Characterization of *AMD*, the AMP Deaminase Gene in Yeast. Production of *amd* Strain, Cloning, Nucleotide Sequence, and Properties of the Protein†

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ABSTRACT: The structural gene for AMP deaminase (*AMD*) from *Saccharomyces cerevisiae* has been cloned and characterized. A yeast strain deficient in AMP deaminase activity was produced and shown to be deficient in AMP deaminase protein by Western blot analysis. The gene for AMP deaminase was located in a λgt11 library of yeast genomic DNA, and a DNA fragment from the λgt11 clone was used to locate homologous DNA in a yeast genomic library in the centromeric plasmid YCp50, a yeast–*Escherichia coli* shuttle vector. One plasmid was selected for its ability to restore AMP catalytic activity to the deficient strain. Yeast deficient in AMP deaminase or those overproducing the enzyme grow at near normal rates. The open reading frame corresponding to *AMD* codes for a protein of 810 amino acids, molecular weight 93 286. The yeast *AMD* transcript is 3.0 ± 0.2 kb, and the transcriptional initiation sites have been identified. Western blot analysis of extracts prepared from actively growing yeast indicates a major band at approximately 96 000 molecular weight with several bands at lower molecular weight, including 83 000. When the *AMD* gene is expressed in *E. coli*, the large M_r form of AMP deaminase is produced. These results show that the purified enzyme (M_r = 83 000) is a truncated form of the full-length translation product. No adenine nucleotide binding sites were located based on the consensus sequence from other nucleotide binding proteins. No overall homology was found between yeast AMP deaminase and *E. coli* AMP nucleosidase. Although their metabolic roles and regulatory mechanisms are similar, these enzymes have arisen from separate ancestral proteins.

AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) catalyzes the hydrolytic cleavage of AMP to produce IMP and ammonia. It is found in a variety of eukaryotes, including mammals, birds, amphibians (Zielke & Suelter, 1971), plants (Turner & Turner, 1961), and yeast (Yoshino et al., 1979), and has been proposed to play a role in the regulation of adenylate energy charge and pool size (Chapman & Atkinson, 1973; Yoshino & Murakami, 1981), in the control of the purine nucleotide cycle (Lowenstein, 1972), and in the regulation of phosphofructokinase (Yoshino & Murakami, 1982a,b). In prokaryotes, AMP nucleosidase (AMP phosphoribohydrolase, EC 3.2.2.4) is involved in stabilizing the adenylate energy charge (Schramm & Leung, 1973; Schramm & Lazorik, 1975). No eukaryotes are known to contain AMP nucleosidase, and no prokaryotes have been demonstrated to contain the specific AMP deaminase. Both enzymes are allosterically activated by ATP and inhibited by P_i (Chapman & Atkinson, 1973; Schramm, 1974; Yoshino et al., 1979; Leung & Schramm, 1980). The similarities between AMP deaminase and AMP nucleosidase in both their metabolic roles and allosteric regulation suggested that the two enzymes may be evolutionarily related. The preceding paper reports the sequence of AMP nucleosidase from *Escherichia coli* (Leung et al., 1989). To look for homology between the prokaryotic AMP nucleosidase and eukaryotic AMP deaminase, we chose to clone AMP deaminase from the lower eukaryote *Saccharomyces cerevisiae*.

This paper describes the isolation of a yeast strain deficient in AMP deaminase and its use in cloning the structural gene *AMD*. The gene was cloned by screening a λgt11 yeast DNA library with oligonucleotide probes designed from the amino acid sequence of an internal tryptic peptide. The portion of the coding sequence so isolated was used to locate a plasmid from a yeast shuttle vector library that restored full activity to the deficient mutant. Comparison of the sequence of the yeast AMP deaminase with *E. coli* AMP nucleosidase revealed no obvious homology, suggesting that the similarities in role and regulation between these two enzymes is more likely due to parallel evolution following the division of prokaryotes and eukaryotes.

Experimental Procedures

Materials. Restriction endonucleases, DNA polymerase I, polynucleotide kinase, M-MuLV-reverse transcriptase, and nucleotide mixes for ^{35}S-labeled DNA sequencing were purchased from New England Biolabs. Cloned Klenow fragment and Sequenase DNA sequencing kit were obtained from U.S. Biochemical Corp. Double-stranded replicative form M13mp18 and M13mp19 DNA was purchased from Pharmacia. [α-^{32}P]dATP, [γ-^{32}P]ATP, and [α-^{35}S]dATPαS were acquired from Amersham Corp. Carrier-free ^{125}I was obtained from New England Nuclear. Nitrocellulose and nytran (nylon membrane) were from Schleicher and Schuell. Goat anti-rabbit IgG peroxidase conjugate was acquired from Sigma. All other chemicals were of reagent grade from various sources. AMP deaminase was purified from Red Star yeast and was provided by Dr. D. Merkler (personal communication).

Strains and Media. Yeast strain S150-2B (*Mat a*, *leu*2-3, 112, *trpl*-289, *ura*3-52, *his*3-Δ1) was acquired from Dr. L. Friedman, University of Rochester. *E. coli* strain Y1090 (Young & Davis, 1983), used for λgt11 library screening, was acquired from Dr. M. Snyder, Stanford University. Strain

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CSH-5 (Miller, 1972) was used for lysogeny of λgt11 recombinants and was obtained from the Cold Spring Harbor Institute. Strain HB101 (Boyer & Roulland-Dussoix, 1969) from Dr. Manfredi, University of North Carolina at Chapel Hill, was used for yeast shuttle vector amplification. Strain JM105 (Yanisch-Perron et al., 1985), used as a host for pUC plasmids and M13 phage, was obtained from Pharmacia. Yeast strains were grown on complete (YPD) or minimal media (SD) (Sherman et al., 1983). *E. coli* strains were either grown on LB medium or plated on H medium for phage propagation (Miller, 1972). Ampicillin at 60 μg/mL was added when required. The yeast strain S150-2B was made deficient in AMP deaminase and is designated SM4-40 (*amd*) (this paper). The mutant strain, SM4-40 (*amd*), was transformed with the plasmids YCp*AMD*5-2 or YEp*AMD*-16, and the *AMD* gene was expressed (Table I). The construction of both plasmids is discussed (see Results).

Transformation. Bacteria were transformed by the calcium chloride method described in Maniatis et al. (1982). Yeast were transformed by the lithium acetate procedure of Ito et al. (1983).

Libraries. A yeast DNA library in the vector λgt11, prepared from randomly sheared 2–8 kb[1] fragments of DNA from yeast strain S288C, was kindly provided by Dr. Michael Synder, Stanford University. The yeast DNA library RB236, containing 10–15 kb *Sau*3A partial-digest DNA inserts from yeast strain DBY918 in the centromere shuttle vector YCp50, was kindly provided by Dr. David Botstein, MIT.

Mutagenesis and AMP Deaminase Activity Screen. Yeast strain S150-2B was mutagenized by resuspending cells from a fresh agar slant in 0.1 M sodium phosphate, pH 7.0, exposed to 50 μg/mL nitrosoguanidine, and grown on YPD plates at room temperature. AMP deaminase activity was detected by an activity assay. Cells from a portion of each colony were resuspended in 200 μL of a reaction mixture composed of 50 mM triethanolamine, pH 7.0, 0.1 M KCl, 1 mM EDTA, 10 mM AMP, and 5% by volume of toluene/ethanol (1:4 v/v). The mixture was shaken for 5 min at room temperature (Serrano et al., 1973), followed by incubation of the covered tubes at 30 °C overnight. Ammonia production was detected by the method of Giusti and Galanti (1974). IMP production was also occasionally measured by HPLC separation of IMP from AMP, monitored at 248.5 nm, using a Waters C_{18} μBondapak column run with an isocratic system of 0.1 M ammonium phosphate, pH 5.0 (Parkin et al., 1984).

Antibody Production. Two female New Zealand white rabbits were injected subcutaneously above each limb four times with 0.5 mg of yeast AMP deaminase in either complete (first two injections) or incomplete (second two injections) Freund's adjuvant at 10–14-day intervals, followed by bleeding 7 days after the last injection. The pooled serum was precipitated with 50% saturated ammonium sulfate, resuspended in 0.1 M Tris, pH 8.0, to give a solution of 87 mg/mL protein, and stored at −70 °C.

Western Blotting and Immunodetection. Purified proteins and cell extracts were separated by SDS–polyacrylamide gel electrophoresis by the procedure of Laemmli (1970) and electroblotted onto nitrocellulose (Towbin et al., 1979). AMP deaminase was immunologically detected by the procedure of Huynh et al. (1985) used for screening λ libraries, using antiserum diluted 1:100 in 50 mM Tris, pH 7.5, containing 0.15 M NaCl and 3% bovine serum albumin. ^{125}I-Labeled protein A was prepared by the solid-phase method of Fraker and Speck (1978) to a specific activity of 5–9 × 10^6 cpm/μg. For some experiments, the antiserum was first affinity-purified as follows. One milliliter of antiserum (30.7 mg/mL protein) diluted 1:20 by volume with 10 mM potassium phosphate, pH 7.4, containing 0.15 M NaCl, was applied to a 1-mL column of purified yeast AMP deaminase attached to cyanogen bromide activated Sepharose 4B (Cuatrecasas, 1970; 3 mg of enzyme/mL of resin). Bound antibodies were eluted with 50 mM glycine, pH 2.5, and immediately adjusted to pH 7.0 with 100 mM Tris base. Affinity-purified antiserum (0.6 mg/mL protein) was diluted 1:200 for use in immunodetection and the antibody reaction visualized by incubating the nitrocellulose blot first with goat anti-rabbit IgG peroxidase conjugate diluted 1:1000 in 10 mM potassium phosphate, pH 7.4, containing 0.15 M NaCl, followed by incubation with the same dilution buffer containing 2.8 mM 3,3′-diaminobenzidine, 0.13% hydrogen peroxide, and 0.9 mM NaCl.

Enzyme Assays. Yeast strains were grown to an A_{600} = 1.0, harvested by centrifugation, resuspended in 20 mM potassium phosphate, pH 7.0, 0.2 M KCl, 0.1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride, and transferred to an Eppendorf tube to which one-third volume of glass beads (0.2–0.3-mm diameter) was added, and the yeast were broken open by vortexing for four 1-min intervals. Tubes were centrifuged for 5 min in an Eppendorf microfuge in the cold, and the AMP deaminase activity of the supernatants was assayed spectrophotometrically at 30 °C by monitoring the increase in absorbance at 235 nm (0.05 cm path length) in a reaction mixture containing 50 mM triethanolamine, pH 7.0, 0.1 M KCl, 0.1 mM dithiothreitol, 0.1 mM ATP, and 4 mM AMP. Protein was measured by the dye-binding method of Bradford (1976) using the dye reagent from Bio-Rad.

Peptide Analysis. Peptides for amino acid analysis were prepared from yeast AMP deaminase purified from commercial bakers' yeast by using a modification (personal communication) of the phosphocellulose adsorption chromatographic method of Yoshino et al. (1979). Ten milligrams of purified enzyme in 6 M guanidine hydrochloride was citraconylated (Atassi & Habeeb, 1972), dialyzed extensively against 0.1 M ammonium bicarbonate, pH 8.0, and then digested with 0.1 mg of trypsin for 48 h at 37 °C. Following addition of $^1/_{10}$ volume of 70% formic acid and lyophilization, these peptides soluble in 0.1% trifluoroacetic acid were separated by HPLC on a Vydak C18 TP reverse-phase column using a 96-mL linear gradient (0.7 mL/min) from 5 to 45% acetonitrile in 0.1% trifluoroacetic acid, pH 2.5 (Hermodson & Mahoney, 1983). Peptides were detected by monitoring absorbance at 214 nm. Individual peaks were collected, rechromatographed, and subjected to Edman degradation to determine the N-terminal amino acid sequence. Edman degradation of the intact protein was also performed. Edman degradations were performed by Dr. M. Hermodson, Purdue University.

Oligonucleotides. Oligonucleotides for radioactive probes, primer extension, and sequencing were prepared by using an Applied Biosystems Model 380A and B DNA synthesizer. The oligonucleotide probes were end-labeled with [γ-^{32}P]ATP by using polynucleotide kinase (Zoller & Smith, 1983) to a specific activity of 3.1–3.8 × 10^6 cpm/pmol and purified electrophoretically on an 8 M urea, 20% polyacrylamide gel (Maniatis et al., 1982). The band corresponding to the full-length oligonucleotide was eluted overnight at 37 °C in 0.5 M ammonium acetate, 10 mM magnesium acetate, 0.1

[1] Abbreviations: kb, kilobase(s); SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 15 mM sodium citrate, pH 7.0; bp, base pair; ss, single stranded; SSPE, 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4; DTT, dithiothreitol.

mM EDTA, and 0.1% SDS. Labeled oligonucleotide (10^7 cpm/5 mL) was hybridized in a mixture of 6× SSC (0.90 M NaCl, 90 mM sodium citrate, pH 7.0), 1× Denhardt's solution (Maniatis et al., 1982), 100 μg/mL denatured salmon sperm DNA, and 0.05% sodium pyrophosphate overnight at the indicated temperature and then washed in 6× SSC containing 0.05% sodium pyrophosphate as indicated following several changes at room temperature. For sequencing and primer extension, the tritylated oligonucleotide was purified by HPLC separation using a Waters C_{18} μBondapak column with a 40-mL gradient, 1 mL/min, from 10 to 70% acetonitrile in 0.1 M triethylamine acetate, pH 7.0 (Fritz et al., 1978). Oligonucleotides were monitored at 254 nm, and the center of the appropriate peak was collected and detritylated by incubating 20 min with 80% acetic acid.

Plasmid Library Screening. Library RB236 was plated, replicated, and screened as described by Hanahan and Meselson (1980). The DNA fragment used as the radioactive probe was isolated after agarose gel electrophoresis and nick-translated with using DNA polymerase I and [α-^{32}P]-dATP to a specific activity of 5–9 × 10^8 cpm/μg by using the spun-column method to remove unincorporated nucleotide (Maniatis et al., 1982).

DNA Isolation, Blotting, and Hybridization Analysis. λ DNA was isolated (Davis et al., 1980) from high-titer λ stocks prepared following heat induction of lysogenized CHS-5 (Maniatis et al., 1982). Small-scale isolation of bacterial plasmid DNA was carried out by a modification of the procedure of Davis et al. (1980), adding two phenol and one chloroform/isoamyl alcohol (24:1 v/v) extractions prior to the ethanol precipitation of the DNA. Larger amounts of plasmid DNA were isolated by the cesium chloride gradient method of Maniatis et al. (1982). Yeast genomic DNA was isolated as described by Cryer et al. (1975). DNA separated by agarose gel electrophoresis was transferred to nitrocellulose (Southern, 1975) and hybridized with radioactive DNA probes (Maniatis et al., 1982), labeled as described above.

RNA Studies. Total yeast RNA was isolated from yeast strain SM4-40 containing plasmid YEp*AMD*-16, which was grown in SD media with amino acid supplements (see Table I) to a Klett of 50 units (A_{600} = 0.86) (Sherman et al., 1983). The 5′-end of the *AMD* transcript was determined by primer extension using a HPLC-purified synthetic oligonucleotide (5′-GAGAAAGGTCGTTAAGCCTCT-3′) that is complementary to nucleotides 20–40 in the coding region of *AMD*. The primer was annealed to the RNA at 65 °C for 3 h in 10 mM Tris buffer, pH 8, containing 0.1 mM EDTA and 0.3 M NaCl. The ethanol-precipitated and dried RNA pellet was resuspended in 100 μL of 50 mM Tris buffer, pH 8.3, containing 60 mM NaCl, 8 mM $MgCl_2$, 10 mM DTT, and 40 μM dNTP with 250 μCi of [α-^{35}S]dATPαS as incorporated label. M-MuLV-reverse transcriptase was added, and the reaction was incubated for 1 h at 41 °C. After RNase digestion followed by phenol/chloroform extraction, the primer-extended products were dissolved in 5 μL of formamide loading buffer. The extension products and a sequencing ladder for size markers were loaded on a DNA sequencing gel. After autoradiography, the sizes of the extension bands were determined by comparison to the standard sequencing ladder.

To determine the size of *AMD* transcript, Northern analysis was carried out by electrophoresis of the total yeast RNA (25 μg) on 1.2% formaldehyde denaturing gel according to the method of Ausubel et al. (1987) except 6% formaldehyde was added to the running buffer and the buffer was recirculated during the electrophoresis. The fractionated RNA and RNA standards were transferred from the agarose gel to nylon membrane according to manufacturer's instructions. The nylon membrane was prehybridized at 52 °C for 3 h with 3 mL of 5× SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA), 5× Denhardt's (Maniatis et al., 1982), 0.5% SDS, 7.5 mM sodium pyrophosphate, and 0.1 mg/mL denatured salmon sperm DNA. To the prehybridization solution, 50 ng of ^{32}P end labeled oligonucleotide (primer extension 21-mer) was added, and the solution was incubated overnight at 52 °C. The nylon membrane was washed twice with 5× SSPE/0.1% SDS for 15 min at room temperature followed by a third wash for 15 min at 52 °C. The blot was exposed to film between two lightning plus (Du Pont) intensifying screens for 1–2 days at −70 °C. To visualize the ribosomal standards, the nylon membrane was soaked in 0.3 M sodium acetate containing 0.02% methylene blue for 10 min and then rinsed in 1× SSPE for 15 min.

DNA Sequencing. Restriction fragments of the cloned inserts were subcloned into either M13mp18 or M13mp19 (Yanisch-Perron et al., 1985) and sequenced by using the Sanger dideoxy termination method (Sanger et al., 1977), as modified for [α-^{35}P]dATP (Biggin et al., 1983). Either the 17-base oligonucleotide from New England Biolabs or 17-base oligonucleotides synthesized from known sequence information were used as primers. Management of the DNA sequence information was aided by the GENED, SEQ, and PEP programs of the BIONET system, which is funded by the National Institutes of Health, Grant P41 RR01685 (Smith et al., 1986). Comparison of protein sequences was carried out by using the GENALIGN program.

Results

AMP Deaminase Activity Screen. To locate a yeast strain deficient in AMP deaminase, a specific screening procedure was developed. Since purified AMP deaminase functions normally in the presence of EDTA, 1 mM EDTA was added to the reaction mixture to inhibit enzymes requiring free divalent cations. As seen in Figure 1, separation of the reaction products by HPLC demonstrates that IMP is the sole nucleotide product of AMP degradation with prolonged incubation of the reaction mix with permeabilized yeast. In addition, the amount of ammonia detected colorimetrically is equivalent to the amount of IMP produced, demonstrating that this procedure specifically detects AMP deaminase activity.

Production of AMP Deaminase Deficient Mutant. Yeast strain S150-2B was mutagenized with 50 μg/mL nitrosoguanidine for varying times up to 1 h. Less than 0.1% survivors were present following 30-, 45-, or 60-min exposures. Survivors from all times were grown on YPD plates and screened for AMP deaminase activity. One colony of the 664 screened was found to be deficient in AMP deaminase activity. This *amd* strain [SM4-40(*amd*)] was stable and thus can be transformed by most yeast vectors. Permeabilized cells or extracts prepared from this strain produced no detectable IMP in overnight incubations with the reaction mixture. On the basis of the sensitivity of the HPLC detection of IMP, the specific activity in this strain is $<4.1 \times 10^{-5}$ μmol min^{-1} (mg of protein)$^{-1}$, which is <0.04% of the wild-type activity. The decrease in activity was shown to be due to an absence of immunodetectable AMP deaminase protein in a Western blot of an extract from the *amd* strain following reaction with rabbit anti-yeast AMP deaminase antiserum (Figure 2B, lane 2).

Partial Amino Acid Composition of Yeast AMP Deaminase. To generate amino acid sequence information required for the preparation of oligonucleotide probes and the verification of DNA sequencing, purified yeast AMP de-

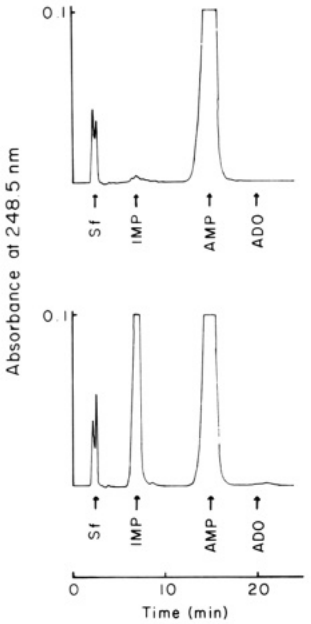

FIGURE 1: HPLC profile of AMP degradation products from permeabilized yeast cells. Yeast colonies were resuspended in 200 μL of reaction mixture (see Experimental Procedures), permeabilized with 10 μL of toluene/ethanol (1:4 v/v), and incubated at 30 °C. A 5-μL aliquot was separated by HPLC at a flow rate of 1.5 mL/min. (Top) Reaction mixture plus permeabilized yeast cells at zero time. (Bottom) Reaction mixture plus permeabilized yeast cells after 18 h. The AMP peak is purposely off-scale for maximum sensitivity in visualizing any minor peaks. Peak identification was from separate runs of standards. The amount of ammonia produced (determined on a separate aliquot) was 91% of the amount of IMP on the basis of triplicate determinations of both. Sf, solvent front; IMP, inosine monophosphate; AMP, adenosine monophosphate; Ado, adenosine.

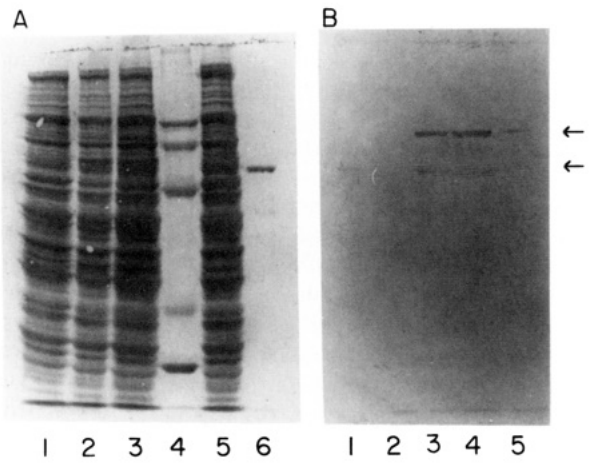

FIGURE 2: Western blot of yeast extracts showing an immunoreactive protein correlated with activity levels of a larger size than the active purified protein. A 7.5% SDS–polyacrylamide gel was electroblotted onto nitrocellulose, reacted with affinity-purified rabbit anti-yeast AMP deaminase, and visualized with peroxidase-conjugated goat anti-rabbit IgG and peroxidase staining. (A) (Total protein stained with Amido-Schwarz) (lane 1) 60-μg S150-2B extract, specific activity 0.1 unit/mg; (lane 2) 50-μg SM4-40 (YEp*AMD*-16) extract, specific activity 1.5 units/mg; (lane 3) 30-μg SM4-40 (YEp*AMD*-16) extract, specific activity 4.8 units/mg; (lane 4) protein standards (from top to bottom) β-galactosidase, phosphorylase B, bovine albumin, egg albumin, and carbonic anhydride (M_r = 116000, 97400, 66000, 45000, and 29000, respectively); (lane 5) 85-μg SM4-40 extract, no activity; (lane 6) 0.4 μg of purified yeast AMP deaminase. (B) (Lane 1) 20 ng of purified yeast AMP deaminase; (lane 2) 85-μg SM4-40 extract, no activity; (lane 3) 50-μg SM4-40 (YEp*AMD*-16) extract, specific activity 1.5 units/mg; (lane 4) 30-μg SM4-40 (YEp*AMD*-16) extract, specific activity 4.8 units/mg; (lane 5) 60-μg S150-2B extract, specific activity 0.1 unit/mg. Activity units are micromoles of IMP formed per minute.

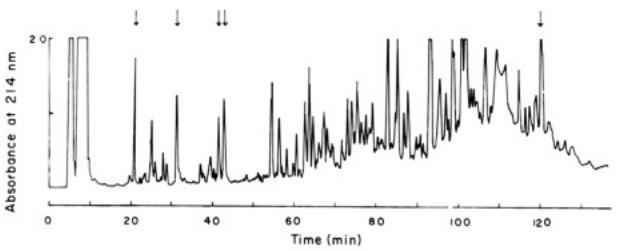

FIGURE 3: HPLC profile of tryptic peptides of citraconylated yeast AMP deaminase. Peptides were prepared and separated as described under Experimental Procedures. Arrows indicate peptides whose amino acid sequence was determined by Edman degradation (sequences are shown in Figure 7).

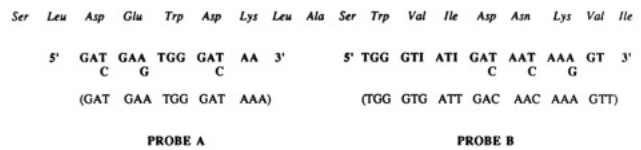

FIGURE 4: Oligonucleotide probes used to isolate yeast AMP deaminase. The top line shows the amino acid sequence of the peptide eluting near 122 min (Figure 3) that was used to generate two oligonucleotide probes, designated probe A and probe B, shown just below it. Where two bases are at one position, both were added in equimolar amounts at that synthesis cycle. The actual nucleotide sequence found in *AMD* is shown in parentheses. I, deoxyinosine.

aminase was used to determine the N-terminal sequence of both the intact protein and selected peptides derived from a tryptic digest of citraconylated enzyme. The HPLC profile of peptides soluble in 0.1% trifluoroacetic acid is shown in Figure 3. Those peptides whose amino acid sequence was determined by Edman degradation are designated with an arrow.

Oligonucleotide Screening for Yeast AMP Deaminase Gene. The amino acid sequence of the peptide eluting near 122 min was used to generate the two oligonucleotide probes shown in Figure 4. The amino acid sequence was translated into the corresponding DNA-coding sequence, with deoxyinosine incorporated at the third position in those codons where three or four different bases code for a particular amino acid (Young & Davis, 1983). A yeast DNA library in λgt11 (Ohtsuka et al., 1985) was screened with the ^{32}P-labeled probe A (Figure 4). Of approximately 12000 phage screened, 6 positively hybridizing λ clones were isolated. *Eco*R1 digestion of DNA isolated from these phage suggested that five of the six were independent clones, i.e., having inserts with different restriction patterns (data not shown). When these six were probed with the ^{32}P-labeled probe B (Figure 4), only one of the six hybridized to the second probe (data not shown). Southern blot analysis of this λ clone, designated λgt11(25A), localized the hybridization with both probes to an 176-bp *Eco*R1, *Hind*III fragment of the λ insert (data not shown). This fragment was subcloned into M13mp18 and M13mp19. Nucleotide sequencing verified that the peptide sequence from AMP deaminase was represented in the DNA fragment.

Screening for the AMD Gene and Its Expression in Yeast. The insert in λgt11(25A) was analyzed by both restriction mapping and sequencing; it did not contain the complete gene. Therefore, the 1.6-kb *Eco*R1 fragment, approximately two-thirds of which contains coding information, was labeled by nick translation and used to probe RB236, a yeast DNA library in the centromere shuttle vector YCp50 (Kuo & Campbell, 1983). Of the approximately 16000 colonies screened, 15 clones were isolated that contained all or part of the *Eco*R1 fragment used as the probe. Several of these were transformed into the *amd* yeast strain, and one (YCp*AMD*5-2,

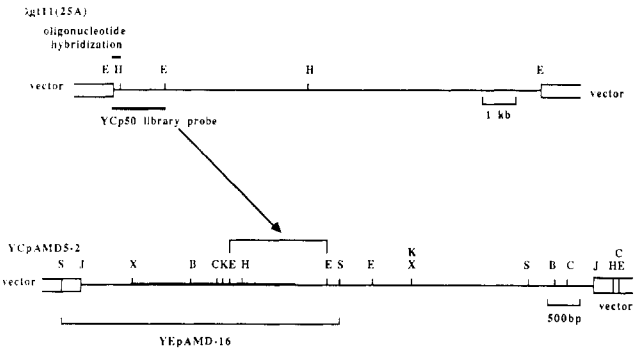

FIGURE 5: Isolation of entire gene from RB236 library. Restriction map of λgt11(25A) shows the position of insert fragment used to screen RB236 yeast DNA library (see Experimental Procedures) and the restriction map of the plasmid that was isolated containing the entire *AMD* gene (YCp*AMD*5-2). The denoted *Sal*I fragment was sufficient to result in overproduction of AMP deaminase when inserted into multi copy number plasmid YEp24 (YEp*AMD*-16). The thicker black line represents the coding region, transcription proceeding from left to right. B, *Bam*H1; C, *Cla*I; E, *Eco*R1; H, *Hin*dIII; J, *Bam*H1/*Sau*3A junction; K, *Kpn*I; S, *Sal*I, X, *Xba*I.

Table I: Growth Properties of Yeast Strains with Varying AMP Deaminase Activities

strain	specific activity[a] (μmol of IMP formed min^{-1} mg^{-1})	doubling time[b] (min)	
		YPD[c]	SD[d]
S150-2B	0.32	78	132
SM4-40(*amd*)	<0.0004	90	144
SM4-40(YCp*AMD*5-2)	0.42		
SM4-40(YEp*AMD*-16)	4.86 ± 0.26		156

[a] Activities are either the average of duplicate determinations or the mean ± SE of three determinations. [b] The doubling time was determined from the linear portion of a logarithmic plot of the increase in A_{600} of an actively growing culture. [c] YPD is a complete media (Sherman et al., 1983). [d] SD is a minimal media, to which 30 μg/mL leucine, 20 μg/mL each of tryptophan and histidine, and 2% glucose were added (Sherman et al., 1983).

Figure 5) restored full AMP deaminase activity to the *amd* mutant (Table I). To isolate a smaller insert containing the gene, the 4.2-kb *Sal*I fragment was subcloned into YEp24, a multicopy number shuttle vector (Botstein et al., 1979; Figure 5). Transformation of the *amd* strain with YEp24 containing the 4.2-kb *Sal*I fragment gave a strain designated SM4-40 (YEp*AMD*-16) with a specific activity 15-fold that of the *AMD* control (Table I).

Growth of Yeast Strains with Varying AMP Deaminase Activities. The doubling times of the *amd* mutant in both YPD and SD media were only slightly longer than those of the parental strain. In addition, the yeast strain SM4-40 (YEp*AMD*-16), which had a specific activity 15-fold that of *AMD* strain, had a doubling time in SD media 118% of the parental strain (Table I). In yeast, therefore, an absence or an excess of AMP deaminase has little effect on growth rates.

Nucleotide Sequence of Yeast AMD Gene. Figure 6 depicts the sequencing strategy and Figure 7 shows the nucleotide sequence of the yeast AMP deaminase structural gene. For the region encoding the protein, 97% of the nucleotide sequence was determined at least twice from independent clones with 97% confirmed from the complementary strand (Figure 6). Sequences of all restriction sites used for subcloning were confirmed from an overlapping fragment. The sequence from nucleotide 1792 to nucleotide 3354 was determined from DNA isolated from λgt11(25A), while the sequence from nucleotide 1 to nucleotide 1970 was obtained from DNA isolated from YCp*AMD*5-2. The 180-bp overlapping region sequenced from

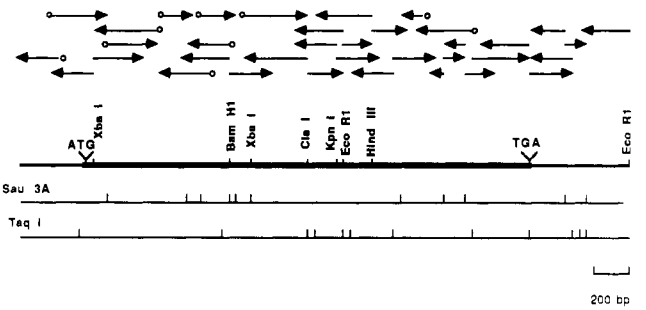

FIGURE 6: Sequencing strategy for yeast *AMD* gene. Arrows represent the direction and extent of each sequencing experiment; those arrows with a circle at the beginning represent sequencing reactions primed with custom-made primers. The thicker line between the ATG and TGA codons designates the coding region.

the two yeast strains was in complete agreement, except for nucleotide 1867, which was a C in the former and a T in the latter. This difference was in the third base of an amino acid codon and did not alter the amino acid sequence. The nucleotide sequence encodes an open reading frame of 2430 bp, beginning with the ATG initiation codon at nucleotide +1 and ending with a TGA termination signal. The amino acid sequence information derived from the purified protein was found in this reading frame and is underlined in Figure 7.

Characterization of Yeast AMD Coding Region. The 2430-nucleotide open reading frame of *AMD* encodes a protein with a molecular weight of 93 286, which is considerably larger than the reported size for the AMP deaminase subunit of 83 000 (Yoshino et al., 1979). In addition, the N-terminal amino acid sequence derived from the intact protein as isolated in this laboratory begins with the tyrosine at amino acid 193, which is 21 amino acids from the nearest methionine (Figure 7). This suggested that the protein purified from commercial yeast by us (personal communication) and others (Yoshino et al., 1979) is probably a proteolytic fragment of the full-sized gene product. This was supported by Western blot analysis of various yeast extracts which showed an immunoreactive band of approximately 96 000 molecular weight, larger than the purified enzyme, that varied with the enzyme activity (Figure 2). In other studies, Western blot analysis of yeast extracts from commercial yeast or strain S150-2B showed two immunoreactive bands at 96 000 and 83 000 molecular weights (data not shown). This higher molecular weight band disappeared in the *amd* mutant and reappeared prominently in extracts from the *amd* mutant transformed with the *AMD* gene, confirming that the size of the protein in extracts from freshly grown yeast is larger than that of the enzyme purified from compressed yeast obtained commercially.

The codon usage for the protein coding region is relatively nonselective. Codon usage can also be expressed by the codon bias index defined by Bennetzen and Hall (1982), which is a measure of the use of the codons preferred in highly expressed yeast genes, and the codon adaptation index of Sharp and Li (1987), which takes into account the frequency of use of all codons of the gene against a reference set of highly expressed yeast genes. For the *AMD* gene, the codon bias index is 0.19 and the codon adaptation index is 0.18, suggesting that the *AMD* gene is low to moderately expressed (Bennetzen & Hall, 1982b) which is consistent with the low abundance of AMP deaminase protein in yeast (Yoshino et al., 1979).

RNA Studies. To characterize the control region of *AMD*, the 5′-end of the mRNA was identified by primer extension. A 21-mer oligonucleotide primer designed to anneal to nucleotides 20–40 in the coding region of *AMD* was hybridized to total yeast RNA isolated from strain SM4-40 containing

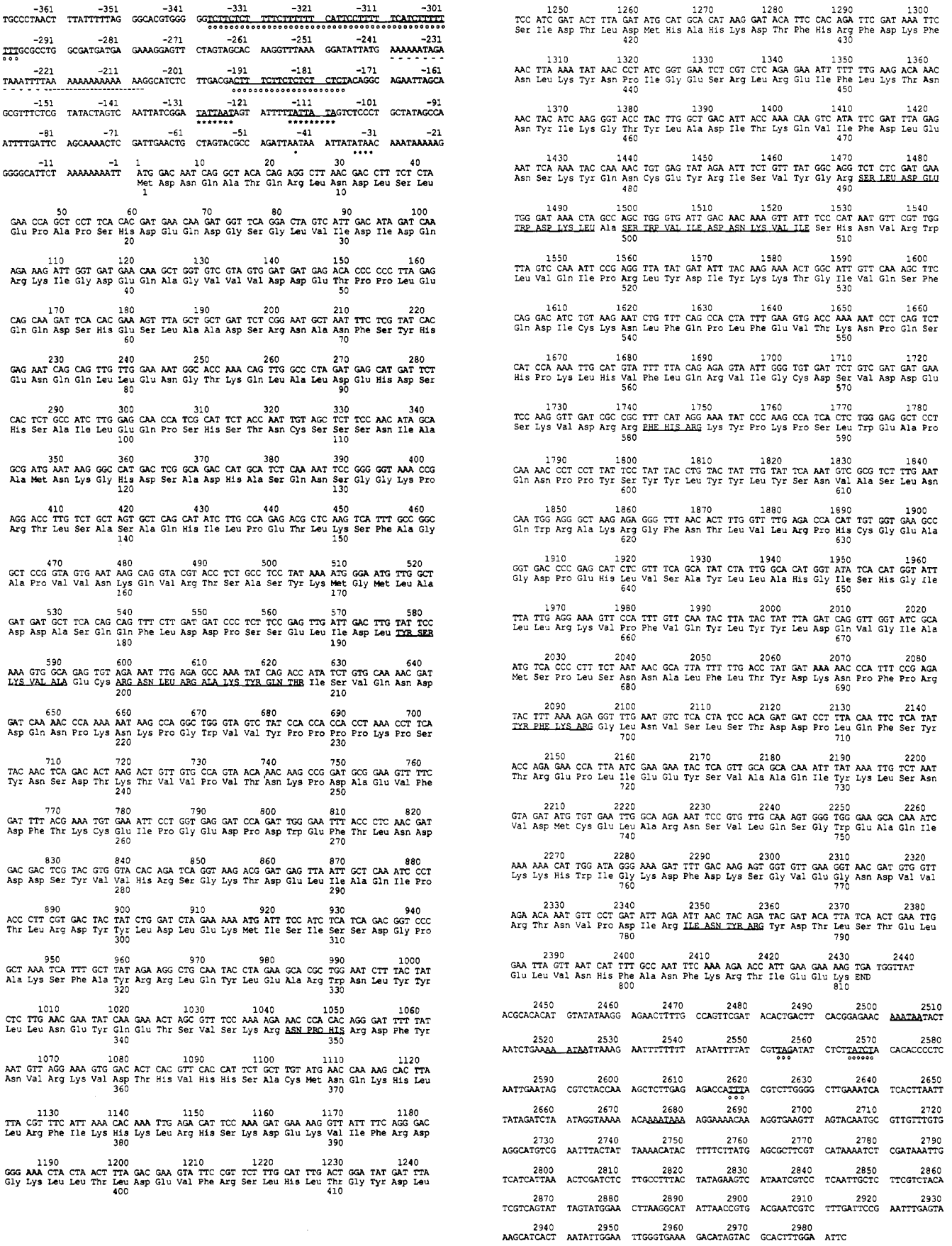

FIGURE 7: Nucleotide sequence and deduced amino acid sequence of yeast AMP deaminase gene. Nucleotide sequence of the mRNA-identical (plus) strand of a contiguous 3354-bp region encompassing the yeast AMP deaminase structural gene shows the deduced amino acid sequence and the DNA sequence of the 370 bp 5′ and 551 bp 3′ to the protein-encoding region. The nucleotide number is above the line, and the amino acid number is below. The amino acid sequences derived from sequencing the peptides are in all capitals and underlined. Specific features in the flanking regions are underlined. In the 5′-flanking region, the TATA boxes are denoted with asterisks, the transcriptional start sites are marked with solid underdots, the pyrimidine-rich regions are shown by hollow underdots, and the dA homopolymer is underlined, with the possible extension of this putative constitutive expression element denoted with dashes. In the 3′-flanking region, the sequence homologous to the consensus polyadenylation/termination signal TAG...TATGT...TTT is denoted by hollow underdots, while the consensus polyadenylation signals AAATAA are underlined.

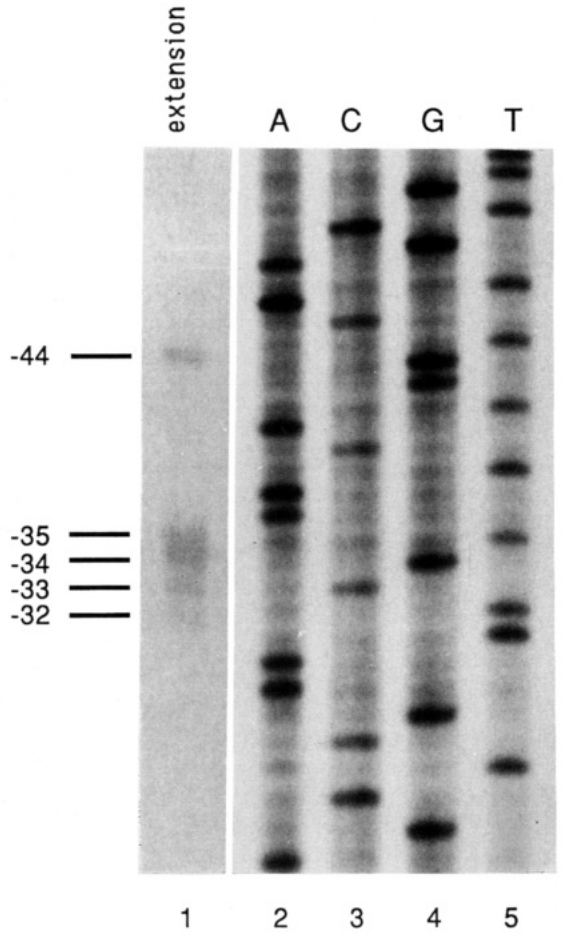

FIGURE 8: Primer extension mapping of transcriptional start sites of yeast AMP deaminase. In lane 1, unlabeled synthesized oligonucleotide (21-mer) (0.5 μg) was hybridized with total yeast RNA (50 μg) and then extended with reverse transcriptase (100 units) in the presence of [α-^{35}S]dATPαS (0.25 nmol). The base numbers on the left margin denote the transcriptional start sites for RNA polymerase on *AMD*. Lanes 2–5 represent the sequencing ladder of M13mp18 ss template DNA annealed to M13 universal primer (17-mer).

the plasmid YEp*AMD*-16. After primer extension with reverse transcriptase, the sizes of the extension products were determined by electrophoresis on a DNA sequencing gel and compared to a standard DNA sequencing ladder. Five extension products were found of 55, 56, 57, 58, and 67 nucleotides in length (Figure 8). These products correspond to transcriptional start sites at bases −32, −33, −34, −35, and −44 upstream of the ATG translational initiation codon. No additional start sites were seen within 500 bases upstream of the ATG codon.

To determine the approximate size of the *AMD* transcript, Northern blot analysis was conducted with total yeast RNA isolated from yeast strain SM4-40 containing the plasmid YEp*AMD*-16. When the ^{32}P end labeled oligonucleotide (primer extension 21-mer) was hybridized to the nylon membrane at T_D −10°C, a single band was observed at 3.0 ± 0.2 kb on the basis of comparison to ribosomal RNA standards (both *E. coli* and yeast) and RNA standards (0.125–1.26 kb) (data not shown).

Comparison of Yeast AMP Deaminase Amino Acid Sequence with That of E. coli AMP Nucleosidase and Rat Muscle AMP Deaminase. The amino acid sequence of yeast AMP deaminase was aligned with the amino acid sequences of *E. coli* AMP nucleosidase (Leung et al., 1989) and rat muscle AMP deaminase (Sabina et al., 1987) by using the GENALIGN program of the BIONET system. The *E. coli* AMP

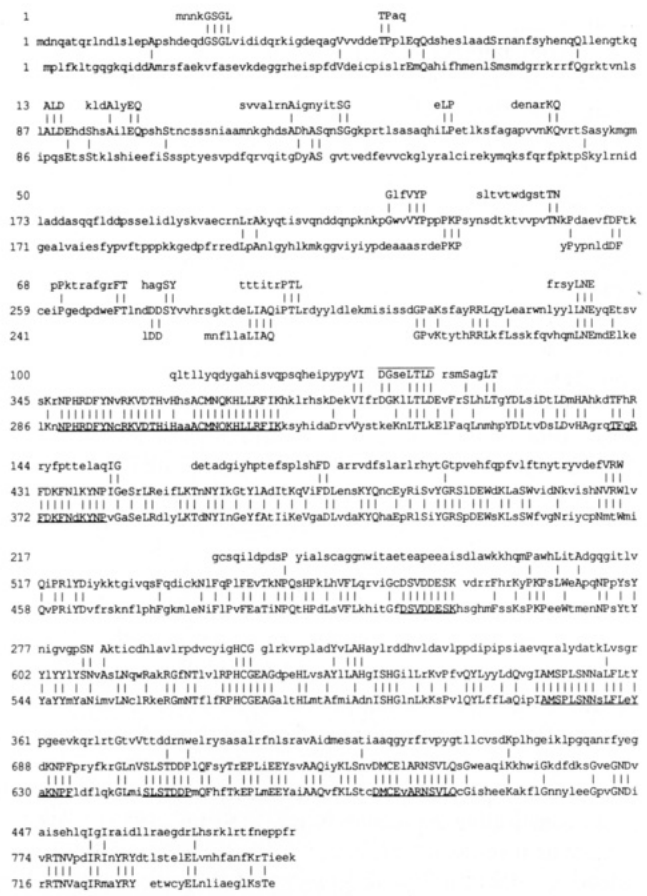

FIGURE 9: Comparison of the amino acid sequence of yeast AMP deaminase with that of *E. coli* AMP nucleosidase and rat muscle AMP deaminase. The amino acid sequence of yeast AMP deaminase (middle line) was aligned separately with that of *E. coli* AMP nucleosidase (top line) (preceding paper) and rat muscle AMP deaminase (bottom line) (Sabina et al., 1987) by using the GENALIGN program of the BIONET system (Smith et al., 1986). Homologous amino acids are in capitals with a dashed line between the two sequences that show the homology. The number of the first amino acid of the line is on the left. The overlined sequence denotes the region of maximum homology between yeast AMP deaminase and *E. coli* AMP nucleosidase, and the underlined sequences are regions of 85% or greater homology between yeast and rat muscle AMP deaminase. a, alanine; c, cysteine; d, aspartic acid; e, glutamic acid; f, phenylalanine; g, glycine; h, histidine; i, isoleucine; k, lysine; l, leucine; m, methionine; n, asparagine; p, proline; q, glutamine; r, arginine; s, serine; t, threonine; v, valine; w, tryptophan; y, tyrosine.

nucleosidase had no apparent overall homology, with only 76 of the 483 amino acids of the nucleosidase being aligned with the 810 amino acids of yeast AMP deaminase (16%), this being possible only by introducing numerous gaps in the *E. coli* sequence (Figure 9). There was one region of eight amino acids, however, that seemed to be similar in the two proteins, with six of the eight amino acids being identical (Figure 9).

The carboxy-terminal portion of the rat muscle AMP deaminase amino acid sequence had significant homology with yeast AMP deaminase (51%), with a number of regions having greater than 85% identity (Figure 9).

DISCUSSION

To establish the extent of homology between AMP deaminase in eukaryotes and AMP nucleosidase in prokaryotes and to establish the amino acid sequence, the gene for yeast AMP deaminase was cloned. Evidence that the isolated gene encodes AMP deaminase includes the following: (1) The predicted amino acid sequence contains all the peptide sequences derived from pure enzyme. (2) Transformation with the cloned gene restores AMP deaminase activity to an *amd*

yeast mutant. (3) Transformants with the cloned gene on a multi copy number plasmid have increased AMP deaminase activity and protein. (4) There is a relatively unbiased codon usage consistent with the low abundance of AMP deaminase in yeast.

5′- and 3′-Flanking Nucleotide Sequence of Yeast AMD. The sequence of 370 nucleotides 5′ to the ATG translational initiation codon was examined for homology to known yeast promoter elements (Figure 7). Although there was no sequence that exactly matched the canonical yeast TATA element sequence (TATAAA; Struhl, 1986), two adjacent regions whose sequences match known functional yeast TATA elements were present (Nakao et al., 1986; Larkin et al., 1987). The TATA box, which is presumed to be involved in the regulation of transcription initiation in most yeast genes, is usually found from 40 to 180 bp from the mRNA initiation site [within 200 bp of the ATG codon (Sentenac & Hall, 1982)]. The two probable TATA elements at nucleotides −130 to −123 and −115 to −109 are within a reasonable space constraint for RNA polymerase. The first region containing the sequence TATTAA, which differs by only one base from the consensus sequence, is the most likely candidate.

The constant specific activity of AMP deaminase in yeast grown on different media (Table I), during both logarithmic and stationary growth phases (data not shown), suggests that it is expressed constitutively. The upstream promoter element consisting of poly(dA/dT) sequences that has been correlated with constitutive expression in yeast (Struhl, 1985) is located either as a sequence of 14 A's from nucleotide −222 to nucleotide −209 or as the 32-base-pair sequence from nucleotide −240 to nucleotide −209 containing 25 A's (Figure 7). Also observed in this 5′-region are two pyrimidine-rich sequences, as described in other yeast promoters (Montgomery et al., 1980; Bennetzen & Hall, 1982; Dobson et al., 1982; Burke et al., 1983). One is a smaller stretch of 17 pyrimidines located from nucleotide −193 to nucleotide −177 (59% T, 41% C), and the other is a larger region of 41 nucleotides containing only 2 purines (both A's) from nucleotide −338 to nucleotide −298 (73% T, 23% C) (Figure 7). Pyrimidine-rich regions between the TATA box and the mRNA initiation site, which are followed shortly by a CAAG sequence, have been proposed to be involved in high levels of gene expression (Dobson et al., 1982). If such pyrimidine-rich segments are involved in regulating expression levels, the positioning of these sequences 5′ instead of 3′ to the putative TATA box in *AMD* may be a factor in its low level of expression.

Termination and polyadenylation signals are not as well-defined in yeast as in higher eukaryotes (Bennetzen & Hall, 1982; Zaret & Sherman, 1982). The consensus sequence TAG...TATGT...TTT has been proposed by Zaret and Sherman (1982) to be a signal in yeast for transcription termination/polyadenylation. A similar sequence, TAG... TATCT...TTT, begins at nucleotide 2554, +121 bases from the 3′-end of the coding region (Figure 7). The sequence AATAAA has also been proposed to specify a polyadenylation site in yeast (Bennetzen & Hall, 1982; Burke et al., 1983). That sequence and two similar ones (AAATAA) are located in the yeast *AMD* gene starting at nucleotides 2501, 2519, and 2674 (+68, +86, and +241, respectively, from the translation stop codon) (Figure 7). From the size of the *AMD* transcript (3 ± 0.2 kb) determined by Northern analysis, any of the three proposed polyadenylation sites could be functional for *AMD*.

Deduced Protein Structure from AMD. Assignment of the indicated ATG as the start of the protein coding region was based on several criteria. First, there is an in-frame TAA termination codon 27 nucleotides upstream of this ATG, and the nearest upstream ATG in any frame is 243 nucleotides away. The subunit size of 96 000 estimated from Western blot analysis of extracts from freshly grown yeast agrees well with the size predicted from the deduced protein sequence of 93 286. Initiation of translation at the next downstream in-frame methionine codon would result in a protein with a predicted molecular weight of only 82 105. In addition, almost all yeast genes have an A −3 bases to the ATG initiation codon (Dobson et al., 1982; Hamilton et al., 1987), with a bias toward A's at positions −5, −7, and −9 (Hamilton et al., 1987), which is the case for the indicated start codon.

The discrepancy between the size of the purified enzyme (M_r = 83 000) and the polypeptide encoded by *AMD* (M_r = 93 286) was resolved by Western blot analysis of yeast extracts prepared in the presence of protease inhibitors. The immunoreactive band that varied with enzyme activity corresponded to the size predicted from the deduced amino acid sequence. The low molecular weight form is present in only a small amount of the initial extracts of yeast. Normal cellular processing of the higher molecular weight form or, more likely, the action of proteases when the yeast is broken, digests the native protein to the size observed for purified enzyme. The finding of proteolytic degradation of enzymes during purification is not unusual in yeast as both phosphofructokinase (Nadkarni et al., 1982) and hexokinase (Gazith et al., 1968) are proteolytically cleaved during purification. The purified enzyme has catalytic and regulatory properties similar to those of AMP deaminases from other eukaryotes, including allosteric activation by ATP (Yoshino et al., 1979; Murakmai, 1979). The catalytic functions of rat muscle AMP deaminase are located near the carboxy-terminal portion of enzyme (Marquetant et al., 1987).

AMP deaminase contains binding sites for both AMP and ATP. The consensus sequence for nucleotide binding sites seems to be composed of two portions: (1) the phosphoryl binding sequence, which is proposed to be either GXXXXGK (Moller & Amons, 1985) or that sequence plus a nearby DXXG (Dever et al., 1987), on the basis of regions of homology between different nucleotide binding proteins and from X-ray crystallographic studies of adenylate kinase (Pai et al., 1977) and (2) a portion conferring nucleotide specificity. For adenine nucleotides, the latter appears not to be confined to a given sequence, but instead has been correlated by Rossmann et al. (1974) with the protein's secondary structure (β loop α β). There were no sequences in the yeast AMP deaminase that conformed to the phosphoryl binding consensus sequence, suggesting that such a motif is not utilized by all nucleotide binding proteins. In addition, the secondary structure predictions for AMP deaminase based on Chou and Fasman (1978) (data not shown) revealed no region that could be clearly interpreted as β loop α β, with many sections having characteristics of more than one structure.

Comparison of Yeast AMD and E. coli amn. Eukaryotic AMP deaminase and prokaryotic AMP nucleosidase appear to play similar metabolic roles, both being allosterically activated by ATP and inhibited by phosphate. No overall homology was found between the amino acid sequences of yeast AMP deaminase and *E. coli* AMP nucleosidase, suggesting that they did not evolve from a common ancestral gene. One small region of amino acid identity, noted in Figure 9, could be an AMP, an ATP, or a phosphate binding site, all of which are common between the two enzymes. A database search of protein sequences found a portion of the *E. coli pho*E gene with amino acid sequence similar to the small common

sequence. There were no proteins in the data bank that showed significant homology to yeast AMP deaminase other than the C-terminal region of rat muscle AMP deaminase.

Comparison of Yeast and Rat Muscle AMD. The amino acid sequences of the C-terminal portions of these two enyzmes have significant homology (51% comparing amino acids 313–810 of yeast AMP deaminase with amino acids 254–747 of the rat muscle enzyme; Figure 9). In the N-terminal portions of these proteins, the degree of similarity is only 11%. Since the various tissue-specific forms of rat AMP deaminase appear to be generated by alternate mRNA splicing (Sabina et al., 1987) and the C-terminal portion of rat muscle AMP deaminase is involved in binding myosin (Marquetant et al., 1989), presumably not a function of the yeast enzyme, the abrupt change in the degree of similarity suggests that the N-terminal portion of the rat muscle enzyme is derived from an exon unrelated to the yeast AMP deaminase gene.

There are a number of regions with degrees of homology of 85% or greater interspersed throughout this C-terminal region, including one 32 amino acid sequence (Figure 9). These conserved sequences are likely to correspond to regions involved in AMP, ATP, or phosphate binding or to regions involved in intersubunit interactions. A database search using those highly conserved sequences of 12 amino acids or greater in length did not locate any proteins with significant homology to these sequences. Radioactive labeling of yeast AMP deaminase with 8-azido-[8-^{32}P]ATP resulted in labeling near this region (Merkler & Schramm, 1988).

Function of Yeast AMD. AMP deaminase has been proposed to be involved in maintaining the energy charge and in regulating phosphofructokinase by regulating cellular ammonia levels (Chapman & Atkinson, 1973; Yoshino & Murakami, 1981, 1982a,b). Since the *amd* mutant, which has severely reduced or no AMP deaminase activity, appears to grow almost as well as wild-type yeast (Table I), these functions must not be essential for yeast growth in complete or minimal media. Although the original *amd* strain could have had a compensating mutation allowing normal growth, preliminary studies with a yeast strain with a specific interruption of the *AMD* gene, abolishing AMP deaminase activity, also indicated near-normal growth (unpublished observations). Further studies are required to establish whether a lack of AMP deaminase would be detrimental to yeast in unfavorable environments.

AMP deaminase deficiency is known to occur in mammalian muscle and causes a benign disorder that prevents vigorous exercise (Sabina et al., 1989). No deficiency of the enzyme is known in liver or other organs, which contain different isoforms. The near-normal growth rate of yeast deficient in AMP deaminase establishes the nonessential role of the enzyme in dividing yeast. The phenotype of yeast deficient in AMP deaminase under more restrictive conditions of growth should be useful in understanding the role of AMP deaminase in adenylate metabolism.

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