) proteins, where *X* was either arginine, aspartic acid, glutamic acid, asparagine, or glutamine. Previous work (Bachmair *et al.*, 1986; Bachmair and Varshavsky, 1989) has shown that a nascent Ub-*X*-βgal fusion protein is efficiently deubiquitinated *in vivo* regardless of the nature of the amino acid residue *X* at the ubiquitin-βgal junction. In contrast to the function of ubiquitin at later stages of the degradative pathway (Chau *et al.*, 1989), the role of ubiquitin in the ubiquitin-βgal fusion approach is simply to allow the generation of the desired *X*-βgal test proteins through the action of a ubiquitin-specific processing protease (Bachmair *et al.*, 1986; Gonda *et al.*, 1989). Physiological functions of this protease include the analogous processing of natural ubiquitin precursors (Özkaynak *et al.*, 1987; Finley *et al.*, 1988, 1989).

To compare the metabolic stabilities of Arg-, Asp-, Glu-, Asn-, and Gln-βgal in the *ATE1* and *ate1*-Δ genetic backgrounds, we determined the concentrations of these *X*-βgal proteins in cells by measuring the enzymatic activity of βgal in crude extracts (Fig. 7). Since all of these *X*-βgal proteins, which differ exclusively in their amino-terminal residues, were expressed using the same vector (Bachmair *et al.*, 1986), the levels of βgal activity in different transformants growing under identical conditions and sampled at the same stage of exponential growth should reflect the metabolic stabilities of the corresponding *X*-βgal proteins (Bachmair *et al.*, 1986; and data not shown).

Although Arg-βgal, the most metabolically unstable of *X*-βgal proteins in yeast ($t_{½}$ of ~2 min) (Bachmair *et al.*, 1986; Bachmair and Varshavsky, 1989) was present at extremely low steady-state levels in both *ATE1* and *ate1*-Δ genetic backgrounds, the levels of Asn-βgal were strikingly different in the *ATE1* and *ate1*-Δ strains (Fig. 7). Similar but smaller increases of βgal activity in the *ate1*-Δ strain (correlating with the known half-lives of the corresponding *X*-βgal proteins in *ATE1* cells; see the legend to Fig. 7) were also observed for Asp-, Glu-, and Gln-βgal, all of which, unlike Arg-βgal, have either secondary or tertiary destabilizing amino-terminal residues (see the Introduction). The levels of Asp-, Glu-, Asn-, or Gln-βgal in the *ate1*-Δ background were similar to the level of Met-βgal, which has a stabilizing amino-terminal residue and is long lived ($t_{½}$ > 20 h) in both *ATE1* and *ate1*-Δ backgrounds (Fig. 7 and Bachmair *et al.*, 1986). Thus, in the absence of the *ATE1*-encoded Arg-transferase, both the *tertiary* (asparagine, glutamine) and the *secondary* (aspartic acid, glutamic acid) destabilizing residues become *stabilizing* ones *in vivo*, as suggested previously on the basis of *in vitro* data (Gonda *et al.*, 1989).

DISCUSSION

The yeast arginyl-tRNA-protein transferase, whose gene, *ATE1*, has been cloned and functionally characterized in the present work, is a ~58-kDa enzyme whose (inferred) substrate specificity is similar to those of the higher eukaryotic Arg-transferases (Soffer, 1980). The latter enzymes (whose genes have not yet been cloned) have been isolated from protozoans, plants, and several mammalian tissues and cell lines (Deutch *et al.*, 1978; Soffer, 1980; Kato and Nozawa, 1984; Ciechanover *et al.*, 1988). One of the better characterized Arg-transferases, isolated from rabbit reticulocytes, is copurified with Arg-tRNA synthetase and appears to exist as a ~400-kDa heteromeric complex between several molecules of Arg-transferase and Arg-tRNA synthetase (Ciechanover *et al.*, 1988).

As described in the Introduction, the *in vitro* evidence (Gonda *et al.*, 1989; Ciechanover *et al.*, 1988) strongly suggested that Arg-transferase functions in the N-end rule pathway of protein degradation, where it conjugates arginine, a primary destabilizing residue, to amino termini of proteins bearing secondary destabilizing residues aspartic acid or glutamic acid (and also cysteine in reticulocytes). By constructing and analyzing *ate1*-Δ mutants in *S. cerevisiae*, we have provided genetic evidence that the Arg-transferase is essential

for this aspect of the N-end rule pathway. Whether this is the only function of the Arg-transferase remains to be determined.

The lack of *ATE1* homologs detectable by a low stringency Southern hybridization in the *S. cerevisiae* genomic DNA (Fig. 3*B*) and the absence of Arg-transferase activity in extracts from *ate1*-Δ mutants (Fig. 5*C*) suggested that ATE1 is the only aminoacyl-tRNA-protein transferase in this organism. This possibility is also consistent with the strong metabolic stabilization of *X*-βgal proteins bearing secondary and tertiary destabilizing amino-terminal residues in the *ate1*-Δ genetic background (Fig. 7). On the other hand, in higher eukaryotes such as mammals, fishes, and mollusks, a variety of amino acids, in addition to arginine, have been reported to be post-translationally conjugated to proteins (Laughrea, 1982; Ingoglia *et al.*, 1983; Jackson and Hunt, 1983; Shyne-Athwal *et al.*, 1986). In none of these "non-Arg" conjugation reactions (with the exception of the conjugation of tyrosine to the carboxyl terminus of tubulin (Kumar and Flavin, 1981; Nath *et al.*, 1989)) have the relevant enzymes been purified or otherwise characterized (see also Rapaport *et al.*, 1985). The functions of these putative conjugation reactions and physiological substrates involved remain to be addressed.

Bacteria such as *E. coli* lack Arg-transferase but contain a leucyl,phenylalanyl-tRNA-protein transferase which is apparently absent from eukaryotes. *In vitro,* this enzyme can conjugate either leucine, phenylalanine, or methionine to amino-terminal arginine, lysine, or histidine in proteins or peptides (Leibowitz and Soffer, 1970; Soffer, 1980; Belitsina, 1988). *E. coli* mutants deficient in the Leu,Phe-transferase are viable but abnormal in several respects (Soffer, 1980). If bacteria possess the N-end rule pathway (a possibility being tested),[2] the function of the Leu,Phe-transferase might be analogous to that of the Arg-transferase in eukaryotes, with the amino-terminal arginine, lysine, and histidine now being *secondary* destabilizing residues, conjugated to the *primary* destabilizing residues leucine or phenylalanine (in contrast to the arginine, lysine, and histidine being *primary* destabilizing residues in the eukaryotic N-end rule pathway (Gonda *et al.*, 1989)).

Physiologically relevant substrates of Arg-transferases remain to be identified. All of the currently used substrates of Arg-transferases are either deliberately engineered proteins bearing amino-terminal aspartic acid, glutamic acid, or cysteine (Gonda *et al.*, 1989) or natural *compartmentalized* proteins (Ciechanover *et al.*, 1988; Soffer, 1980) whose amino-terminal residues are often aspartic acid or glutamic acid, but whose normal intracellular location is expected to preclude their contact with the cytosolic Arg-transferase and the rest of the N-end rule pathway *in vivo*. Physiological functions of the N-end rule pathway, of which the Arg-transferase is a component, are also unknown (see discussion by Gonda *et al.*, 1989). Mutational inactivation of the N-end rule pathway in *S. cerevisiae* has recently been shown to result in viable cells,[3] implying that the *in vivo* degradation of physiological substrates of the N-end rule pathway (which are expected to include substrates of the Arg-transferase) is either nonessential for cell growth and division or, alternatively, can take place in the absence of the N-end rule pathway because the short-lived proteins involved may each carry more than one degradation signal. A precedent for this possibility is provided by the recent analysis of the yeast MATα2 repressor that has been shown to possess two degradation signals operating via distinct pathways.[4] One function of the N-end rule pathway which is consistent with the available evidence may involve selective destruction of otherwise long-lived proteins that mislocalize into cytosol from vesicular compartments such as endoplasmic reticulum, Golgi, and vacuoles or lysosomes. It has been noted previously by Bachmair *et al.* (1986) that the amino-terminal residues of compartmentalized proteins are largely of the destabilizing type according to the N-end rule, in striking contrast to the amino-terminal residues of non-compartmentalized proteins which are almost exclusively of the stabilizing type. Biochemical and genetic searches for physiological substrates of the Arg-transferase and the N-end rule pathway are in progress.

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Cloning and functional analysis of the arginyl-tRNA-protein transferase gene ATE1 of Saccharomyces cerevisiae.

E Balzi, M Choder, W N Chen, A Varshavsky and A Goffeau

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