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NAT2, an Essential Gene Encoding Methionine N^{α}-Acetyltransferase in the Yeast *Saccharomyces cerevisiae**

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N^{α}-Acetylation is catalyzed by N^{α}-acetyltransferases, which transfer acetyl groups from acetyl coenzyme A to the N termini of most eukaryotic proteins co-translationally. *NAT1* and *ARD1* from the yeast *Saccharomyces cerevisiae* (Mullen, J. R., Kayne, P. S., Moerschell, R. P., Tsunasawa, S., Gribskov, M., Colavito-Shepanski, M., Grunstein, M., Sherman, F., and Sternglanz, R. (1989) *EMBO J.* 8, 2067–2075) were previously shown to encode the major N^{α}-acetyltransferase, which act on certain proteins having serine, glycine, and alanine but not methionine termini (Sherman, F., Moerschell, R. P., Tsunasawa, S., and Sternglanz, R. (1993) in *Methods in Protein Sequence Analysis* (Imahori, K., and Sakiyama, F., eds) pp. 173–181, Plenum Publishing Corp., New York). We have identified a second gene, *NAT2*, that may correspond to the N^{α}-acetyltransferase acting on a subset of proteins having methionine termini. Crude extracts of a series of heat-sensitive mutants (Ts^{-}) were screened for acetylation of a 24-amino acid synthetic peptide Met-Asn-Asn- *in vitro*. One mutant, *nat2-1*, out of 115 strains examined, lacked acetyltransferase activity, and the mutation co-segregated as a single gene with the heat-sensitive phenotype. The *nat2-1* mutants were deficient in the ability to acetylate Met-Asn-Asn- and Met-Glu-Arg-peptides but were able to N^{α}-acetylate Ser-Glu-Phe- and Ser-Tyr-Ser- peptides *in vitro*. The *NAT2* wild-type gene was cloned by complementation of the *nat2-1* mutant, and the DNA sequence revealed an open reading frame of 288 amino acids. Gene disruption demonstrated that *NAT2* is an essential gene, and hybridization analysis indicated that it is located on chromosome VII. Furthermore, there was limited, but significant identities between the yeast N^{α}-acetyltransferases Nat1, Ard1, Nat2, and Mak3, although no common motifs could be identified. We propose that *NAT2* encodes the major N^{α}-acetyltransferase acting on certain proteins with only methionine termini, and that N^{α}-acetylation of some of these proteins is essential for viability.

MATERIALS AND METHODS

Nomenclature—The standard phenotypic designations Ura$^+$ and Ura$^-$ denote normal and mutant strains, respectively, that are able and unable to grow on media lacking uracil. The phenotypic designations Ts$^+$ and Ts$^-$ are used in this paper to denote, respectively, normal strains and heat-sensitive strains that do not grow at the restrictive temperature of 37 °C, in contrast to growth at the permissive temperature of 23 °C. Nat$^+$ and Nat$^-$ denote, respectively, normal and mutant strains that are deficient in N^α-acetyltransferase activity. *NAT2* or *NAT2*$^+$ denote the wild-type allele that encodes an N^α-acetyltransferase acting on at least some peptides with methionine termini. The *nat2-1* designates a recessive mutant allele that produces a presumably heat-sensitive enzyme, whereas *nat2*-Δ denotes a deletion of *NAT2*. We have employed the standard designations, where, for example, *NAT2*, etc., denote genes, and Nat2, etc., denote gene products.

Media, Yeast Genetics, and Molecular Biological Methods—Standard YPD and SD media and LB media were used, respectively, for the growth of yeast (22) and *Escherichia coli* (23).

The general yeast genetics procedures, including testing of mutants and sporulation and dissection of tetrads, have been described (22, 24), as have the general yeast procedures for DNA transformation (25) and gene disruption (26). Other standard molecular biological procedures were employed, including Southern blot hybridization (23).

Both strands of the *NAT2* gene were sequenced by the dideoxy chain termination method (27) using [α-^{32}P]dATP and custom-made oligonucleotide primers.

Filters containing the ordered library of *S. cerevisiae* genomic inserts was obtained from Dr. M. Olson (28). The gel with separated *S. cerevisiae* chromosomes was provided by Dr. E. Rustchenko. The 2.9-kb[1] *Xba*I-*Eco*RI fragment from pAB994 used for hybridization, described under "Results," was isolated from a low melting agarose gel, and was labeled with ^{32}P using a Random Primed DNA labeling kit (U. S. Biochemical Corp.).

Yeast Strains—The strains of *S. cerevisiae* used in this study are listed in Table I. A364A, as well as the collection of Ts$^-$ mutants (13), were kindly provided by Dr. J. S. Butler. A364A-115 and A364A-65 are members from the collection. B-7457, B-7723, and B-7889 are strains from our collection. B-8861, used to clone *NAT2*, is a meiotic segregant from the D-2125.

Construction of Plasmids—The plasmids used in this study are listed in Table II. The original plasmid pAB1027 obtained after transforming B-8861 with the YCp50 library (29) contained a 14-kb insert. Plasmid pAB991 was constructed by transferring a 8.4-kb *Nur*I-*Eco*RI fragment, encompassing *NAT2*, from pAB1027 to the YCp50 plasmid (29) (Fig. 1). The plasmid pAB992 was constructed by transferring a 2.9-kb *Xba*I-*Eco*RI fragment, encompassing *NAT2* (Fig. 1), to pAB625, a pBluescript phagemid with yeast CEN and *URA3* fragments. The plasmid pAB994 was made by inserting the same 2.9-kb *Xba*I-*Eco*RI fragment in the pAB621 plasmid, a pBluescript phagemid. The plasmids pAB991 and pAB994 were used for double-stranded DNA sequencing of *NAT2* gene.

The plasmid pAB993, which contained a *Hin*dIII-*Eco*RI fragment with *NAT2* replaced by *URA3*, was used for *NAT2* gene disruption (Fig. 1). The *URA3* gene was inserted at the *Kpn*I-*Sac*II site in the *NAT2* gene (Fig. 1) by the following steps. Plasmid pAB991, containing *NAT2*, was doubly digested with several pairs of restriction endonucleases. Plasmid pAB707, containing *URA3*, was digested with *Eco*RI-*Hin*dIII to liberate the *URA3* fragment, and pAB208 was cut with *Eco*RI-*Hin*dIII. All digests were electrophoresed on low melting gels and the following fragments were pooled and ligated: *Eco*RI-*Sac*II fragment from

pAB991, *Kpn*I-*Sac*II fragment from pAB707 containing *URA3*, *Kpn*I-*Hin*dIII fragment from pAB991, and the vector pAB208 cut with *Hin*dIII-*Eco*RI.

The plasmid pAB993 was digested with *Bss*HIII-*Sna*B1 (Fig. 1*C*), and digest was used to transform B-7723, a haploid *ura3-52* strain, and diploid strain D-2126, a homozygous *ura3-52 CYC1*$^+$ strain as described under "Results." The replacement of one of the *NAT2*$^+$ alleles by *nat2*-Δ in the diploid strain was confirmed by polymerase chain reaction amplification.

pAB995, used to overproduce Nat2 in strain B-8862, was constructed by transferring a 2.9-kb *Xba*I-*Eco*RI fragment, encompassing *NAT2*, from pAB991 into the *Xba*I-*Eco*RI site of the high copy number plasmid YEp355R (30).

Peptides and Protein Substrates—The three synthetic peptides used in this study for acetylating substrates (Table III) were synthesized by Dr. N. Bransom, using conventional methods with an Applied Biosystems system. The purity of the peptides was established by high performance liquid chromatography analysis. As summarized in Table III, the sequences of the Met-Asn-Asn- and Ser-Glu-Phe- peptides correspond to termini, respectively, of the *CYC1-345-H* and *CYC1-793* mutant forms of iso-1-cytochrome *c* that are normally acetylated (2, 3). The sequence of Ser-Tyr-Ser- corresponds to the terminus of adenocorticotrophic hormone. Met-Glu-Arg- denotes the *E. coli* tryptophan synthetase subunit α-chain having an unacetylated terminus in its native form (31). (A sample of the tryptophan synthetase subunit α-chain was generously provided by Dr. S. Tsunasawa.)

N^α-Acetyltransferase Assay of Crude Extracts—Ts$^-$ yeast mutants were grown at 23 °C in 15 ml of YPD to a density of 1×10^7 cells/ml. The cell pellet was collected by centrifugation, washed with distilled water, and then washed with 0.5 ml of a solution of 10 mM dithiothreitol and 100 mM Tris-HCl, pH 7.4. The cells were resuspended in the above solution and incubated for 10 min at 30 °C. Following incubation, cells were washed and resuspended in a 0.5-ml solution of 1.2 M sorbitol, 20 mM potassium phosphate, pH 7.2. Spheroplasts were formed by gently shaking the cell suspension with 200 μg/ml zymolyase-100T (ICN Biochemicals) at room temperature. The spheroplasts were collected by centrifugation at 4 °C for 5 sec, washed three times with 1.2 M sorbitol and 20 mM potassium phosphate, pH 7.2, and subsequently lysed in resuspension buffer (200 mM sodium phosphate, pH 7.6, 1 M NaCl, 2 mM EDTA, 10 mM dithiothreitol, and 2% Triton X-100). The crude extracts were frozen at −70 °C until ready to use. Total protein in the crude extracts was measured by the Bradford assay (32).

Extracts were assayed for N^α-acetyltransferase activity by the procedure described by Lee *et al.* (33). Briefly, [^{3}H]acetyl coenzyme A (4.0 Ci/mmol; Amersham Corp.) was a donor of a [^{3}H]acetyl group to a substrate of peptides in an *in vitro* assay buffer. Portions of the lysates were added to a reaction mixture of 50 mM HEPES, pH 7.4, 150 mM EDTA, 25 mM [^{3}H]acetyl coenzyme A (0.5 mCi) and 50 mM of the synthetic peptide substrate with an adjusted final volume of 100 μl. The mixture was incubated at 30 °C for 30 min and then acidified by adding 17 μl of 0.5 N acetic acid. The mixture was filtered through SP membrane disc filters (a strong cation exchange membrane with dextran matrix; Cono, Inc.), which were previously soaked overnight in 0.5 N acetic acid. The membranes were washed with 0.5 N acetic acid, and radioactivity on the filters was quantitated. One unit of enzyme activity was defined as the amount of enzyme able to transfer 1 pmol of [^{3}H]acetyl group from [^{3}H]acetyl coenzyme A to the peptide substrate in the assay.

Comparisons of Protein Sequences—Protein sequences were searched and compared with a number of computer programs, including FASTA, BLAST (34, 35), BESTFIT (36), and PILE-UP (37).

TABLE I
Yeast strains

Abbreviation	Strain no.	Genotype	Phenotypes
	A364A	*MAT***a** *ade1 ade2 ura1 his7 gal1 lys2*	Nat^+Ts^+
	A364A-115	*nat2-1 MAT***a** *ade1 ade2 ura1 his7 gal1 lys2*	Nat^-Ts^-
NAT2$^+$	A364A-65[a]	*MAT***a** *ade1 ade2 ura1 his7 gal1 lys2*	Nat^+Ts^-
nat2-1	B-8861	*MAT***a** *nat2-1 ura3-52 his7 tyr1*	Nat^-Ts^-
nat2-1 p(*NAT2*$^+$)$_n$	B-8862	*MAT***a** *nat2-1 ura3-52 his7 tyr1* pAB995	Nat^+Ts^+
nat2-1 (FOA)[b]	B-8908	*MAT***a** *nat2-1 ura3-52 his7 tyr1*	Nat^-Ts^-
nat2-1 p(*NAT2*$^+$)$_1$	B-8879	*MAT***a** *nat2-1 ura3-52 his7 tyr1* pAB991	
	B-7457	*MAT***a** *cyc1-1 ura3-52 trp1-1 his3*	
	D-2125	B-7457 × A364A-115	
	B-7889	*MAT*α *cyc1-31 cyc7-67 can1-100 ilv3 leu2-3 leu2-112 ura3-52*	
	B-7723	*MAT***a** *cyc1-872 cyc7-67 ura3-52 lys5-10*	
	D-2126	B-7723 × B-7889	

[a] Contains a undefined heat-sensitive mutation.
[b] FOA, 5-fluoroortic acid.

TABLE II
Plasmids

Plasmid *no.*	Pertinent yeast genes	Origin	Origin or function
pAB1027	*NAT2*$^+$ *URA3*	YCp50	Original plasmid from B-8861
pAB991	*NAT2*$^+$ *URA3*	YCp50	Single copy, complementation, sequencing
pAB992	*NAT2*$^+$ *URA3*	pAB625	Single copy, complementation
pAB993	*nat2-Δ URA3*	pAB288	Disruption
pAB994	*NAT2*$^+$ *URA3*	pAB621	Sequencing
pAB995	*NAT2*$^+$ *URA3*	YEp355R	Overproduction

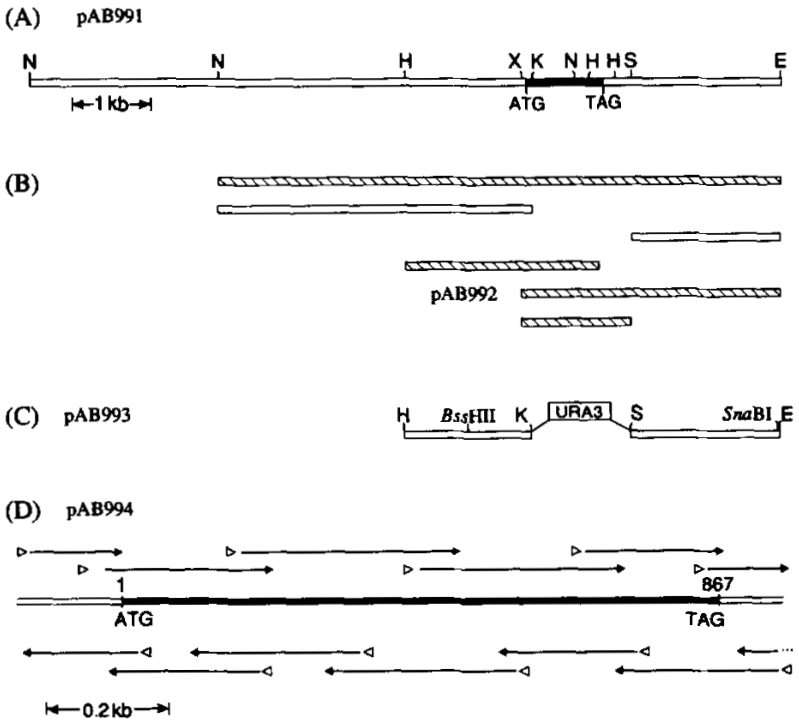

FIG. 1. **The physical structures of *NAT2* and adjacent regions, and the sequencing strategy of the *NAT2* gene.** *A*, the 8.4-kb fragment in pAB991, showing the *NAT2* gene (*filled-in bar*) and the following restriction endonuclease sites: *N*, *Nru*I; *H*, *Hin*dIII; *X*, *Xba*I; *K*, *Kpn*I; *S*, *Sac*II; *E*, *Eco*RI. *B*, localization of *NAT2* gene by complementation. Different size yeast DNA fragments from pAB991 were transferred to YCp50 and tested for complementation of *nat2-1*. The *bars* with *cross-hatched lines* and the *open bars* represent the fragments, respectively, which complemented and did not complement the *nat2-1* mutation. pAB992 contains the 2.9-kb *Eco*RI-*Xba*I fragment of yeast DNA encompassing the *NAT2* gene. Below is the smallest size fragment containing *NAT2*, as deduced from the complementation analysis. *C*, the *Hin*dIII-*Eco*RI fragment used for disrupting the *NAT2*. Most of the *NAT2* open reading frame of this fragment was replaced with the yeast *URA3* gene. *D*, DNA sequence strategy of the *NAT2* gene. The extent and direction of sequencing are shown by the length and direction of each *arrow*. The *open triangles* indicate the custom-made oligonucleotide primers. The position of the longest open reading frame is indicated by the *thick line*.

TABLE III

Synthetic peptides and a protein used for substrates

Abbreviation	Sequence	Length (amino acids)	Description
Met-Asn-Asn-	MNNLAGSAKKGATLFKTRSLQSHT	24	*CYC1-345-H* terminal region
Ser-Glu-Phe-	SEFLAGSAKKGATLFKTRSLQSHT	24	*CYC1-793* terminal region
Ser-Tyr-Ser-	SYSMEHFRWGKPVGKKRRPVKVYP	24	Adenocorticotrophic hormone
Met-Glu-Arg-	MERYE . . .	268	Tryptophan synthetase subunit α-chain

TABLE IV

N^a-acetylation of synthetic peptides and a protein by crude extracts from NAT2$^+$ and nat2-1 strains

Peptide or protein[a]	Activity[b]	
	NAT2$^+$ (A364A-65)	*nat2-1* (B-8861)
Met-Asn-Asn-	18	0.6
Ser-Glu-Phe-	13	8
Ser-Tyr-Ser-	11	9
Met-Glu-Arg-	12	0.5

[a] See Table III.

[b] Activity units: pmol/mg total protein; 1 unit is defined as the amount of enzyme able to transfer 1 pmol of [^{3}H]acetyl group from [^{3}H]acetyl coenzyme A to the substrate in the assay (33).

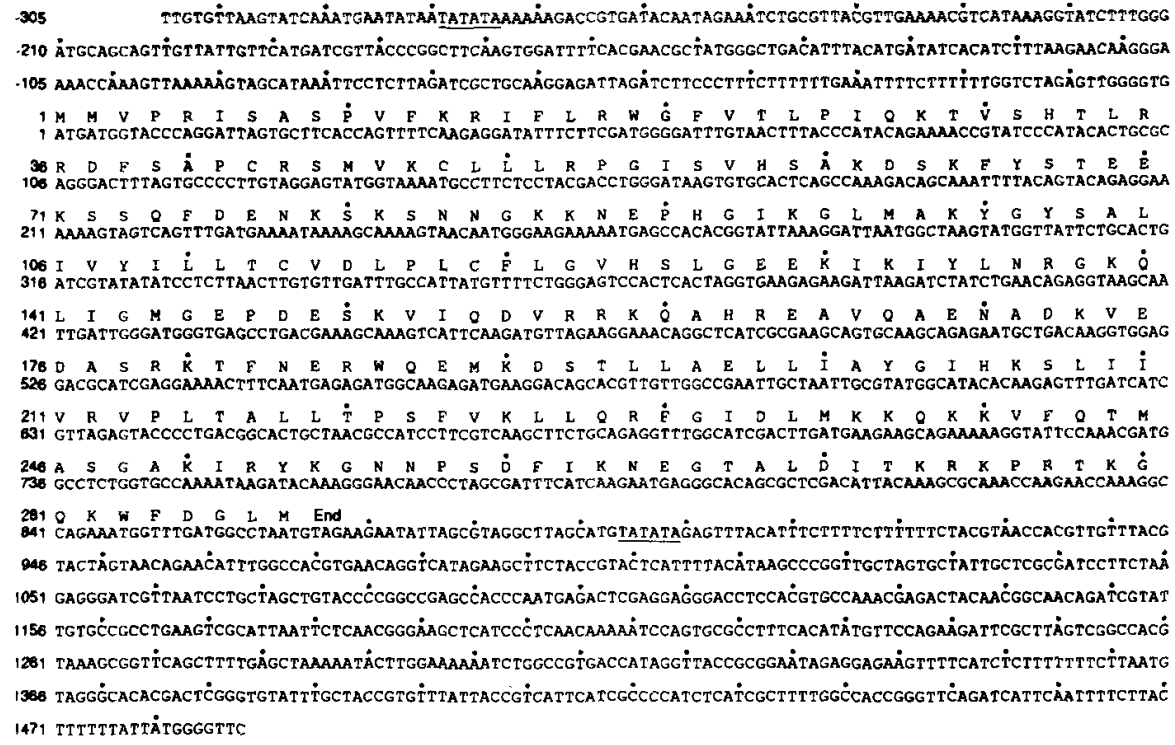

FIG. 2. **Nucleotide sequence of *NAT2* gene, the deduced amino acid sequence, and the 5′- and 3′-flanking regions.** The A of the ATG initiator codon and the initiator methionine residue have been assigned position 1. A putative TATA element, observed at nucleotide position −277, and a putative TATATA 3′ end-forming signal (39), observed at nucleotide position 897, are *underlined*.

TABLE V

N^{α}-acetylation of synthetic peptides by crude extracts from various strains

Strain no.	Pertinent genotype	Phenotypes	N-terminal sequence[a]	
			Met-Asn-Asn-	Ser-Tyr-Ser-
B-8861	*nat2-1*	Nat^- Ts^-	0.6	9
B-8879	*nat2-1* p($NAT2^+$)$_1$	Nat^+ Ts^+	14	
B-8908	*nat2-1* (FOA)[b]	Nat^- Ts^-	0.8	
B-8934	*nat2-1* p(YCp50)	Nat^- Ts^-	1.2	
B-8862	*nat2-1* p($NAT2^+$)$_n$	Nat^+ Ts^+	21	7
B-8933	$NAT2^+$	Nat^+ Ts^+	18	
B-8935	$NAT2^+$ p($NAT2^+$)$_n$	Nat^+ Ts^+	18	

[a] Activity units: pmol/mg total protein; 1 unit is defined as the amount of enzyme able to transfer 1 pmol of [^{3}H]acetyl group from [^{3}H]acetyl coenzyme A to the substrate in the assay (33).

[b] FOA, 5-fluoroortic acid.

TABLE VI

Percent identities between various N^{α}-acetyltransferases from yeast and E. coli

The percent identities were determined with the BESTFIT program (36).

	Nat1	Ard1	Nat2	Mak3	CAT
Nat1	100				
Ard1	18	100			
Nat2	20	16	100		
Mak3	19	30	23	100	
CAT	17	17	16	22	100
Random	12	12	13	8	14