

Properties of Nat4, an N^α -Acetyltransferase of *Saccharomyces cerevisiae* That Modifies N Termini of Histones H2A and H4[∇]

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Nat4, also designated NatD, was previously shown to acetylate the N termini of histones H2A and H4, which have SGGKG and SGRGK N termini (O. K. Song, X. Wang, J. H. Waterborg, and R. Sternglanz, J. Biol. Chem. 278:38109–38112, 2003). The analysis of chimeric proteins with various N-terminal segments of histone H4 fused to iso-1-cytochrome *c* revealed that efficient acetylation by NatD required at least 30 to 50 amino acid residues of the N terminus of histone H4. This requirement for an extended N terminus is in marked contrast with the major N-terminal acetyl transferases (NATs), i.e., NatA, NatB, and NatC, which require as few as two specific residues and usually no more than four or five. However, similar to the other NATs, NatD is associated with ribosomes. The *nat4-Δ* strain showed several minor phenotypes, including sensitivity to 3-aminotriazole, benomyl, and thiabendazole. Moreover, these *nat4-Δ* phenotypes were enhanced in the strain containing K5R K8R K12R replacements in the N-tail of histone H4, suggesting that the lack of N-terminal serine acetylation is synergistic to the lack of acetylation of the H4 N-tail lysines. Thus, N-terminal serine acetylation of histone H4 may be a part of an essential charge patch first described for the histone H2A.Z variant in *Tetrahymena* species.

N-terminal acetylation is one of the most common protein modifications in eukaryotes, occurring on approximately 50% of yeast soluble proteins and about 80 to 90% of mammalian proteins (10, 13, 18). Eukaryotic cytosolic proteins initiate with methionine, which is cleaved from nascent chains of most proteins. Subsequently, N-terminal acetylation occurs on certain proteins either containing or lacking the methionine residue (10, 13, 18, 25, 27). Proteins susceptible to N-terminal acetylation have a variety of different N-terminal sequences, with no simple consensus motifs and with no dependence on a single type of residue (25, 27). Earlier workers attempted to determine the N-acetylation rules, and some distinguishing features were found in the sequences, mainly between residues 1 and 10 and fewer features between residues 16 and 24 and residues 30 and 40, but the precise nature of these features was not determined (2). Proteins with serine and alanine termini are the most frequently acetylated, and these residues, along with methionine, glycine, and threonine, account for over 95% of the N-terminal acetylated residues. However, proteins having these amino acids at their N termini are not always acetylated and none of these N-terminal residues guarantees acetylation, indicating that the enzymes recognize some structural characteristics of the N-terminal portion in addition to a particular amino acid at the N terminus (25).

N-terminal acetylation of proteins is catalyzed by N-terminal acetyltransferases (NATs) that transfer acetyl groups from acetyl coenzyme A to termini of α -amino groups. We have established that *Saccharomyces cerevisiae* contains the three major NATs, NatA, NatB, and NatC, with each having differ-

ent catalytic subunits, Ard1, Nat3, and Mak3, respectively, and with each acting on a different group of proteins (Table 1) (28, 29). As previously summarized (21, 24, 25), subclasses of proteins with Ser, Ala, Gly, or Thr termini are acetylated by NatA; proteins with Met-Glu or Met-Asp termini and subclasses of proteins with Met-Asn and Met-Met termini are acetylated by NatB; subclasses of proteins with Met-Ile, Met-Leu, Met-Trp, or Met-Phe termini are acetylated by NatC.

NatA activity requires two subunits, the catalytic subunit Ard1p and the auxiliary subunit Nat1 (22). *nat1* and *ard1* mutants were unable to N-terminally acetylate *in vivo* the same subset of 24 normally acetylated proteins, including those with Ala and Ser termini (1). In addition to lacking NAT activity, both *nat1-Δ* and *ard1-Δ* mutants exhibited slower growth, derepression of the silent mating-type gene *HMLα*, and failure to enter Go, due in part to the lack of acetylation of Sir3 and Orc1 (15, 45). Overexpression of both Ard1 and Nat1 subunits are required for increased NAT activity *in vivo* (22), and both interact with each other to form an active complex *in vitro* (23). A putative N-terminal acetyltransferase, Nat5, homologous to catalytic subunits Ard1, Nat3, and Mak3, was shown to copurify with NatA at about a 1:1:1 ratio (14). Although the substrates of Nat5 have not been identified, the *nat5-Δ* strain produces a phenotype that is distinct from those found in NatA, NatB, NatC, and NatD mutants (see below), and Nat5 is not required for NatA activity, suggesting that Nat5 forms a distinct NAT, which we have denoted NatE (Table 1).

NatB acetyltransferase is composed of at least two subunits, the catalytic subunit Nat3 (29) and the auxiliary subunit Mdm20 (16), which also is required for acetylation of the NatB substrates (30, 38). The corresponding deletion mutants have similar phenotypes, including slow growth, temperature and osmotic sensitivity, calcium and caffeine sensitivity, deficiency in utilization of nonfermentable carbon sources, reduced mat-

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TABLE 1. The five types of yeast N-terminal acetyltransferases

NAT type	Catalytic subunit	Auxiliary subunit(s)	Substrates ^c	No. of substrates
NatA	Ard1 ^a	Nat1 ^{a,b}	Ser, Ala, Gly, Thy	>2,000
NatB	Nat3 ^a	Mdm20 ^a	Met-Glu, Met-Asp, Met-Asn, Met-Met	>600
NatC	Mak3 ^a	Mak10, ^a Mak31	Met-Ile, Met-Leu, Met-Trp, Met-Phe	Low
NatD	Nat4 ^a		Ser-Gly-Gly-Lys, Ser-Gly-Arg-Gly	At least 2
NatE	Nat5 ^a	Nat1 ^{a,b}	Unknown	Unknown

^a Subunits that were shown to be associated with ribosomes.

^b We suggest that the auxiliary subunit Nat1p is common to both NatA and NatE and that *nat1*-Δ mutants are deficient in both acetyltransferases.

^c Acetylation occurs only on subclasses of proteins containing the indicated termini, except for Met-Glu and Met-Asp termini, which are apparently always acetylated.

ing efficiency, sensitivity to antimetabolic drugs, and susceptibility to DNA-damaging agents (30).

NatC contains three subunits, Mak3, Mak10, and Mak31 (34). Tercero et al. (41) described the *MAK3* gene, which encodes the catalytic subunit of NatC and is required for the N-terminal acetylation of the viral major coat protein, *gag*, with an Ac-Met-Leu-Arg-Phe terminus (42). We demonstrated that each of the Mak3, Mak10, and Mak31 subunits are required for acetylation of the NatC-type sequences in vivo (26). In addition, all three deletion strains had similar phenotypes, including slower growth on nonfermentable carbon sources at elevated temperature.

Recently, an additional NAT, Nat4 (NatD), was shown to acetylate the N termini of highly conserved histones H2A and H4, which have Ser-Gly-Gly-Lys-Gly and Ser-Gly-Arg-Gly-Lys N termini, respectively (39). Song et al. (39) also demonstrated that Nat4 acetylates in vitro a 23-amino-acid-long synthetic peptide corresponding to the N terminus of histone H4, but not an H3 peptide, which is not normally acetylated, nor an adrenocorticotropin peptide, which is an NatA substrate. However no obvious *nat4*-Δ phenotype was reported (39). So far, there is no evidence that Nat4 acetylates any other yeast protein, as judged by two-dimensional (2D) gel analysis of the soluble proteins prepared from normal and *nat4*-Δ mutant strains (39). We have designated this NAT activity as NatD, to be consistent with our established nomenclature.

In this work we investigated the NatD requirements for N-terminal acetylation and uncovered *nat4*-Δ phenotypes. Surprisingly, NatD differs from the other NATs in its requirement for an extended N-terminal region for efficient acetylation. Mutant forms of iso-1-cytochrome *c* with extensions with as many as 23 amino acid residues corresponding to the N terminus of H4 histone were only partially acetylated by Nat4. In contrast, only five or fewer N-terminal amino acid replacements were necessary to acetylate mutant forms of iso-1-cytochrome *c* (iso-1) by NatA, NatB, or NatC. For example, iso-1 with eight N-terminal amino acids corresponding to the N-terminal region of H2B histone was sufficient for acetylation by NatA (29). In addition, we have investigated the site of action of NatD in the cell and demonstrated that Nat4 is a cytoplasmic protein that colocalizes with mono- and polyribosomes in a sucrose density gradient. Also, we have uncovered *nat4*-Δ phenotypes by using a variety of special media, and these phenotypes include sensitivity to 3-aminotriazol (3-AT), benomyl, salt, and thiabendazole (TBZ). Interestingly, *nat4*-Δ showed a synthetic growth defect in the strain with an altered histone H4 protein having K5R K8R K12R replacements and

therefore lacking acetylated lysine residues in the N-terminal region.

MATERIALS AND METHODS

Yeast strains. The strains of *S. cerevisiae* used in this study are listed in Table 2. The analysis of N-terminal acetylation was carried out with several isogenic series which were derived from the parental strain B-7528 (*MATα cycl-31 cyc7-67 ura3-52 lys5-10*) (29). The following parental strains were used for genetic manipulations and protein expression: B-11679 (*MATα ura3-52*); B-10190 (*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52*); B-14276 (*MATα his3-Δ0 leu2-Δ0 lys2-Δ0 ura3-Δ0*). Normal and histone H4 mutant strains were obtained from M. Grunstein (University of California at Los Angeles) and were isogenic to UKY412 (*MATα ade2-101 his3-201 leu2-3,112 lys2-801 trp1-901 ura3-52 hhf1::HIS3 hhf2::LEU2/pUK499 [URA3 CEN4 ARS1 HHF2]*). PKY501 contains *HHF2*, one of the two wild-type genes encoding H4; PKY821 contains the mutant allele *hhf2-100*, which encodes H4 with K5R, K8R, and K12R replacements, whereas LJY305 contains the mutant allele *hhf2-101*, which encodes H4 with a K16G replacement (17). B-7928 (*MATα ade5 cyh2 leu1 lys5 met15 trp5*) was used as a tester strain in mating experiments with the histone H4 strain series.

Media. Standard media, yeast extract-peptone-dextrose (YPD), YP-glycerol (YPG), yeast extract-0.2% dextrose-3% glycerol (YPDG), yeast extract-peptone-ethanol (YPE) and synthetic dextrose (SD) containing appropriate supplements have been described elsewhere (36). Unless stated otherwise, yeast strains were grown at 30°C. Certain phenotypes of the *nat4*-Δ strains were determined with YPD medium containing the following amounts of different agents: 1 M potassium chloride (KCl), 1 M sodium chloride (NaCl), 0.3 M calcium chloride (CaCl₂), 0.15% caffeine, 10 to 30 μg/ml benomyl, 50 to 75 μg/ml TBZ, 250 mM dinitrobenzene (DNB), 6% diethyleneglycol, 0.02 to 0.1% methyl methanesulfonate. Other phenotypes were determined with synthetic complete medium containing the following: 50 to 75 mM 3-AT, 75 or 100 mM hydroxyurea, and 4 or 10 μg/ml camptothecin in medium containing 0.25% dimethyl sulfoxide.

Determining strain mating efficiencies. The frequencies of mating were quantitatively determined by plating serial dilutions of logarithmically growing yeast cells onto SD plates containing a lawn of tester mating strain B-7928 and determining the frequencies of prototrophic diploid colonies arising after incubation for 3 days. Dilutions of the haploid strains were also plated on YPD plates and on various omission synthetic media to determine total numbers of cells for both mating strains. Mating efficiencies were expressed as the number of diploid colonies divided by the number of haploid cells plated on the lawn of the tester strain.

Construction of deletion mutants. Standard molecular biological procedures were performed as described previously (29). The *NAT4* gene was disrupted by replacing the segment of the gene corresponding to the open reading frame with the *kanMX4* gene produced by PCR and then using the appropriate fragment for yeast transformation (29). For the *nat4*-Δ::*kanMX4* disruption in various strains, a pair of primers, Oligo1 and Oligo2 (Table 3), was used to prepare the PCR fragment for transformation with yeast genomic DNA made from a *nat4*-Δ::*kanMX4* strain, B-13859 (Open Biosystems, Huntsville, AL), as a template. A correct disruption among the transformants was identified by PCR, using the same set of primers.

Construction of mutationally altered *CYC1* and *HHF2* alleles. We used the method of direct transformation with synthetic oligonucleotides to construct the yeast strains containing mutationally altered *CYC1* alleles (47). Strain B-7528, which contains the *cycl-31* mutation (21), was transformed with Oligo3 and Oligo4; functional transformants were selected on YPDG plates, and the sequences of desired *CYC1* alleles were confirmed by DNA sequencing of the

TABLE 2. Yeast strains used in this study

Strain	Genotype	Synonym	Reference or source
B-10190	<i>MATa his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52</i>		This study
B-14070	<i>MATa his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 nat4-Δ::kanMX4</i>		This study
B-14381	<i>MATa CYC1-1390 cyc7-67 lys5-10 ura3-52</i>		This study
B-14382	<i>MATa CYC1-1391 cyc7-67 lys5-10 ura3-52</i>		This study
B-14449	<i>MATa CYC1-1390 cyc7-67 lys5-10 ura3-52 nat4-Δ::kanMX4</i>		This study
B-14450	<i>MATa CYC1-1391 cyc7-67 lys5-10 ura3-52 nat4-Δ::kanMX4</i>		This study
B-14446	<i>MATa CYC1-1390 cyc7-67 lys5-10 ura3-52 ard1-Δ::kanMX4</i>		This study
B-14447	<i>MATa CYC1-1391 cyc7-67 lys5-10 ura3-52 ard1-Δ::kanMX4</i>		This study
B-15195	<i>MATa CYC1-1390 cyc7-67 lys5-10 ura3-52 NAT4::3xHA</i>		This study
B-15196	<i>MATa CYC1-1391 cyc7-67 lys5-10 ura3-52 NAT4::3xHA</i>		This study
B-15274	<i>MATa CYC1-1390 cyc7-67 lys5-10 ura3-52 nat4-Δ::kanMX4 p[2μm URA3 NAT4-3xHA]</i>		This study
B-15279	<i>MATa CYC1-1390 cyc7-67 lys5-10 ura3-52 nat4-Δ::kanMX4 p[CEN URA3 NAT4-3xHA]</i>		This study
B-14276	<i>MATα his3-Δ leu2-Δ lys2-Δ ura3-Δ</i>		Open Biosystems
B-13859	<i>MATα his3-Δ leu2-Δ lys2-Δ ura3-Δ nat4-Δ::kanMX4</i>		This study
B-15329	<i>MATα his3-Δ leu2-Δ lys2-Δ ura3-Δ NAT4 -TAP::HIS3</i>		Open Biosystems
B-11679	<i>MATa ura3-52</i>		29
B-14324	<i>MATa ura3-52 nat4-Δ::kanMX4</i>		This study
B-7528	<i>MATa cyc1-31 cyc7-67 lys5-10 ura3-52</i>		29
B-15341	<i>MATa cyc1-31 cyc7-67 lys5-10 ura3-52 p[2μm URA3 CYC1-1398] (8-aa H4 iso-1)</i>		This study
B-15342	<i>MATa cyc1-31 cyc7-67 lys5-10 ura3-52 p[2μm URA3 CYC1-1399] (23-aa H4 iso-1)</i>		This study
B-15343	<i>MATa cyc1-31 cyc7-67 lys5-10 ura3-52 p[2μm URA3 CYC1-1400] (52-aa H4 iso-1)</i>		This study
B-15196	<i>MATa cyc1-31 cyc7-67 lys5-10 ura3-52 nat4-Δ::kanMX4</i>		This study
B-15367	<i>MATa cyc1-31 cyc7-67 lys5-10 ura3-52 nat4-Δ::kanMX4 p[2μm URA3 CYC1-1398] (8-aa H4 iso-1)</i>		This study
B-15368	<i>MATa cyc1-31 cyc7-67 lys5-10 ura3-52 nat4-Δ::kanMX4 p[2μm URA3 CYC1-1399] (23-aa H4 iso-1)</i>		This study
B-15369	<i>MATa cyc1-31 cyc7-67 lys5-10 ura3-52 nat4-Δ::kanMX4 p[2μm URA3 CYC1-1400] (52-aa H4 iso-1)</i>		This study
B-7928	<i>MATα ade5 cyh2 leu1 lys5 met15 trp5</i>		This study
B-15949	<i>MATa ade2-101 his3-201 leu2-3,112 lys2-801 trp1-901 ura3-52 hhf1::HIS3 hhf2::LEU2 pUK499 p[URA3 CEN4 ARS1 HHF2]^a</i>	PKY501	17
B-15952	<i>MATa ade2-101 his3-201 leu2-3,112 lys2-801 trp1-901 ura3-52 hhf1::HIS3 hhf2::LEU2 pUK499 p[URA3 CEN4 ARS1 hhf2-100]</i>	PKY821	17
B-15953	<i>MATa ade2-101 his3-201 leu2-3,112 lys2-801 trp1-901 ura3-52 hhf1::HIS3 hhf2::LEU2 pUK499 p[URA3 CEN4 ARS1 hhf2-101]</i>	LJY305	17
B-15954	<i>MATa ade2-101 his3-201 leu2-3,112 lys2-801 trp1-901 ura3-52 hhf1::HIS3 hhf2::LEU2/pUK499 p[URA3 CEN4 ARS1 hhf2-100] nat4-Δ::kanMX4</i>		This study
B-15955	<i>MATa ade2-101 his3-201 leu2-3,112 lys2-801 trp1-901 ura3-52 hhf1::HIS3 hhf2::LEU2 pUK499 p[URA3 CEN4 ARS1 HHF2] nat4-Δ::kanMX4</i>		This study
B-16142	<i>MATa ade2-101 his3-201 leu2-3,112 lys2-801 trp1-901 ura3-52 hhf1::HIS3 hhf2::LEU2/pUK499 p[URA3 CEN4 ARS1 HHF2] pAB3435 p[TRP1 CEN6 ARS4 hhf2-102]</i>		This study
B-16143	<i>MATa ade2-101 his3-201 leu2-3,112 lys2-801 trp1-901 ura3-52 hhf1::HIS3 hhf2::LEU2/pAB3435 p[TRP1 CEN6 ARS4 hhf2-102]</i>		This study
B-16163	<i>MATa ade2-101 his3-201 leu2-3,112 lys2-801 trp1-901 ura3-52 hhf1::HIS3 hhf2::LEU2 nat4-Δ::kanMX4 pAB3435 p[TRP1 CEN6 ARS4 hhf2-102]</i>		This study
B-16144	<i>MATa cyc1-31 cyc7-67 lys5-10 ura3-52 p[2μm URA3 CYC1-1402]</i>		This study
B-16145	<i>MATa cyc1-31 cyc7-67 lys5-10 ura3-52 p[2μm URA3 CYC1-1402] nat4-Δ::kanMX4</i>		This study

^a The alleles *hhf2-100*, *hhf2-101*, and *hhf2-102* encode altered forms of histone H4 with R5K R8K R12K replacements, a G16K replacement (17), and a V1S replacement, respectively.

appropriate PCR product. The resulting strains B-14381 and B-14382 contained the alleles *CYC1-1390* and *CYC1-1391* encoding the iso-1 proteins with eight N-terminal residues corresponding to the N termini of histones H2A (SGGKG GKA) and H2B (SAKAEKKP), respectively.

The strains containing *CYC1* alleles that encoded the chimeric iso-1 proteins with extended N termini for 8, 23, or 52 amino acid residues corresponding to the N terminus of histone H4 were made with DNA fragments amplified by PCR, using the normal yeast genomic DNA. The following oligonucleotides were used to prepare DNA fragments from the *HHF1* gene, which encodes histone H4, and in which the open reading frame ATG initiation codon is assigned position +1: (i) Oligo5 and Oligo6, resulting in a fragment from nucleotide (nt) -167 to +24 and an 8-amino-acid extension; (ii) Oligo5 and Oligo7, resulting in a fragment from nt -167 to +69 and a 23-amino-acid extension; (iii) Oligo5 and Oligo8, resulting in a fragment from nt -167 to +156 and a 52-amino-acid extension (Table 2). Note that Oligo5 was

designed to introduce a BglII restriction site and Oligo6, Oligo7, and Oligo8 were designed to have EcoRI restriction sites. Also, EcoRI was introduced into Oligo6, Oligo7, and Oligo8 such that the *HHF1* DNA fragment would be in frame with the *CYC1* gene. Subsequently, all three PCR products were cut with BglII and EcoRI and each fragment was inserted into BamHI- and EcoRI-digested plasmid pAB458 (12) containing the entire native *CYC1* gene. The resulting plasmids, pAB3065, pAB3066, and pAB3067, contained the chimeric genes that encoded iso-1 with extensions of 8, 23, and 52 amino acid residues, respectively, of the H4 N terminus, under regulation of the *HHF1* promoter. The three genes encoding iso-1 with the extensions were designated, respectively, *CYC1-1398* (8-amino-acid extension), *CYC1-1399* (23-amino-acid extension), and *CYC1-1400* (52-amino-acid extension). Strains B-7528 (*cyc1-31*) and B-15196 (*cyc1-31 nat4-Δ*) were transformed with the three plasmids, resulting in the following two sets of strains, respectively (Table 2): B-15341, B-15342, and B-15343, and B-15367, B-15368, and B-15369.

TABLE 3. Oligonucleotides used in construction and testing for the genes

ORF	Oligonucleotide	Sequence (5'→3') ^a
<i>NAT4</i>	Oligo1	(nt -92) CCTTTTTTCCACATATGTAATGCG
<i>NAT4</i>	Oligo2	(nt +954) GGTCTCCTCAGTCGGAGTCAAG
<i>CYC1</i>	Oligo3	(nt -17) ACACACTAAATTAATAATGTCTGGAGGAAAGGCGGTAAAGCTAAGAAAGGTGCTACACTTTTC
<i>CYC1</i>	Oligo4	(nt -17) ACACACTAAATTAATAATGTCTGCAAAAGCGGAGAAA AAACCT AAGAAAGGTGCTACACTTTTCAAG
<i>HHF1</i>	Oligo5	(nt -168) CATTGGGTAAAGATCTATTTGATGGATAAAATGGTTG
<i>HHF1</i>	Oligo6	(nt +41) CCTTTACCGAATTCCTTACCACCTTTACCTCTACC
<i>HHF1</i>	Oligo7	(nt +86) CCTTGGATGAATTCCTTAGAATCAACCTTTCTGTGACG
<i>HHF1</i>	Oligo8	(nt +170) GCTCTGACGAATTCGTAGATCAAACCCAGAAATACG
<i>NAT4</i>	Oligo9	(nt +793) GGTGGGGGACGTGTAGTCGTGCCCTCGATCCGCTTTAT TACGTATATAGGGAACAAAAGCTGG
<i>NAT4</i>	Oligo10	(nt +887) TTTTATCGCGCGTTGTCCCTGTCCGCTTTCACGGCATG TGAAGGCATATAGGGCGAATTGG
<i>NAT4</i>	Oligo11	(nt +602) CCAGCATACCCACAATTCCG
<i>HHF2</i>	Oligo12	(nt -7) GTAAAATATGTCCGCTGCAGCTGCAGCTGCTGCAGCTCTAGGTAAAGGT
<i>HHF2</i>	Oligo13	(nt +42) ACCTTACCTAGAGCTGCAGCTGCAGCTGCAGCTGCAGCGACATATTTTAC
<i>HHF2</i>	Oligo14	(nt -10) ATAGTAAAATATGGTCGGTAGAGGTAAA
<i>HHF2</i>	Oligo15	(nt +18) TTTACCTCTACCGACCATATTTACTAT

^a The position of the first nucleotide is presented, where A of the ORF ATG initiation codon is assigned position 1. The underlined sequences are homologous to the following regions: in Oligo3 and Oligo4, they correspond to the altered *CYC1* alleles, *CYC1-1390* and *CYC1-1391*, encoding 8-amino-acid residues of histones H2A and H2B, respectively; in Oligo5, it introduces a BglII site in the sequence of the *HHF1* gene (histone H4); in Oligo6, Oligo7, and Oligo8 they introduce an EcoRI site into *HHF1*; in Oligo9 and Oligo10, they correspond to the template plasmid pMPY3 ξ HA (pAB1868); in Oligo12 and Oligo13, they correspond to the altered *CYC1-1402* allele, encoding chimeric iso-1 with a 52-amino-acid extension of the H4 histone but in which eight N-terminal residues were changed to alanines; in Oligo14 and Oligo15, they replace the N-terminal serine with valine in histone H4. Oligo11 was used for sequencing of the *NAT4* fusion genes.

As stated above, *CYC1-1400* encodes iso-1 with a 52-amino-acid N-terminal extension of H4, which has an N-terminal sequence of SGRGKGGK. *CYC1-1400* was further altered by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), resulting in *CYC1-1402*, which contained SAAAAAAA at the N terminus instead of SGRGKGGK. *CYC1-1402* (plasmid pAB3437) was made using the *CYC1-1400* allele (plasmid pAB3067) as a template and Oligo12 and Oligo13 (Table 3). Subsequently, strain B-7528 was transformed with pAB3437, which contained *CYC1-1402*, resulting in strain B-16144 (Table 2). B-16145 was obtained by deletion of *NAT4* in B-16144.

The QuikChange site-directed mutagenesis kit also was used to make a mutant histone H4 in which the N-terminal serine was replaced with valine; this altered form, denoted *hhf2-102*, was constructed by PCR with Oligo14 and Oligo15 (Table 3) and with the plasmid pUK499 as a template, which was recovered from the strain B-15949. The resulting plasmid, pAB3435, containing *hhf2-102*, was transformed into strain B-15949 (Table 2) to produce B-16142; subsequently, the pUK499 plasmid containing the wild-type *HHF2* was removed by plasmid shuffle, via selection on 5-fluoroorotic acid, resulting in strain B-16143, which was compared to *nat4*- Δ strains in 1/10 serial dilution experiments.

Construction of a *NAT4-3xHA* epitope-tagged strain. The *NAT4-3xHA* epitope-tagged strain was made by using PCR-based techniques as described earlier (35). Oligonucleotides Oligo9 and Oligo10 (Table 3) were designed to generate a PCR product to make the *NAT4* gene in frame with the 3xHA epitope; plasmid pMPYx3HA (pAB1868) was used as a template in the PCR. After transformation with the PCR product, transformants were screened for correct insertion; subsequently, a correct transformant was plated on 5-fluoroorotic acid medium to evict the *URA3* marker gene. The *NAT4* locus of the resulting strain, B-15195, was checked by sequencing with Oligo11 to confirm in frame the 3xHA fusion. In addition, the *NAT4-3xHA* gene was inserted into a multicopy plasmid to produce B-15275 (Table 2), which had enhanced protein expression for better detection by Western blotting.

The *NAT4-TAP* strain, B-15329, was obtained from Open Biosystems.

Biochemical fractionation for subcellular protein localization. The biochemical fractionation of mitochondria and other subcellular components was performed essentially as described by Zinser and Daum (48). Briefly, cells of the *NAT4-TAP* strain, B-15329 (Table 2), were grown to early log phase, collected, washed, and treated with Zymolyase. Spheroplasts were disrupted with a Dounce homogenizer (10 to 15 strokes) and the homogenate was centrifuged at 3,000 \times g for 5 min. A portion of the resulting pellet was used as the pellet fraction. The supernatant was spun at 9,000 \times g for 10 min and a portion of the resulting supernatant was loaded on a protein gel as the cytosolic fraction. The pellet was washed several times and a final precipitate was taken for protein analysis as the mitochondrial fraction.

Preparation and fractionation of polyribosomes. Yeast polyribosomes were fractionated by sucrose density gradient centrifugation essentially as described by Baim et al. (3). Briefly, various yeast strains were grown in YPD medium to an optical density at 600 nm (OD₆₀₀) of approximately 1.5. Cultures were incubated on ice for 5 min, harvested at 5,000 \times g for 5 min, and washed twice with a solution containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 30 mM MgCl₂.

MgCl₂ was omitted in all solutions in experiments when we tested the NAT subunit association with dissociated ribosomal particles. The cells were resuspended in a small volume of the same buffer and homogenized with glass beads by vortexing 10 times for 30 s with 30-s intervals on ice. Crude extracts were centrifuged at 10,000 \times g for 10 min and portions of supernatants corresponding to 90 OD₂₆₀ units were layered onto 11.0 ml of a 7 to 47% linear sucrose gradient containing 50 mM Tris-acetate (pH 7.0), 50 mM NH₄Cl, and 1 mM dithiothreitol (made with or without MgCl₂). The gradients were centrifuged at 41,000 \times g for 2 h at 5°C (SW-41 rotor; Beckman L7 ultracentrifuge), and the 500- μ l fractions were collected sequentially from the bottom (polyribosomal fractions) of the gradient to the top (cytosolic proteins). Protein levels in the fractions were estimated by measuring the OD₂₅₄.

Purification and characterization of the yeast Nat4p-TAP. The potential Nat4p protein complex was purified by the tandem affinity purification (TAP) method (34), using the *NAT4-TAP* strain B-15329, obtained from Open Biosystems (Table 2). The normal strain with no TAP tag, B-14276, served as a negative control. After a second affinity purification step, the eluted Nat4p-TAP was concentrated by speed vacuum and dry centrifugation and loaded onto a 4% to 20% gradient Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After gel electrophoresis, the protein bands were visualized by using PageBlue Coomassie staining (Fermentas, Vilnius, Lithuania) or the Silver Staining Plus kit (Bio-Rad, Hercules, CA).

Western immunoblotting. Portions of the samples from the polyribosome fractions, as well as from the total cell extract, were loaded on 4 to 20% Tris-glycine SDS-PAGE gels, and the separated proteins were transferred to enhanced chemiluminescence (ECL) nitrocellulose membranes (GE Healthcare/Amersham, Piscataway, NJ) with a standard running condition of 2 h at 75 V. Subsequently, the membranes were probed with the following antibodies: mouse anti-hemagglutinin (HA) monoclonal antibody (Neomarkers, Fremont, CA) for Nat4p-3xHA detection; rabbit anti-glucose-6-phosphate dehydrogenase (anti-G6PDH; Zwf1p; Sigma, St. Louis, MO); rabbit anti-Rpl3p (ribosomal protein L3, large subunit) and anti-Rps3p (ribosomal protein S3, small subunit) for polyribosomes and ribosomal subparticle detection (both gifts from Y.-H. Chang, Washington University, St. Louis, MO) (44); rabbit peroxidase-antiperoxidase (PAP) antibody (Sigma) to detect Nat4p-TAP. A rabbit polyclonal anti-iso-1-cytochrome *c* antibody was described previously (11). After Western blotting and incubation with various antibodies, the membranes were treated with ECL reagents (GE Healthcare) as recommended by the manufacturer.

Iso-1-cytochrome *c* content. The levels of iso-1-cytochromes *c* in yeast strains were estimated by low-temperature (-196°C) spectroscopic examinations of intact cells (37) and by comparing the intensities of the C _{α} bands at 547 nm to the corresponding bands of strains having known amounts of cytochrome *c*.

Purification of iso-1-cytochrome *c*. Iso-1 proteins were purified as previously described by using two subsequent rounds of weak cation exchange BioRex70 column chromatography, with 100/200 mesh and 200/400 mesh (Bio-Rad, Hercules, CA), respectively, in potassium phosphate buffer, pH 7.0, with a 0 to 1.0 M potassium chloride linear gradient (29). For mass spectrometry (MS) analysis the

iso-1 proteins were dialyzed against H₂O and, if necessary, concentrated in a vacuum speed-dry centrifuge.

Protein isoelectric focusing using the ZOOM IPGRunner system and Triton-acetic acid-urea gel separation. Isoelectric focusing of cell extracts or purified chimeric iso-1-cytochromes *c* was performed using the ZOOM IPGRunner system (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Approximately 50 µg of cell extract or 0.5 µg of each purified sample was used to rehydrate the immobilized pH gradient (IPG) strips (pH 9 to 11). After protein separation, the strips were equilibrated in a buffer containing 0.1 M sodium dodecyl sulfate, 0.05 M dithiothreitol, and 0.2 M Tris for 30 min and subsequently in transfer buffer (1× electrode buffer with 20% methanol) for 10 min. The plastic backing was removed from the strips, and the proteins were transferred to ECL membranes. Membranes were probed with anti-iso-1-cytochrome *c* antibody and treated as described above in "Western immunoblotting." Separation of histones using Triton-acetic acid-urea polyacrylamide gels was conducted as described at <http://www.koko.gov.my/CocoaBioTech/Protein%20Detection17.html>.

Mass spectrometry analysis and N-terminal protein sequencing. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) samples were analyzed at the MicroChemical Protein/Peptide Core (University of Rochester) and were prepared essentially as previously described (29) by mixing 1 part of a protein sample with 1 part of a sinapinic acid matrix at a concentration of 10 mg/ml in 30% acetonitrile and applying 1 µl of this mixture to the sample probe. Positive ion mass spectra were recorded on a Voyager-DE STR linear time-of-flight mass spectrometer (PE Biosystems, Framingham, MA) using 25 keV of total acceleration energy and a grid voltage of 93.5%. In addition, some MALDI-TOF spectra were obtained at the Mass Spectrometry Facility, Department of Chemistry, Louisiana State University (Baton Rouge).

N-terminal protein sequencing was performed by the Edman degradation procedure at the MicroChemical Protein/Peptide Core Facility, University of Rochester. Purified iso-1 proteins were separated by SDS-PAGE and transferred to a polyvinylidene membrane (Millipore, Bedford, MA), and amino acids were sequentially cleaved from the N terminus via Edman degradation (phenylthiocyanate reaction) and analyzed using an Applied Biosystems model 473A instrument.

RESULTS

Nat4p does not acetylate an altered iso-1-cytochrome *c* with short NatD type N termini. It was previously shown (39) that NatD acetylates the N termini of histones H2A and H4, which have SGGKG and SGRGK termini, respectively, and also acetylates the N terminus of a peptide corresponding to the N-terminal tail of H4. However, NatD neither acetylates the H3 peptide, which is normally not acetylated, nor the adrenocorticotropin peptide, which is a NatA substrate (39). Although there are certain proteins with similar N-terminal sequences, there is no evidence that Nat4p acetylates any other yeast protein as judged by 2D gel analysis of the soluble proteins prepared from normal and *nat4*-Δ mutant strains (39). Thus, NatD is different from the major NATs, NatA, NatB, and NatC, which acetylate a variety of certain N-terminal sequences (27, 29). Also, we previously showed that by altering just several amino terminal residues in iso-1, we were able efficiently convert a NAT-type substrate, for example, NatA to NatB or NatC (26, 29). To investigate the sequence requirements for NatD acetylation, we constructed a series of N-terminal altered iso-1-cytochrome *c* proteins with amino termini mimicking the H2A sequence, a potential NatD substrate, and the H2B sequence, a potential NatA substrate. By transforming the *cyc1-31* strain directly with synthetic oligonucleotides (21, 47), we produced altered *CYC1* alleles encoding iso-1 with various N-terminal sequences (oligonucleotide sequences are presented in Table 3). B-14381 contained *CYC1-1390*, which encodes iso-1 with an SGGKGGKA N terminus, a predicted NatD substrate; B-14382 contained *CYC1-1391*, which encodes iso-1 with an SAKAEKKP N terminus, a predicted

NatA substrate. In addition, we produced the two *ard1*-Δ and *nat4*-Δ deletions, which lacked, respectively, NatA and NatD activities, in both *CYC1-1390* and *CYC1-1391* backgrounds (Table 2). It should be noted that iso-1 proteins in the *CYC1-1390* and *CYC1-1391* strains were found to be functional and present at the normal level, as indicated, respectively, by growth on media containing nonfermentable carbon sources and by low-temperature spectroscopic examinations of intact cells (data not presented).

The iso-1 proteins were purified from both set of strains by ion exchange chromatography and then subjected to MALDI-TOF mass spectrometry. As shown in Fig. 1, the analysis of MS data revealed that the iso-1 proteins prepared from the isogenic normal *CYC1-1391* strain and the *CYC1-1391 nat4*-Δ mutant had molecular masses of 12,804 and 12,806 Da, respectively, values that were in close agreement with the calculated molecular mass of the acetylated Cyc1-1391 protein (12,805 Da). On the other hand, iso-1 from the *CYC1-1391 ard1*-Δ mutant had a molecular mass of 12,765 Da (Fig. 1). The reduced molecular mass of the protein from the *CYC1-1391 ard1*-Δ mutant was 40 Da lower, which closely corresponds to the mass of an acetyl group (42 Da), and indicated that the Cyc1-1391 protein was not acetylated in the *ard1*-Δ strain and that this Cyc1-1391 protein is a NatA substrate.

In contrast, the iso-1 proteins purified from the isogenic normal *CYC1-1390* strain and the corresponding *ard1*-Δ and *nat4*-Δ mutants all had very similar molecular masses of 12,563, 12,565, and 12,568 Da, respectively (Fig. 1), values that were all close to the calculated mass of the unacetylated Cyc1-1390 protein (12,566 Da), thus indicating that the altered iso-1 was not acetylated in any of the strains. To confirm that the N terminus of the Cyc1-1390 protein is not acetylated and that it accurately corresponds to the designed amino acid sequence, we subjected the protein purified from the normal strain, B-14381, to N-terminal Edman degradation. Protein microsequencing demonstrated that the Cyc1-1390 protein was not blocked, that the first 12 amino acid residues, SGGKGGKAK KGA, perfectly matched the expected N-terminal sequence encoded by designed Oligo3 (Table 3), and that the first eight residues (underlined) corresponded to the histone H2A amino terminus (primary microsequencing data not shown). Thus, an alteration of the eight N-terminal amino acid residues was not sufficient to effectively acetylate the Cyc1-1390 protein by NatD. A summary of the results, including the previous data of Song et al. (39), is presented in Table 4.

NatD requires a significantly longer N-terminal sequence than the major NATs to acetylate a proper substrate. In order to determine the Nat4p sequence requirements for acetylation, we designed a series of chimeric iso-1 proteins containing 8, 23, and 51 residues of the H4 N-terminal region. The corresponding proteins, Cyc1-1398, Cyc1-1399, and Cyc1-1400, were expressed using *CEN* plasmid vector and a native *HHF1* (histone H4) promoter. The complete technical procedure for this approach is described in Materials and Methods, the oligonucleotide sequences for making the constructs are provided in Table 3, and the resulting strains, B-15431, B-15432, and B-15433, containing the *CYC1* alleles *CYC1-1398*, *CYC1-1399*, and *CYC1-1400*, respectively, are listed in Table 2. It should be noted that all three strains expressed the functional cytochrome *c* and all grew on the media with nonfermentable

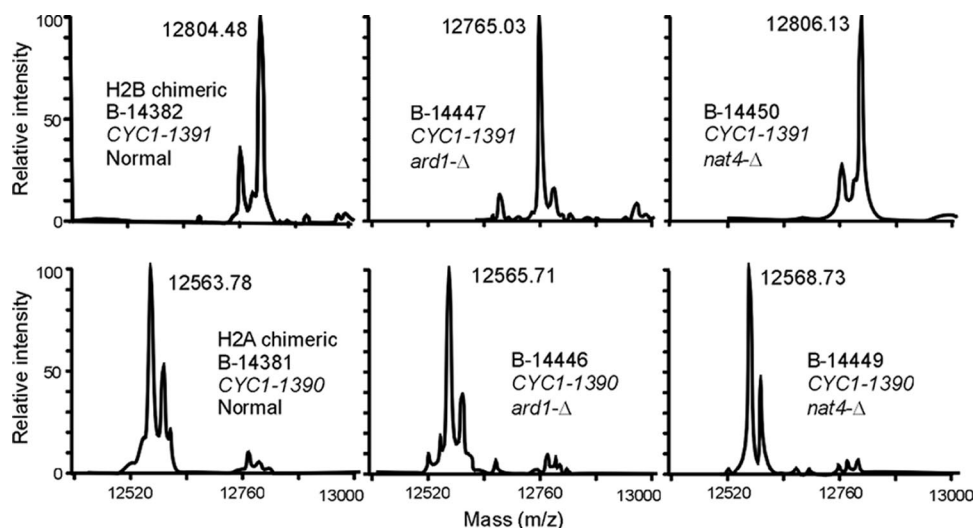


FIG. 1. NatA acetylates an altered Cyc1-1391, a designed NatA substrate, while NatD does not acetylate an altered Cyc1-1390, a potential NatD substrate. MALDI-TOF mass spectra are from the altered iso-1 proteins prepared from the following strains: top, the *CYC1-1391* strain series having the eight N-terminal residues of histone H2B, (Met) Ser-Ala-Lys-Ala-Glu-Lys-Lys-Pro; bottom, the *CYC1-1390* strain series having the eight N-terminal residues of histone H2A, (Met) Ser-Gly-Gly-Lys-Gly-Gly-Lys-Ala. The molecular masses of the altered iso-1 proteins are shown next to each MS peak. The diminished mass of iso-1 from B-14447 (*CYC1-1391 ard1-Δ*) indicated the lack of N-terminal acetylation. Minor peaks on MS data are nonspecific. The masses were determined with the Voyager-DE STR linear time-of-flight mass spectrometer.

carbon sources, such as glycerol or ethanol (results not shown). Also, all three chimeric proteins were expressed at similar rates and were imported into mitochondria, as indicated by Western blot analysis of the crude mitochondrial preparations and by probing with anti-cytochrome *c* antibody (results not shown). However, while the *CYC1-1398* strain, with an 8-residue H4 extension, and the *CYC1-1399* strain, with a 23-residue extension, produced holo-cytochrome *c* at levels comparable to the normal *CYC1* strain, the *CYC1-1400* strain, with a 51-residue extension, produced a much lower level of holo-iso-1 protein, constituting less than 10% of the normal level, as estimated by low-temperature spectroscopic examinations of intact cells and comparing the intensities of the C_{α} bands at 547 nm to the corresponding bands of strains having known amounts of cytochrome *c* (results not shown). In addition, strains B-15367 (*CYC1-1398 nat4-Δ*), B-15368 (*CYC1-1399 nat4-Δ*), and B-15369 (*CYC1-1400 nat4-Δ*) were prepared by deleting *NAT4* in all three of these strains. The growth on YPG and YPE and the levels of iso-1 in these strains were similar to those of the parental strains (data not shown).

The chimeric iso-1 proteins were purified from both sets of strains by ion exchange chromatography, and molecular masses of the proteins were determined by MALDI-TOF MS. The MS peak values obtained for proteins from the normal

CYC1-1398 strain and corresponding *nat4-Δ* mutant were similar and both were close to 13,375 Da, the predicted molecular mass of the unmodified protein (data not shown), indicating that the Cyc1-1398 protein was not acetylated. Analysis of MS iso-1 spectra from the *CYC1-1399* strain set showed that the Cyc1-1399 protein from the normal strain had two peaks with molecular masses of 15,040 Da and 14,998 Da (major peak) and Cyc1-1399p from the *nat4-Δ* mutant had two peaks with molecular masses of 14,999 Da and 14,957 Da (Fig. 2A). Considering that the calculated molecular mass of the unacetylated Cyc1-1399 protein is 15,001 Da and that both samples contained proteins possibly modified by amidation, we suggest that the Cyc1-1399 protein was only partially acetylated in the normal strain.

In contrast, iso-1s from the normal *CYC1-1400* strain and the corresponding *nat4-Δ* mutant had a distinct difference in molecular masses of approximately 39 Da, from 16,771 Da to 16,732 Da, respectively (Fig. 2B). It should be noted again that the Cyc1-1400 protein is unstable and that it appeared to lack heme and possibly another modification, methylation, which usually occurs during cytochrome *c* maturation. The reduced molecular mass of the protein from the *CYC1-1400 nat4-Δ* strain indicated that the Cyc1-1400 protein was not acetylated in the *nat4-Δ* strain and that this Cyc1-1400 protein is a NatD

TABLE 4. N-terminal acetylation (Ac-Ser) and free N termini (Ser) of proteins from the normal, *ard1-Δ*, and *nat4-Δ* strains

Gene(s)	Protein	N terminus			N-terminal sequence	Acetylated by:	Reference or source
		Normal	<i>ard1-Δ</i>	<i>nat4-Δ</i>			
<i>HHF1, HHF2</i>	Histone H4	Ac-Ser-	Ac-Ser-	Ser-	SGRGKGGK	NatD	39
<i>HTA1, HTA2</i>	Histone H2A	Ac-Ser-	Ac-Ser-	Ser-	SGGKGGKA	NatD	39
<i>CYC1-1390</i>	Cyc1-1390	Ser-	Ser-	Ser-	SGGKGGKA	None	This work
<i>HTB1, HTB2</i>	Histone H2B	Ac-Ser-	Ser-	Ac-Ser-	SAKAEKKP	NatA	39
<i>CYC1-1391</i>	Cyc1-1391	Ac-Ser-	Ser-	Ac-Ser-	SAKAEKKP	NatA	This work

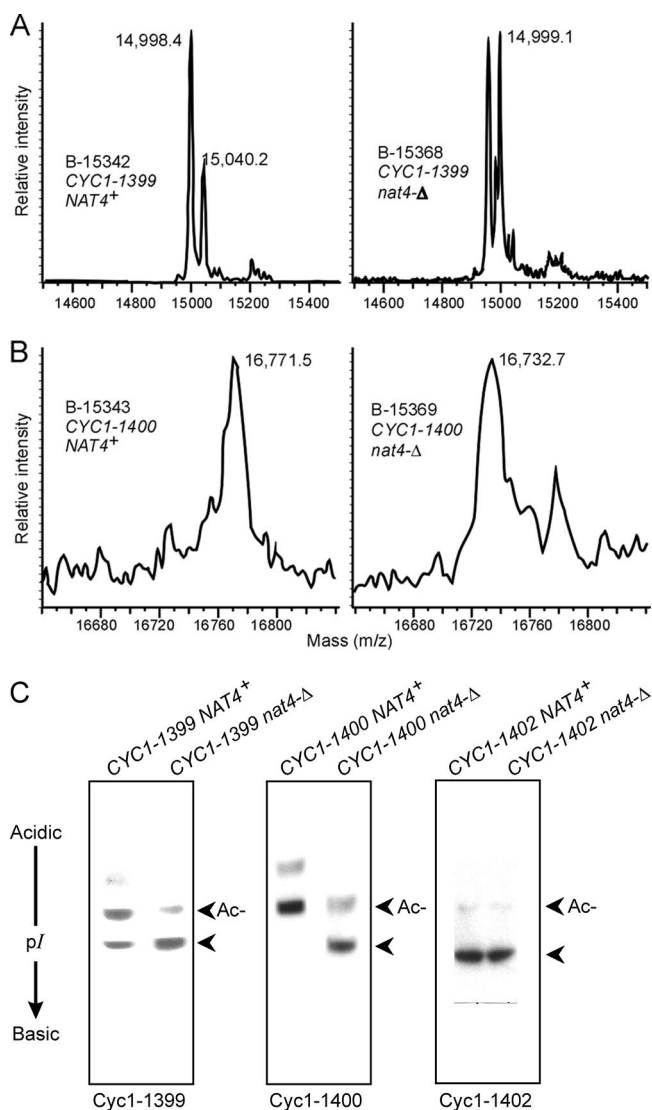


FIG. 2. Only a chimeric protein, Cyc1-1400, containing an N-terminal extension of 51 amino acid residues corresponding to the amino terminus of histone H4, is efficiently acetylated by NatD. (A and B) MALDI-TOF mass spectra of the altered iso-1 proteins prepared from the *CYC1-1399* strains and the *CYC1-1400* strains, respectively. The molecular masses of the altered iso-1s are shown above each MS peak. The diminished masses of iso-1s from B-15368 (*CYC1-1399 nat4*-Δ) and B-15369 (*CYC1-1400 nat4*-Δ) indicated the lack of N-terminal acetylation. Minor peaks in MS data are nonspecific. (C) Western blot analysis of isoelectrically focused iso-1s having extensions of either 23 amino acids (Cyc1-1399) or 51 amino acids (Cyc1-1400), corresponding to the amino terminus of the histone H4. The Cyc1-1402 protein is similar to the Cyc1-1400 protein, except that the N-terminal sequence, SGRGKGGKGL, was replaced with SAAAAAAAAA. Proteins were prepared from the normal (*NAT4*⁺) and *nat4*-Δ cells and separated using the ZOOM system (Invitrogen) and IPG strips, pH 9 to 11, and then transferred to an ECL membrane and probed with anti-cytochrome *c* antibody. The positions of the acetylated and unacetylated forms are denoted by the arrowheads with or without Ac-, respectively. Cyc1-1399 is partially acetylated in the *NAT4*⁺ strain, and the proportion of acetylation is greatly diminished in the *nat4*-Δ strain. Cyc1-1400 is completely acetylated in the *NAT4*⁺ strain; the proportion of acetylation is greatly diminished in the *nat4*-Δ strain. Only a minor portion of the Cyc1-1402 protein is acetylated. The top band in the *CYC1-1400 NAT4*⁺ lane is an unidentified contaminant or isoform.

substrate. The partial acetylation of the Cyc1-1399 protein and acetylation of the Cyc1-1400 protein in *NAT4*⁺ strains and the lack of acetylation in *nat4*-Δ strains were confirmed by another approach, a 2D gel Western procedure that included isoelectric focusing of cell extracts from the normal and *nat4*-Δ strains using the ZOOM IPGRunner system and then testing the separated proteins with anti-cytochrome *c* antibody. Cell extracts were separated on IPG strips, pH 9 to 11, and the proteins were subsequently transferred to an ECL membrane and probed with rabbit anti-cytochrome *c* antibody. The results (Fig. 2C) clearly showed that the Cyc1-1400 protein from the *nat4*-Δ strain exhibited a distinct shift of its position toward a basic side, indicating the loss of an N-terminal acetyl group that normally neutralizes a positive charge of an amino group. These experiments also showed a shift toward a basic side for a portion of the Cyc1-1399 protein, confirming a partial acetylation of this Cyc1-1399 protein in the normal *NAT4*⁺ strain. Thus, NatD requires a significantly longer N-terminal sequence to efficiently acetylate a proper substrate, between 23 and 51 amino acid residues, compared to just several for NatA, NatB, or NatC.

The amino-terminal sequences of histones H4 and H2A, which are NatD substrates, and the related histones H2B and H3, which are not NatD substrates, were analyzed with computational protein analysis tools for possible secondary structures. NatD acetylation occurs cotranslationally (see below), and this acetylation occurs when NatD substrates protrude from the ribosome by approximately 40 to 50 residues; thus, we assumed that proteins are rather unfolded but may form secondary structures. In addition to the protein primary sequences, the predicted secondary structures of histones H2A and H4 are similar to each other, but differ from H2B, which is acetylated by NatA, and differ to a lesser extent from H3, although H3 is not acetylated (Fig. 3). The 50-amino-acid N-terminal regions of both H4 and H2A have two predicted α -helix regions of 8 to 12 amino acid residues, separated and surrounded by coiled structures (Fig. 3B). In contrast, H2B is predicted to have a longer coiled-coil region of over 35 residues, except for the four N-terminal residues. We considered the possibility that a certain secondary structure is required for effective NatD acetylation. Although a detailed analysis of such requirements was beyond the scope of this study, it should be pointed out that the region from position 1 to 9 in both H2A and H4 is similar in both primary sequence and in predicted secondary structure. Another region, located at the position from residues 34 to 41, is also similar but less so (Fig. 3B).

To test if the secondary structures of N termini are important for NatD acetylation, the SGRGKGGKGL segment of the Cyc1-1400 protein, which contains 51 N-terminal residues of H4 and which is acetylated by NatD, was changed to SAAAA AAAA, thus disrupting the putative coiled structure and converting it to an α -helix (Fig. 3B). The resulting Cyc1-1402 protein was purified and the 2D gel Western procedure showed that a major protein band was positioned closer to the basic (+) side of the gel, whereas only a trace amount of the protein was positioned closer to the acidic pI (Fig. 2C), indicating that the Cyc1-1402 protein is a poor NatD substrate and suggesting that the H4 amino-terminal region encompassing residues 1 to 9 is important for efficient acetylation by NatD.

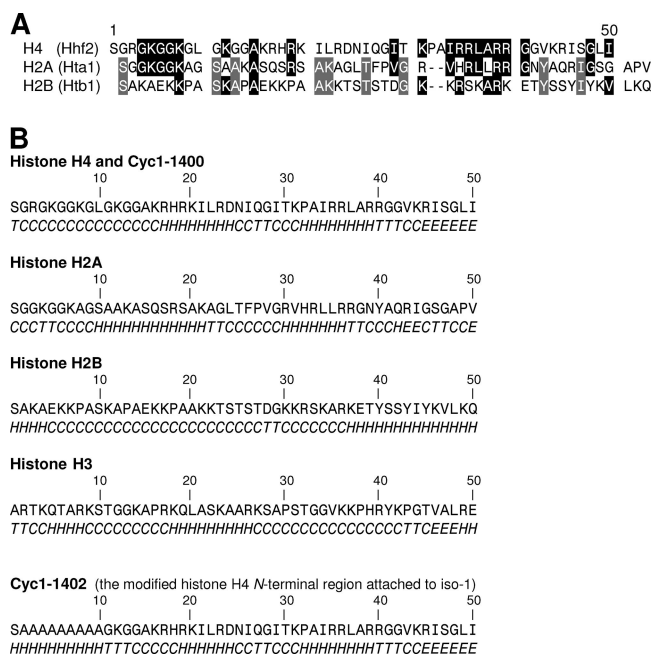


FIG. 3. Comparison of the primary amino-terminal sequences and secondary structure predictions of the yeast histones and altered iso-1-cytochromes *c* (only the 50 N-terminal residues). (A) Protein primary sequence alignments of histones H2A, H2B, and H4. Protein sequences were aligned with Multalin version 5.4.1 (7). Highly conserved residues are highlighted in black, whereas moderately conserved residues are highlighted in gray. (B) Secondary structure predictions of histones H2A, H2B, H3, and H4 and altered iso-1-cytochromes *c*, Cyc1-1400, and Cyc1-1402. Predictions were obtained by using the program Network Protein Sequence Analysis (IBCP, Lyon, France; http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html), as described by Combet et al. (6). *C*, a predicted random coil; *E*, extended strand; *H*, α -helix; *T*, β -turn.

Thus, segments positioned throughout the 50-amino-acid region appear to be important for NatD acetylation.

NatD has no auxiliary subunit. All major NATs have at least one auxiliary subunit in addition to the catalytic subunit. To test whether NatD has any other interacting subunit, we used strain B-15329, containing the *NAT4-TAP* gene. TAP tagging and other TAP procedures were previously used to identify the subunits of NatB (30), NatA (31), and NatC (34). The Nat4-TAP complex, which was purified by a standard procedure, did not contain any protein at a stoichiometric amount other than Nat4 (data not shown). Also, no validated genetic or physical interaction was reported for Nat4 in multiple genome-scale experiments (information from SGD [<http://db.yeastgenome.org/cgi-bin/locus.pl?locus=nat4>]). Thus, Nat4 differs from the other NATs in that it does not require interaction with any protein for activity. In agreement with this conclusion, Nat4 solely overexpressed in a bacterial system was sufficient to acetylate its substrates, histones H2A and H4, in an in vitro assay (39).

Nat4 colocalized with mono- and polyribosome fractions in a sucrose density gradient. Gautschi et al. (14) previously demonstrated with peptide cross-linking that NatA is bound to ribosomes. Also, Nat1 was found in close proximity to nascent polypeptides, independent of whether they were substrates for

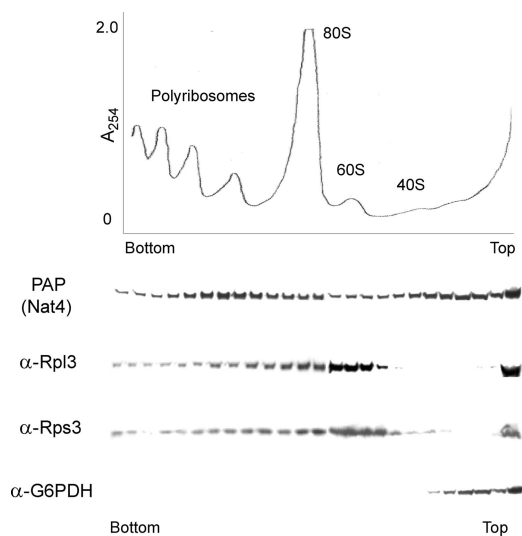


FIG. 4. Nat4-TAP protein colocalizes with mono- and polyribosomes. The polyribosome fractions were prepared from strain B-15329 containing *NAT4-TAP* and were analyzed by Western blot analysis with the following antibodies: PAP (Nat4), PAP complex for detection of the TAP-tagged Nat4; anti-Rpl3 (α -Rpl3), anti-ribosomal protein L3 antibody; anti-Rps3, anti-ribosomal protein S3 antibody; anti-G6PDH (Zwf1) antibody, to determine the location of free cytoplasmic proteins. Fractions are shown from the bottom (left) to the top (right) of 7 to 47% linear sucrose gradients. The results with total cell extracts, serving as positive controls, are shown in the last line on the right. A typical protein absorbance profile at A_{254} of the sucrose gradient fractions is shown on the top.

N-acetylation or not. However, longer nascent polypeptides were required for interaction with the polypeptide-associated complex (NAC) or with Ssb1/Ssb2, which is involved in protein folding. In separate studies, biochemical fractionation in sucrose density gradients revealed that NatA, NatB, NatC, and NatE subunits are associated with mono- and polyribosomes and act cotranslationally (31).

In this regard, the association of NatD with ribosomes was determined by preparing and examining polyribosome fractions from sucrose density gradients of the cell extract prepared from the *NAT4-TAP* strain, B-15329 (Table 2). Although the *nat4*- Δ strain shows only minor phenotypes (see below), the TAP-tagged strain grew similar to the *NAT4*⁺ normal strain on media that were used to characterize the *nat4*- Δ mutant, suggesting that the Nat4-TAP protein was completely functional (data not shown). The cell extract prepared from B-15329 was fractionated in sucrose gradients, the fractions were loaded on 4 to 20% gradient SDS-PAGE gels, and the separated proteins were subsequently transferred onto an ECL membrane and probed with various antibodies. Nat4-TAP localization in the gradient was determined with PAP antibody (Fig. 4). The anti-Rpl3 and the anti-Rps3 antibodies were used to localize polyribosomal fractions, as well as ribosomal subunit fractions; the anti-Zwf1 antibody was used to detect Zwf1 (G6PDH), a cytoplasmic protein.

The results of the experiments, presented in Fig. 4, showed that the majority of Nat4 colocalized to the positions of the ribosomal proteins Rpl3 and Rps3, which corresponded to the positions of the mono- and polyribosome fractions at the lower

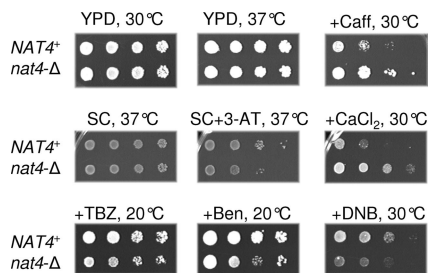


FIG. 5. Phenotypes of the *nat4-Δ* strain. Serial 1/10 dilutions of the isogenic normal *NAT4*⁺ (B-10190) and *nat4-Δ* (B-14070) strains grown for 3 days at 30°C and 37°C on YPD and YPD media containing one or another of the following reagents: 0.15% caffeine (Caff), 0.3 M calcium chloride (CaCl₂), 20 μg/ml benomyl (Ben), 50 μg/ml TBZ, or 250 mM DNB. Synthetic complete medium was used for the plates containing 75 mM 3-AT.

portion of sucrose gradient. On the other hand, a known cytosolic protein Zwfl was found only at the top of the gradient. Similar results were obtained with polyribosome fractions prepared from the strain B-15274 containing HA-tagged *NAT4* (data not shown). Thus, NatD is also ribosome associated, as are NatA, NatB, NatC, and NatE, although a portion of Nat4 is located in the free cytoplasm.

Phenotypes of *nat4-Δ* strains. No obvious phenotype was previously reported for *nat4-Δ* by testing the growth of the mutant strain on common media (39). Therefore, we compared the growth of several series of isogenic *NAT4*⁺ and *nat4-Δ* strains (Table 2) on media with additions of various chemicals or drugs. The *nat4-Δ* mutant did not show reduced growth at 30°C or 37°C on YPD, YPG, or synthetic complete medium. However, by using series of 1/10 serial dilutions of cell suspensions, the *nat4-Δ* strains exhibited reduced growth on the following media at the indicated temperatures: 3-amino-1,2,4-triazole, a general inhibitor of transcription, 37°C; benomyl and thiabendazole, both antimitotic microtubule-destabilizing drugs, 20°C; dinitrobenzene, a nonspecific membrane inhibitor, 30°C (Fig. 5). Although the growth differences were minor, the results were reproducible. Also, the mutant cells grew better than the normal strain on media containing caffeine or calcium chloride, both of which produce multiple effects.

These *nat4-Δ* phenotypes may be caused by either or both of the following two mechanisms: (i) the lack of N-acetylation of as-yet-unidentified proteins is directly or indirectly responsible for these traits; (ii) the diminished activity of histones H2A and H4 due to the lack of N-terminal acetylation, a condition that is expected to affect expression of numerous genes, is responsible for these traits. Although no other NatD substrate was detected by 2D gel electrophoresis (39), it is not excluded that less-abundant proteins could be acetylated by NatD. Therefore, we have investigated whether abnormal forms of histone H4 can enhance and produce *nat4-Δ* phenotypes. In this regard, it should be noted that histone H4 is encoded by two identical genes, *HHF1* and *HHF2* (SGD information [<http://www.yeastgenome.org>]).

We tested whether the mutant allele *hhf2-100*, which encodes a histone H4 with K5R K8R K12R replacements (17), would affect the phenotypes of *nat4-Δ* strains. Normally K5,

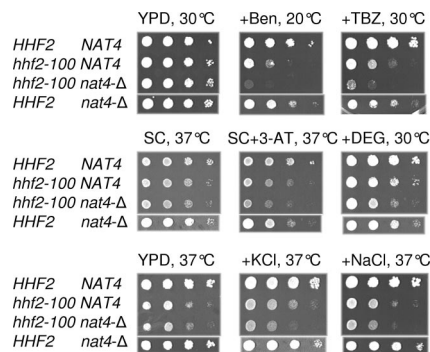


FIG. 6. The lack of N-terminal serine acetylation (*nat4-Δ* deletion) causes a synthetic growth defect with the lack of histone H4 N-tail lysine acetylation (R5K R8K R12K replacements in histone H4; *hhf2-100*). Serial 1/10 dilutions of the following isogenic strains were evaluated: B-15949, *NAT4 HHF2*; B-15955, *nat4-Δ HHF2*; B-15952, *NAT4 hhf2-100*; and B-15954, *hhf2-100 nat4-Δ* mutant. The strains were grown for 3 days at 30°C or 37°C on YPD and on YPD media containing the following reagents: 1 M potassium chloride (KCl), 1 M sodium chloride (NaCl), 20 μg/ml benomyl, 75 μg/ml TBZ, and 6% diethyleneglycol (DEG). Synthetic complete (SC) complete medium was used for the plates containing 75 mM 3-AT.

K8, and K12 are acetylated in histone H4, and these acetylations are prevented by the arginine replacement. These strains were constructed by disrupting the *NAT4* gene in strain B-15950 (*hhf2-100*) containing such mutations and in the normal strain B-15949 (*HHF2*), resulting in B-15954 (*hhf2-100 nat4-Δ*) and B-15955 (*HHF2 nat4-Δ*), respectively (Table 2). The 1/10 serial dilution experiment clearly showed that the double mutant strain grew much slower on medium containing benomyl and thiabendazole, while a very moderate effect was observed on the plates containing 3-aminotriazole, salt (sodium chloride and potassium chloride, both at 37°C), and diethyleneglycol (Fig. 6). Therefore, the lack of N-terminal serine acetylation further increases the overall positive charge of the H4 N-tail and acts synergistically with the lack of lysine acetylations in the N-tail domain of histone H4, producing a synthetic growth defect.

Also, we compared the mating efficiencies of the normal (*HHF2 NAT4*) and single (*HHF2 nat4-Δ*) and double (*nat4-Δ hhf2-100*) mutant strains with a tester strain, B-7928. With the mating efficiency of the *HHF2 NAT4* strain assigned a value of 1.0, the relative value of the *HHF2 nat4-Δ* strain was 0.8 and that of the *nat4-Δ hhf2-100* strain was 0.65. Thus, there was only a slight diminution of mating with the double mutant strain. In contrast, the mating efficiency of strain B-15953 (*hhf2-101*), containing a G16K replacement in the histone H4 N-tail, was substantially lower, below 1×10^{-4} , compared to the normal control strain and was similar to the value obtained by Johnson et al. (17).

In addition, we considered the extent to which the lack of histone H4 and H2A acetylation contributes to the *nat4-Δ* phenotypes. Based on our results with double *nat4-Δ hhf2-100* strain phenotypes (see above) and numerous publications on the role of H4 and H2A N-tails, we choose H4 (*HHF2*) as a potentially more important NatD substrate for making such N-terminal mutants. For this purpose we used an approach similar to our previously published experiments (27) and we

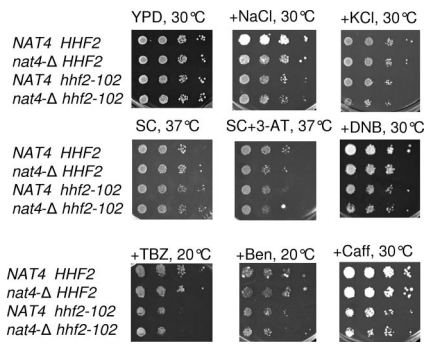


FIG. 7. The strain containing the *hhf2-102* allele (V1S replacement in histone H4) showed a similar growth defect as the *nat4-Δ* deletion strain and double mutant *hhf2-102 nat4-Δ* strain. Serial 1/10 dilutions of the following isogenic strains were evaluated: B-15949, normal, *NAT4 HHF2*; B-15955, *nat4-Δ HHF2*; B-16143, *NAT4 hhf2-102*; B-16163, *nat4-Δ hhf2-102*. The strains were grown as described in the legend for Fig. 5, except benomyl (Ben) was used at 15 $\mu\text{g/ml}$ and DNB was at 500 mM.

replaced the H4 N-terminal residue serine with valine, a residue that usually is not acetylated in yeast (25, 27). This *hhf2-102* allele (S1V), which was made by site-directed mutagenesis, was introduced into the strain with a wild-type *HHF2* gene, which was subsequently removed by plasmid shuffle. Indeed, unlike a wild-type histone, the V1S H4 mutant was not N-terminally acetylated, as the mobilities of H4 mutant protein isoforms prepared from the normal, *ard1-Δ*, and *nat4-Δ* strains and separated in a Triton-acetic acid-urea-polyacrylamide gel were very similar (data not shown). The growth of the resulting strain, B-16143 (*hhf2-102 NAT4*) (Table 2), was compared with strain B-15954 (*HHF2 nat4-Δ*) in 1/10 serial dilution experiments. Both the *hhf2-102* and *nat4-Δ* strains showed minor but similar phenotypes, including sensitivity to 3-aminotriazole, benomyl, thiabendazole, dinitrobenzene, and diethyleneglycol (Fig. 7). The slightly more sensitive phenotype of the *hhf2-102* strain compared to the *nat4-Δ* mutant could be explained not only by the lack of acetylation but also by the lack of phosphorylation of the H4 histone (20). Moreover, the *hhf2-102 nat4-Δ* double mutant strain B-16163 showed a very similar if not identical phenotype to the single *hhf2-102* mutant phenotype (Fig. 7). Although the N-terminal serine of H4 is known to be phosphorylated and may play a regulatory role during sporulation and mitosis (4), contributing to observed phenotypes, acetylation, unlike phosphorylation, is an irreversible modification and thus permanently neutralizes a basic charge of the protein amine group. Therefore, these additional experiments with *hhf2-102* supported the suggestion that the enhanced phenotypes observed in the double *nat4-Δ hhf2-102* strain are not caused by a lack of acetylation of any other protein in the cell.

DISCUSSION

Our studies with N-terminally altered iso-1 proteins, combined with the analysis of certain normal proteins, revealed that the major NATs, NatA, NatB, and NatC, recognize their substrates primarily based on the sequence of the last several N-terminal residues (Table 1) (25, 27). However, some N-

terminally acetylated proteins could not be easily assigned as a substrate to any known NAT, leading us to suggest that new NATs still remain to be uncovered (25), especially for proteins with unusual N-terminal sequences, for example, those with highly basic histone tails (25). This study revealed that efficient acetylation by NatD required at least 30 to 50 amino acid residues of the N terminus of histone H4, a finding that is in contrast with the requirement of only a few specific amino acid residues for efficient acetylation by the major NATs.

It is important to note that an intact histone N-tail and N-terminal serine acetylation are required for normal function of the cell. For example, although deletion of the hydrophilic H4 N terminus (residues 4 to 28) still allows viability, it alters the normal chromatin structure and lengthens the cell cycle, especially the G_2 phase (17, 19). Surprisingly, the lack of the H4 N terminus also causes derepression of the silent mating-type loci, *HML α* and *HMRa*, resulting in diminution of mating. In contrast, deletion of the H2A or H2B N-tails does not affect *HML α* and *HMRa*. (17). The N-terminal serine acetylation may function as a part of the "charge patch" together with N-tail lysine acetylations (32, 33), a mechanism in which acetylation alters the charge of a protein domain rather than affecting a specific site (known as the "histone code") (43). Such modifications that alter the charge can affect chromatin function, regulation of the expression of specific genes by phosphorylation of linker histone H1 (8, 9), and the essential function of histone H2A.Z in *Tetrahymena* species (33). In these cases, the function of the modification is to alter the charge of the domain in which it resides and, unlike the histone code, these changes need not be site specific. Modulation of the charge at any one of a number of clustered sites can have the same effect. If the lack of any single acetylation site, N-serine or N-tail lysine residues, were to promote nucleosome condensation in vivo, it could inhibit transcription, thus enhancing certain phenotypes.

Possible functional interactions involving the state of acetylation of N-serine and the N-tail lysines of histone H4 were explored by determining the sensitivities to various agents of *nat4-Δ* mutants and *hhf2-100* mutants, which contain K5R K8R K12R replacements. Most importantly, the double mutant (*hhf2-100 nat4-Δ*) was more sensitive to the following additives than either of the single *HHF2 nat4-Δ* or *hhf2-100 NAT4* mutants: benomyl, thiabendazole, 3-aminotriazole, salts, and diethyleneglycol (Fig. 6). The increased sensitivity to these agents is clearly caused by diminished activity of histone H4, although the mode of action is completely unknown. The lack of N-terminal serine acetylation of histone H4 acts synergistically with the lack of N-tail lysine acetylations, causing a further increase of the overall positive charge of the protein N-tail and suggesting its role in a charge patch for histone H4. Thus, it appears as if N-terminal serine acetylation results in a part of a charge patch for histone H4. Although we have not excluded the possibility that the lack of acetylation of some unknown proteins in the *nat4-Δ* strains may be also playing a role in determining these mutant phenotypes, the similar sensitivities in *nat4-Δ HHF2*, *NAT4 hhf2-102*, and *nat4-Δ hhf2-102* strains strongly suggest that the mutant phenotypes in *nat4-Δ* strains are primarily due to a defective histone H4. Alternatively, the enhanced synthetic phenotype in the *nat4-Δ hhf2-100* mutant could be explained by further weakened recruitment of transcrip-

tion factors or activators to hypoacetylate chromatin, as some models have proposed that histone hypoacetylation increases the affinity of histone tails for DNA, resulting in more compact chromatin and decreased accessibility of transcription factors to DNA (for example, see the review by Wassarman and Sauer [46]). It should be remembered that transcription factors recognize and bind histone acetylated lysine residues through their bromodomain.

Our studies with mutant forms of iso-1 revealed that acetylation of an N-terminal region depends on both positive, or optimal, residues and negative, or interfering, residues that are situated anywhere on the nascent chain at the time of acetylation (27). The lack of an optimal residue, or replacement with a suboptimal residue, reduces acetylation; the presence of an interfering residue reduces or prevents acetylation of an otherwise-optimal motif. Thus, the degree of acetylation is the net effect of positive optimal or suboptimal residues and negative inhibitory residues. The lack of acetylation could be due to the absence of required residues or the presence of inhibitory residues. Of the inhibitory residues, arginine, lysine, proline, and histidine are the ones most affecting acetylation. Interestingly, the N termini of histone H2A, SGGKGGKA, and of histone H4, SGRGKGGK, are both enriched with lysine residues that potentially inhibit acetylation, thus making acetylation of these sequences by NatA impossible or highly unlikely. In this regard, it should be noted that positively charged N termini with lysine, arginine, proline, or histidine at the penultimate position are more characteristic of prokaryotic proteins, which are rarely acetylated (27).

In this study we demonstrated that an altered iso-1 with the N-terminal sequence SAKAEKKP, which corresponds to the N terminus of histone H2B, was efficiently acetylated by NatA. Also, the following proteins with similar sequences were previously shown to be completely or partially acetylated by NatA: SAKAQ (Rpl11a), SAKSF (Dak1), SAPAA (Gsp1), SAPAQ (Sah1), SAPEA, (Rps2), SAPQA (Rps7a), and SAPTP (Tom40) (Table 2 in reference 27). Although some of these N-terminal sequences are positively charged, as are histone N-tails, they do not contain glycine residues, which allow bending of proteins. In contrast, the following N-terminal sequences that are acetylated by NatD are highly positively charged and include a number of glycine residues: SGGKGGKA (histone H2A), SGRGKGGK (histone H4), and potentially SGKAHGGK (histone H2AZ). Such N-terminal sequences rarely occur on yeast proteins and we were unable to find other proteins having N termini that perfectly match the N termini of histones H2A and H4. Our search revealed that polypeptides with partial similarity, having less-charged and fewer glycines, are usually acetylated by NatA, for example, SGAAAASA (Scf1) and SGYD RALS (Pre6) (27). On the other hand, a number of ribosomal proteins with the following amino termini are not acetylated in vivo (40, 1), although they all contain alanine as the N-terminal residue: APGKKVAP (Rpl8), AKSKNHTA (Rpl29), AKVH GSLA (Rps30), APSAKATA (Rpl25), AGLKDVVT (Rpl31), APVKSQES (Rpl30), and AGKKIAGV (Hom2). It appears that NatD substrates, H2A and H4 N-tails, are indeed unique both in sequences and other structural features and that they specifically evolved to be acetylated by NatD in eukaryotes. Our computer analysis of the primary sequences and the predicted secondary structures of histone H4 and H2A N-terminal

regions (Fig. 3) showed that their characteristics differ from the characteristics of their conserved functional partners, H2B and H3, and that any essential H4 N-tail alteration may affect efficient protein acetylation by NatD (Fig. 1 and 2). Interestingly, when fractionated on a high-performance liquid chromatography reverse C₁₈ column, H2A and H4 eluted very closely to each other and distantly from H3 or H2B.

On the other hand, not only H2A and H4 N-tail structural features are unusual; NatD is unusual in its requirement for a much longer N-terminal sequence for efficient acetylation. In contrast to the major NATs that require just several amino acid residues to recognize a proper substrate, NatD requires between 23 and 51 residues to efficiently acetylate its substrates, histones H4 and H2A. It is possible that such a Nat4 requirement for acetylation is similar to certain lysine acetyltransferases. In this regard, Berndsen et al. (5) reported that efficient acetylation of the ϵ -amine of lysine residues of the histone H4 tail by picNuA4 requires 52 N-terminal residues of H4, which contain helical regions. Moreover, NatD does not require any other subunit for function, unlike the major NATs, although it appears to act cotranslationally. By using polyribosome fractionation in a sucrose density gradient, we demonstrated that the major portion of Nat4p colocalizes with mono- and polyribosome fractions (Fig. 4). Previously we showed that all major NATs, NatA, NatB, NatC, and NatE, are ribosome associated as well (31), with Nat1p, Mdm20p, and possibly Mak10p acting as the anchors to the ribosome. NatD has no auxiliary subunit but is ribosome associated. The lack of an auxiliary subunit might be explained by the limited number of substrates, histones H2A and H4, both having similar N-terminal sequences that could be directly accommodated by only a catalytic subunit. In contrast, NatA, NatB, and NatC acetylate significantly higher numbers of diverse proteins and thus may require more complex machinery for recognition of diverse substrates. Furthermore, the binding to the ribosome could be provided by the N-terminal domain of Nat4. When the catalytic subunits of major NATs, Ard1, Nat3, and Mak3, and of Nat4 are aligned by acetyl coenzyme A binding motifs, Nat4 has an N-terminal extension of 85 amino acid residues compared to Nat3, Mak3, and Ard1 (the protein alignment is not shown). However, no strong similarity to known motifs was found within that N-terminal 85-residue region of Nat4. It remains to be determined whether the N-terminal domain of Nat4 is involved in binding to the ribosome.

This study has addressed the important question of why histones H2A and H4 are not acetylated by NatA, which acetylates over 2,000 different proteins in yeast. The importance of NatD from an evolutionary point of view is evident from the phenotypes of the *nat4*- Δ mutant and by the functional requirement of N-serine acetylation of the essential histones H2A and H4 as discussed above. The inability of NatA to efficiently acetylate H2A and H4 histones has now been explained from our analysis of the N-terminal sequences that can and cannot serve as NatA substrates, mainly by the inhibitory effects of basic residues.

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