An N\textsuperscript{\textasteriskcentered}-Acetyltransferase Responsible for Acetylation of the N-terminal Residues of Histones H4 and H2A* 

Received for publication, August 8, 2003, and in revised form, August 11, 2003
Published, JBC Papers in Press, August 12, 2003, DOI 10.1074/jbc.C300355200

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A yeast gene has been identified that encodes a novel, evolutionarily conserved N\textsuperscript{\textasteriskcentered}-acetyltransferase responsible for acetylation of the N-terminal residues of histones H4 and H2A. The gene has been named NAT4. Recombinant Nat4 protein acetylated a peptide corresponding to the N-terminal tail of H4, but not an H3 peptide nor the peptide adrenocorticotropin. H4 and H2A are N-termi

Acetylation Reactions—Histones or peptides were acetylated in 40-μl reactions with 75 mM Tris-Cl (pH 8.8), 135 mM NaCl, 0.2 mg/ml chicken erythrocyte histones or 50 μg peptide, 1 μCi of [\textsuperscript{\textasteriskcentered}H]acetyl-CoA (Amer

Histone Purification—Yeast histones were purified for sequencing as described previously (11). The histones were separated by electrophoresis on 15% SDS-polyacrylamide gels. After electrophoresis, proteins were blotted onto polyvinylidene difluoride membranes (0.2 μm; Bio

Eukaryotic proteins are subject to two cotranslational modifications as the nascent polypeptides emerge from the ribosome, methionine cleavage by methionine aminopeptidase, and acetylation of the α-amino group of the N-terminal amino acid (1). Removal of the methionine occurs only if the second amino acid is a small one such as serine, alanine, or glycine. Acetylation of the α-amino group on either methionine cleavage, or on the N-terminal residue resulting from methionine cleavage, occurs in the great majority of (but not all) eukaryotic proteins, and is accomplished by one of several N\textsuperscript{\textasteriskcentered}-acetyltransferases (NATs)\textsuperscript{1} (1). A number of years ago we identified the genes for a yeast NAT, now called NatA, that consists of two subunits, Ard1 and Nat1 (2). Subsequent work showed that NatA was responsible for the acetylation of many, but not all, yeast proteins beginning with small residues such as serine, alanine, or glycine (2–5). Yeast ard1 and nat1 mutants are viable and the many proteins that are no longer N-terminally acetylated in the mutant strains are as stable as they are in a wild type strain (2). Thus, there is no evidence that N-terminal acetylation serves to protect proteins from degradation in yeast.

Ard1 protein has an acetyl-CoA binding motif found in members of the GNAT superfamily, enzymes that acetylate amino groups on proteins and other molecules (6). Thus, Ard1 is very likely to be the catalytic subunit of NatA. Two other NATs have been identified in yeast, called NatB and NatC, with catalytic subunits Nat3 and Mak3, respectively (4, 7). NatB and NatC both acetylate proteins with N-terminal methionine residues, although they have different specificities dictated by the nature of the subsequent amino acids (4).

Histone H4 is a highly conserved protein with an N-terminal serine residue that is acetylated in both mammals and yeast (8, 9). In our initial paper on NatA, we noted that H4 from ard1 or nat1 mutants had the same mobility on Triton-acid-urea gels as when isolated from wild type strains (2). We therefore concluded that H4 was not acetylated by NatA. But, as noted above, NatB and NatC only acetylate proteins beginning with methionine, not serine, so the question arose as to whether another yeast NAT existed that was responsible for the N-terminal acetylation of H4. We therefore examined uncharacterized yeast genes that are members of the GNAT superfamily of acetyltransferases (6) to see if one of them might be the NAT that acetylates H4. Here we describe an evolutionarily conserved yeast protein, encoded by the gene we call NAT4, that N-terminally acetylates not only histone H4, but also H2A.

MATERIALS AND METHODS

Nat4 Expression Plasmid and nat4 Mutant—The NAT4 coding sequence was amplified from yeast genomic DNA by PCR and cloned into the expression vector, PET28a, as an EcoRI-XhoI fragment. This led to the expression of full-length Nat4 protein with a His-tag at the N terminus. The protein was expressed well in Escherichia coli and purified by Ni\textsuperscript{\textasteriskcentered} affinity chromatography. Yeast nat4 mutants were constructed by replacing the entire NAT4 open reading frame either with the kanMX6 gene (strain 051) or with the S. pombe his5 gene (strain OS4), as described previously (10).

Histone Purification—Histone H4 and H2A were purified for sequencing as described previously (11). The histones were separated by electrophoresis on 15% SDS-polyacrylamide gels. After electrophoresis, proteins were blotted onto polyvinylidene difluoride membranes (0.2 μm; Bio

‡ This work was supported by National Institutes of Health Grant GM28220 (to R. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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∥ The abbreviations used are: NAT, N\textsuperscript{\textasteriskcentered}-acetyltransferase; HAT, histone acetyltransferase; ACTH, adrenocorticotropin.
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**RESULTS**

The sequence of the peptide encoded by yeast ORF, YMR069wc, has the acetyl-CoA binding motifs characteristic of members of the GNAT superfamily (6). Furthermore, the protein has sequence similarity to the catalytic subunits of the three known NATs, Ard1, Nat3, and Mak3, and is of similar size (Fig. 1). We expressed and purified the peptide encoded by YMR069wc in E. coli. The recombinant protein (that we call Nat4 based on the results to be presented below) was able to acetylate a peptide with a sequence corresponding to the N-terminal 28 amino acids of H4, but not a corresponding H3 peptide or an unrelated peptide, adrenocorticotropic (ACTH; Fig. 2A). We chose ACTH because Nat4 is able to acetylate this peptide in vitro (13). Notably, Nat4 protein could not acetylate chicken histones even though a known histone acetyltransferase (HAT), Hat1, could acetylate both the H4 peptide and chicken histones. Recombinant Nat4 acetylates an unacetylated H4 peptide, but not H3 or ACTH peptides or chicken histones. Recombinant Hat1 acetylates both the H4 peptide and chicken histones. B, recombinant Nat4 acetylates both completely unacetylated (un Ac) and internally tetraacylated (tetra Ac) H4 peptides, whereas Hat1 acetylates only the unacylated peptide.

Next we constructed a mutant with a complete deletion of the ORF for Nat4, YMR069wc. The mutant was viable and grew at a normal rate. Histones were purified from the mutant, as well as from a wild type and an ard1 strain (Fig. 3). The proteins were separated by SDS-PAGE, transferred to a polyvinylidine difluoride membrane, and the bands at the positions of H4 and H3 excised and subjected to Edman degradation and protein sequencing. Histone H3, which is known to be unblocked (i.e. not acetylated) at its N terminus, gave the expected N-terminal sequence, Ala-Arg-Thr-Lys. Histone H4 gave no readable sequence from a wild type and an ard1 strain (Fig. 3). The proteins from wild type and the ard1 strain (Fig. 3). The proteins from wild type and the ard1 strain (Fig. 3). The proteins from wild type and the ard1 strain (Fig. 3). The proteins from wild type and the ard1 strain (Fig. 3).

![Fig. 1. Nat4 shows sequence similarity to three known yeast NATs. The numbers in parentheses are the total number of amino acids in each protein. Motifs A and B refer to the acetyl-CoA binding motif present in members of the GNAT superfamily (6). Asterisks indicate residues identical in all four proteins; colons indicate very similar residues; and periods indicate similar residues.](image)
with the H4 sequencing data described above, this experiment convincingly demonstrates that the H4 N-terminal serine is acetylated by Nat4. H3 exhibited the same electrophoretic mobility in all four strains, as expected, given that H3 is not N-terminally acetylated (Fig. 4). In addition, the electrophoretogram confirmed our previous result that histone H2B is a substrate for Nat1-Ard1 (2). Note that some, but not all, of the H2B isoforms migrate more rapidly in both ard1 mutant strains. This is discussed below.

To our surprise, H2A isoforms also migrated more rapidly when isolated from both nat4 mutant strains as compared with the Nat4 strains (Fig. 4). This suggested that H2A, which has an N-terminal serine residue, just as H4 does, also is a substrate for Nat4. We therefore sequenced purified yeast H2A from wild type and the nat4 mutant. H2A from the wild type strain gave no readable sequence, while H2A from the mutant gave the sequence Ser-Gly-Gly-Lys, which corresponds exactly to the first four amino acids of H2A (Fig. 3 and Table I). Thus, H2A also is N-terminally acetylated by Nat4.

To examine whether other proteins were substrates for Nat4, we labeled yeast proteins with [35S]methionine in both a wild type strain and a nat4 mutant. A protein extract from each strain was then analyzed by two-dimensional gel electrophoresis followed by autoradiography. We used this method previously to show that many yeast proteins gain an extra positive charge, and thus shift to the basic side, in vitro, even one already acetylated (Fig. 2). However, a careful comparison showed that the pattern of protein spots on the gels from the Nat4 and the Nat4 strains were almost identical (data not shown). The one visible difference was that the Nat4 mutant had a missing spot at a molecular weight consistent with the predicted size of Nat4 protein. Thus, we saw no evidence that other proteins were substrates for Nat4. Histones H4 and H2A do not contain methionine and thus would not have been seen in the autoradiogram. Also, highly basic proteins like histones are not well resolved by the usual two-dimensional gel methods (14).

As mentioned above, the Nat4 mutant was viable. The mutant grew normally at all temperatures tested on both rich medium and synthetic medium. The strain grew normally on galactose or glycerol medium. A Nat4/nat4 homozygous diploid underwent normal sporulation and meiosis, leading to four viable haploids per spore. Three experiments failed to uncover any transcription defects for nat4 mutants. First, the strain could grow on a medium lacking inositol. This is a sensitive test, because many viable mutants in genes for HATs or other transcriptional coactivators are defective in transcription of the INO1 gene, leading to an inositol auxotrophy (15). Second, a nat4 mutation was introduced into a strain with an HO-lacZ reporter gene. No defect in lacZ expression was seen. Many mutations affecting the Gcn5 HAT complexes or the Swi/Snf chromatin remodeling complex have a defect in transcription of the HO gene (16, 17). Finally, the nat4 mutation was moved into a strain with a his4 reporter by the method to check for an Spt—phenotype (18, 19). The strain remained His+, indicating the lack of an Spt phenotype.

**DISCUSSION**

Three experiments demonstrated that Nat4 protein acetylates the N terminus of H4. First, the recombinant protein acetylated an H4 peptide in vitro, even one already acetylated on the four acetylatable lysines (Fig. 2). Second, and most convincing, H4 isolated from a nat4 mutant could be sequenced by Edman degradation and yielded the expected N-terminal sequence, whereas H4 from a wild type yeast strain was blocked and could not be sequenced (Fig. 3). Third, H4 from the mutant had an altered mobility on acid-urea gels, consistent with an extra positive charge (Fig. 4). The fact that all the H4 shifted position in the nat4 mutant suggested that all the H4 was acetylated by Nat4 in wild type yeast and that no other NAT could acetylate it.

All of the H2A from the nat4 mutant also shifted position in acid-urea gels (Fig. 4). This prompted us to sequence H2A. As with H4, H2A gave the expected sequence when isolated from the nat4 mutant and gave no sequence from wild type (Fig. 3). Interestingly, H2A has an N-terminal sequence that is quite similar to that of H4 (Table I). Only one other yeast protein is similar to H4 and H2A, comparing the first 9 amino acids, and that is the H2A variant, H2AZ (Table I). It is quite possible...
that H2A.Z is also a substrate for Nat4, but, like H4 and H2A, H2A.Z has no methionine residues and thus could not have been seen in the two-dimensional gel analysis. Interestingly, recombinant H2A.Z purified from *E. coli* (as an H2A.Z-H2B dimer) was an excellent substrate for *in vitro* acetylation by Nat4, while H2B was not acetylated (data not shown). In addition to H4, H2A, and H2A.Z, there are 61 other yeast proteins predicted to begin with (Met)-Ser-Gly. As noted above, we did not observe any mobility shifts comparing proteins from wild type in members of the GNAT superfamily (6).

Since all the H4 and H2A isoforms had an altered mobility on the acid-urea gel, we conclude that all of the H4 and H2A in yeast are acetylated by Nat4 (Fig. 4). But, as noted, some, not all, of the H2B isoforms had an altered mobility on acid-urea gels when isolated from *ard1* mutants (Ref. 2 and Fig. 4). One explanation for this result is that NatA (with the catalytic subunit, Ard1) acetylates H2B inefficiently. Another explanation takes note of the fact that the two yeast H2B genes code for slightly different proteins, one starting with Ser-Ala-Lys and the other starting with Ser-Ala-Thr. Perhaps NatA can only acetylate one of these two H2Bs, and the other one either is not acetylated or is acetylated by another, as yet unidentified, NAT.

Nat4 is well conserved from yeast to mammals, not just in the catalytic CoA binding domain, but throughout the length of the protein (Fig. 5). We predict that those homologs from larger eukaryotes also will acetylate the N terminus of H4 and H2A, and possibly H2A.Z. Both H4 and H2A are known to be blocked from N-terminal sequencing in all species in which they have been examined, and in the case of the bovine proteins, N-terminal acetylation has been directly demonstrated by elegant biochemistry (9). It is surprising that nat4 mutants did not exhibit readily observable phenotypes, especially, since every nucleosome in the mutant cell will have four extra positive charges, one for each H4 and H2A present in the octamer. And yet the mutant grew normally under all conditions tested. Furthermore, the mutant had no observable transcription defects using tests that do exhibit phenotypes for mutants affecting the Swi/Snf chromatin remodeling complex, the Gen5 HAT complexes and the various Spt proteins involved in transcription.

To try to uncover phenotypes, we constructed nat4-ard1 and nat4-hat1 double mutants. We also constructed a strain with the nat4 mutation plus only one copy of the duplicate H3-H4 genes (either *HHT1-HHF1* or *HHT2-HHF2*). None of those strains had phenotypes over and above that of the single mutants (data not shown). Nevertheless, since acetylation of the N-terminal serine on H4 and H2A is conserved, the modification must be important under some environmental conditions. Additional phenotypic studies or a synthetic lethal screen may uncover the role(s) for N-terminal acetylation of H4 and H2A.

**Acknowledgments**—We thank Aaron Neiman for suggesting that this gene might encode a NAT, Tom Fischer of the Center for Analysis and Synthesis of Macromolecules at Stony Brook for protein sequencing, Michael Grunstein for peptides, and Ann Sutton for advice.

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**Fig. 5.** Nat4 homologs in other species. A sequence alignment between *Saccharomyces cerevisiae* Nat4 and proteins from four other species is shown. Residues in red are highly conserved among the five species, and residues in green are moderately conserved. A consensus sequence is shown below the alignment, with capital letters signifying identity among all five proteins. Motifs A and B refer to the acetyl-CoA binding motif present in members of the GNAT superfamily (6).