Importance of the Sir3 N Terminus and Its Acetylation for Yeast Transcriptional Silencing

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ABSTRACT

The N-terminal alanine residues of the silencing protein Sir3 and of Orc1 are acetylated by the NatA N-acetyltransferase. Mutations demonstrate that the N terminus of Sir3 is important for its function. Sir3 and, perhaps, also Orc1 are the NatA substrates whose lack of acetylation in ard1 and nat1 mutants explains the silencing defect of those mutants.

In the budding yeast, Saccharomyces cerevisiae, the MAT locus is expressed while the HML and HMR are kept transcriptionally silent. Transcription at the HM loci is repressed due to cis-acting sequences termed silencers that bind specific proteins, ORC, Rap1, and Abf1, which in turn attract the silent information regulator proteins, Sir1, Sir2, Sir3 and Sir4, to the loci. A Sir2, Sir3, Sir4 protein complex spreads from the silencers onto nearby nucleosomes, forming a heterochromatin-like structure that is transcriptionally silent (Hecht et al. 1996; Hoppe et al. 2002; Rusche et al. 2002). Several recent reviews have been published on yeast transcriptional silencing (Gartenberg 2000; Gasser and Cockell 2001; Rusche et al. 2003).

It is thought that N-terminal acetylation of one or more proteins involved in silencing is important for their function. This is based on the observation that mutations in either ARD1 or NAT1, genes that encode subunits of the N-acetyltransferase now called NatA, cause a noticeable silencing defect (Whiteway et al. 1987; Mullen et al. 1989; Aparicio et al. 1991; Park and Szostak 1992). Specifically, such mutants are completely defective in telomeric silencing and partially defective in silencing at HML. NatA acetylates many, but not all, proteins that begin with small residues such as Ser, Ala, Gly, and Thr (after cleavage of the initiating methionine; Polevoda et al. 1999). The acetylation occurs cotranslationally as the nascent polypeptide emerges from the ribosome (Polevoda and Sherman 2000, 2003). Since Sir3 has an Ala residue at its N terminus, it is a potential substrate for the NatA acetyltransferase and, in fact, we have speculated previously that it might be the relevant substrate that causes the silencing defect of ard1 and nat1 mutants (Stone et al. 1991). Here we show directly that Sir3 is N-terminally acetylated by NatA.

Previous work from our lab suggested that the N terminus of Sir3 is important for the function of the protein. We noted that a Gal4 DNA binding domain (GBD)-Sir3 hybrid protein is only partially functional when GBD is at the N terminus of the protein but is fully functional when GBD is at the C terminus (Chien et al. 1993). The same is true for LexA-Sir3 and Gal4 activation domain-Sir3 hybrids (our unpublished data). Gotta et al. (1998) also noted that N-terminal fusions of Sir3 were not functional. These results suggest that the N terminus of Sir3 needs to be exposed and not blocked by fusion to other proteins for full function. In this report we describe a mutational analysis of the N-terminal Ala residue of Sir3, test the acetylation state of these mutant proteins, and show that such mutants can have a profound negative effect on silencing.

Sir3 is acetylated by the NatA acetyltransferase: Strains used in this study are listed in Table 1. To determine if Sir3 is acetylated by NatA, we expressed a Sir3 fragment (amino acids 1–51) with GST fused to its C terminus in both ARD1 and ard1 mutant strains. This fusion protein was purified from yeast on glutathione beads and then subjected to N-terminal sequencing by Edman degradation. The protein from the wild-type strain gave no sequence, showing that it was blocked at its N terminus, presumably by acetylation. On the other hand, the same protein isolated from the ard1 mutant gave the sequence AKTL that corresponded exactly to the predicted N-terminal sequence of Sir3 (after cleavage of the initiating methionine). It was necessary to use N-terminal fragments of Sir3 because it was not

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The N-terminal residue of Sir3 is important for silencing: To examine the function of the N terminus of Sir3 and its acetylation state in more detail, we introduced various mutations into a plasmid-borne SIR3 gene that changed the N-terminal Ala to Ser, Gly, Thr (all potential NatA substrates), or Gln (not a NatA substrate). The plasmids were introduced into an MATa sir3Δ strain. To measure silencing at HML, we tested the transformants for their ability to mate. Figure 1A shows the results of both patch mating and quantitative mating measurements. An empty vector exhibited no mating, as expected for a sir3Δ mutant, known to be completely defective in silencing. The plasmid carrying the wild-type SIR3 gene restored good mating as did the Ala-to-Ser, -Gly, and -Thr substitutions (A2S, A2G, and A2T). The Ala-to-Gln (A2Q), on the other hand, had an almost complete loss of mating ability, indicating that silencing at HML was severely compromised. It is known that sir1 mutations exacerbate the effects of hypomorphic sir3 mutations (Stone et al. 2000). Therefore, the various SIR3 plasmids were introduced into an MATa sir3Δ sir1Δ double mutant, and mating was measured to assess silencing. In this strain, the A2S mutant still mated normally while the A2G and A2T mutations had a mating defect, demonstrating that these mutations did cause a slight silencing defect that could be detected only in the absence of SIR1. Our result with the sir3 A2T mutation confirms a result obtained previously (Stone et al. 2000).

The same SIR3 plasmids (wild type and the various Ala2 mutants) were also transformed into an MATa sir3Δ strain; in this strain mating assays are a measure of silencing at HMR. All the mutants mated as well as wild type in this strain (Figure 1B). To examine the mutants more closely for silencing defects at HMR, the plasmids were transformed into a sir1Δ derivative of the MATa sir3Δ strain. In this strain the A2S mutant behaved like wild type while the A2G and A2T, and A2Q exhibited no mating, just as was seen in the equivalent MATa sir3Δ sir1Δ strain (Figure 1, B compared to A).

To assess telomeric silencing, plasmids expressing Sir3 and the various Ala2 mutants also were transformed into a sir3Δ strain with a URA3 reporter gene placed near a telomere. The results showed that the A2S mutant was very slightly defective at this locus while the other three mutants, A2G, A2T, and A2Q, gave no silencing (Figure 1C). While the A2S mutant appeared to silence HML and HMR as well as wild-type Sir3, even in the absence of Sir1 (Figure 1, A and B), the more sensitive assay at telomeres demonstrated that this mutant is also slightly defective.

### Table 1

<table>
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<th>Strain</th>
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<td>R. Rothstein</td>
</tr>
<tr>
<td>W303-1b</td>
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<td>W303-1a sir3Δ::kanMX6</td>
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<td>W303-1b sir3Δ::kanMX6</td>
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<tr>
<td>YDS631</td>
<td>W303-1b adh4::URA3-(C1-3-A)α</td>
<td>Chen et al. (1993)</td>
</tr>
<tr>
<td>XRY16</td>
<td>YDS631 sir3Δ::kanMX6</td>
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Deletions of SIR3, SIR1, HTB2, and ARD1 were made by gene replacement using the E. coli kanMX6 gene or the S. pombe his5+ gene as described in Longtine et al. (1998).
Figure 1.—Mutations that alter the Ala2 residue of SIR3 affect silencing. Plasmids encoding wild-type Sir3 or the various sir3 mutations were introduced into the indicated strains. Details about the plasmids and how they were made are available upon request. (A) Silencing at HML as measured by mating of an MATα sir3Δ strain (JCY3) and an MATα sir3Δ sir1Δ strain (JCY8). Both patch mating and quantitative mating results are shown. (B) Silencing at HMR as measured by mating of an MATα sir3Δ strain (JCY4) and an MATα sir3Δ sir1Δ strain (JCY9). (C) Silencing at telomeres assessed in a sir3Δ strain (XRY16) with a URA3 reporter gene near a telomere. Tenfold serial dilutions of the cells were spotted on −trp medium to show the total number of cells plated and on 5-FOA medium to show the fraction of cells in which the URA3 reporter gene is silenced. Good growth on the 5-FOA plates indicates good telomeric silencing.

We also determined the acetylation state of the Sir3 N terminus for three of the mutants shown in Figure 1, the ones that changed the N-terminal Ala residue to Ser, Gly, or Thr. On the basis of previous work on NatA, all three of these amino acids are possible substrates for this N-terminal acetyltransferase (Polevoda et al. 1999). As with wild-type Sir3, we expressed, affinity purified, and sequenced proteins containing residues 1–51 of the mutant Sir3 with GST fused at the C terminus. The proteins were purified from both ARDI and ard1 strains and sequenced. The results are summarized in Table 2. It can be seen that the A2S mutant, which behaved almost like wild type in the silencing assays (Figure 1), was acetylated by NatA; i.e., the protein was blocked in the wild-type strain and gave the expected sequence SKTL in the ard1 mutant. The A2G mutant, slightly defective in silencing, was unblocked in the ARDI strain, giving the expected sequence GKTL. On the other hand, the A2T mutant, also slightly defective in silencing, was a NatA substrate. Table 2 also presents the result predicted for the A2Q mutant. Since Gln is a large residue, the Met will not be cleaved (Polevoda and Sherman 2000). Furthermore, no yeast protein beginning with the sequence Met-Gln is known to be acetylated and therefore it is highly likely that this Sir3 mutant is not acetylated at its N terminus (Polevoda and Sherman 2000, 2003).

It is striking that mutating the N-terminal Ala residue of the 977-amino-acid Sir3 protein can affect its function, in some cases causing a null phenotype. Clearly
this residue and this domain of the protein must be very important for its silencing function. Mutating the Ala residue to Ser caused a very slight defect, whereas mutating the Ala to a somewhat larger residue, Thr, caused an intermediate phenotype (Figure 1). The difference is not due to the acetylation state of the N terminus, because the Sir3 is acetylated whether the N-terminal residue is Ala, Ser, or Thr (Table 2). We think that the silencing defect of the A2T mutant is due to the larger size of the Thr residue, compared to Ala or Ser. The idea that the size of the first residue matters agrees with the result that changing the Ala to Gln (hence leading to a Met-Gln N terminus) caused an almost complete loss of function (Figure 1). Furthermore, changing the Ala to three other large amino acids, Lys, Asp, or Glu, also led to a severe loss of function (data not shown). Our initial impetus for this project was to determine the NatA substrate that is responsible for the silencing defect of nat1 or ard1 mutants. Given that Sir3 is a NatA substrate and given the importance of the N-terminal residue, it is very likely that Sir3 is a relevant substrate. Perhaps Sir3 is the only relevant substrate for silencing, as discussed below. It is instructive to note how similar the phenotypes are for an ard1 or nat1 mutant and the sir3 A2G mutant. The Sir3 A2G mutant protein is not acetylated even in an ARD1 strain (Table 2) and thus is quite similar to a wild-type Sir3 protein from an ard1 mutant. Both mutants are completely defective at telomeric silencing, partially defective at HML silencing, and not defective at HMR silencing (Whiteway et al. 1987; Mullen et al. 1989; Aparicio et al. 1991). Furthermore, the silencing defects of ard1 or nat1 mutants are greatly exacerbated by also deleting SIR1, just as is the case for all the sir3 mutations that alter the N-terminal Ala residue (Stone et al. 1991). This is particularly striking at HMR where the sir3 Ala2 mutants have no phenotype unless SIR1 is also mutated (Figure 1B).

Are silencing proteins other than Sir3 relevant NatA substrates for silencing? Certainly Orc1 might be. Orc1 is a NatA substrate, and the Orc1 N-terminal BAH domain binds Sir1 and is responsible for bringing it to the silencers (Triolo and Sterngranz 1996; Gardner et al. 1999; Zhang et al. 2002; Bose et al. 2004). On the basis of sequences of their N termini, Sir1, Sir4, and Abf1 cannot be NatA substrates (Polevoda and Sherman 2000, 2003). Rap1 has an N-terminal Ser residue and thus might very well be a NatA substrate, but it is highly unlikely to be relevant for silencing because the C terminus of the large Rap1 protein is the domain that interacts with Sir3 and Sir4. Histone H4 and H2A are acetylated on their N-terminal Ser residues by Nat4, and H3 is not acetylated, and thus those three histones cannot be relevant NatA substrates for silencing (Song et al. 2003). In work described elsewhere, we have shown that the acetylation state of H2B also does not influence silencing. We conclude that the most likely NatA substrate to explain the ard1-nat1 silencing phenotypes is Sir3. Since Orc1 has a very similar N-terminal domain (30% identity over the first 214 amino acids) and is also a NatA substrate, its acetylation state may also be important for silencing.

We have shown that both the nature of the N-terminal residue of Sir3 and its acetylation state greatly influence its ability to function. Presumably this domain of Sir3 binds to one or more proteins. It could bind to other Sir proteins in the Sir complex, to nucleosomes, or to Sir3 itself. Further experiments are needed to determine the binding partners of the Sir3 N-terminal domain.

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**LITERATURE CITED**


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