

Pnc1p-Mediated Nicotinamide Clearance Modifies the Epigenetic Properties of rDNA Silencing in *Saccharomyces cerevisiae*

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ABSTRACT

The histone deacetylase activity of Sir2p is dependent on NAD⁺ and inhibited by nicotinamide (NAM). As a result, Sir2p-regulated processes in *Saccharomyces cerevisiae* such as silencing and replicative aging are susceptible to alterations in cellular NAD⁺ and NAM levels. We have determined that high concentrations of NAM in the growth medium elevate the intracellular NAD⁺ concentration through a mechanism that is partially dependent on *NPT1*, an important gene in the Preiss–Handler NAD⁺ salvage pathway. Overexpression of the nicotinamidase, Pnc1p, prevents inhibition of Sir2p by the excess NAM while maintaining the elevated NAD⁺ concentration. This growth condition alters the epigenetics of rDNA silencing, such that repression of a *URA3* reporter gene located at the rDNA induces growth on media that either lacks uracil or contains 5-fluoroorotic acid (5-FOA), an unusual dual phenotype that is reminiscent of telomeric silencing (TPE) of *URA3*. Despite the similarities to TPE, the modified rDNA silencing phenotype does not require the SIR complex. Instead, it retains key characteristics of typical rDNA silencing, including RENT and Pol I dependence, as well as a requirement for the Preiss–Handler NAD⁺ salvage pathway. Exogenous nicotinamide can therefore have negative or positive impacts on rDNA silencing, depending on the *PNC1* expression level.

IN the budding yeast, *Saccharomyces cerevisiae*, there are three general locations that are silenced in the genome, the silent-mating type loci *HML* and *HMR*, the telomeres, and the ribosomal DNA (rDNA) (see RUSCHE *et al.* 2003 for review). Silencing at these locations is dependent on the silent information regulator genes, *SIR1–SIR4*. All four *SIR* genes are required for the efficient establishment, maintenance, and inheritance of silent chromatin structure at the *HM* loci (PILLUS and RINE 1989). *SIR2*, *SIR3*, and *SIR4* are critical for silencing at telomeres (APARICIO *et al.* 1991), but only *SIR2* is required for silencing and suppression of recombination at the rDNA (GOTTLIEB and ESPOSITO 1989; BRYK *et al.* 1997; FRITZE *et al.* 1997; SMITH and BOEKE 1997). *SIR2* encodes a highly conserved NAD⁺-dependent histone deacetylase that is predominantly localized in the nucleolus and in perinuclear foci that harbor the telomeres (GOTTA *et al.* 1997). In the nucleolus, Sir2p is a subunit of the multiprotein deacetylase complex known as RENT. This complex also contains Net1p and Cdc14p, and functions in rDNA silencing and regulation of the exit from mitosis (SHOU *et al.* 1999; STRAIGHT *et al.*

1999), as well as rDNA transcription by RNA polymerase I (SHOU *et al.* 2001). At the telomeres and *HM* loci, Sir2p is a subunit of the SIR complex, which minimally consists of Sir2p and Sir4p (GHIDELLI *et al.* 2001; TANNY *et al.* 2004). Sir3p can also be part of the complex, which is a heterotrimer when purified from insect cells (CUBIZOLLES *et al.* 2006). The sharing of Sir2p by all forms of yeast silencing leads to competition between the various compartments for a limiting amount of Sir2p (SMITH *et al.* 1998). rDNA silencing is especially sensitive to changes in Sir2p levels, as *SIR2* overexpression dramatically strengthens silencing at this locus (FRITZE *et al.* 1997; SMITH *et al.* 1998), and extends replicative life span through the suppression of rDNA recombination (GOTTLIEB and ESPOSITO 1989; KAEBERLEIN *et al.* 1999). Similarly, deletion of *SIR4* releases Sir2p from the telomeres and *HM* loci, causing it to accumulate in the nucleolus and strengthen rDNA silencing (KENNEDY *et al.* 1997; SMITH *et al.* 1998).

The Sir2p family of protein deacetylases (collectively known as sirtuins) utilize NAD⁺ as a cosubstrate. For every lysine that is deacetylated, one molecule of NAD⁺ is hydrolyzed, yielding one molecule each of nicotinamide (NAM) and O-acetyl-ADP-ribose (AAR) (LANDRY *et al.* 2000; TANNY and MOAZED 2001). The consumption of NAD⁺ implies there is a constant need for NAD⁺ production in the cell if sirtuins are to remain active. NAD⁺ production in yeast cells occurs through four

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known pathways (see Figure 1A for schematic). The *de novo* NAD⁺ synthesis pathway converts tryptophan into NAD⁺ through a series of steps also known as the kynurenine pathway that are catalyzed by products of the *BNA* genes (KUCHARCZYK *et al.* 1998). This pathway is cytosolic and does not usually contribute to rDNA silencing regulation unless nicotinic acid in the growth medium is limiting (ANDERSON *et al.* 2002; SANDMEIER *et al.* 2002). A second pathway involves the conversion of imported nicotinamide riboside (NR) into NAD⁺ via the NR kinase (Nrk1p) (BIEGANOWSKI and BRENNER 2004). A third, newly identified pathway involves direct conversion of NR into NAM by a set of NR hydrolases and phosphorylases (BELENKY *et al.* 2007). In the fourth pathway, NAM produced by sirtuin-mediated protein deacetylation or breakdown of NR is converted into nicotinic acid by the nicotinamidase, Pnc1p (GHISLAIN *et al.* 2002), and then converted back into NAD⁺ by the Preiss–Handler pathway, primarily in the nucleus (ANDERSON *et al.* 2002; SANDMEIER *et al.* 2002). The nicotinic acid phosphoribosyltransferase, Npt1p, is a critical step of this pathway (RAJAVEL *et al.* 1998; SMITH *et al.* 2000), as deletion of *NPT1* causes a two- to threefold reduction in the intracellular NAD⁺ concentration (LIN *et al.* 2000; SMITH *et al.* 2000). Collectively, Pnc1p and Npt1p are often referred to as the NAD⁺ salvage pathway, a term that we will use throughout the article.

The nicotinamide produced by Sir2 and the other sirtuins is a potent noncompetitive inhibitor of their deacetylase activity (LANDRY *et al.* 2000; BITTERMAN *et al.* 2002). As a result, researchers commonly use high concentrations of exogenous NAM in the growth medium (0.5–25 mM) to inhibit the various sirtuins in their experiments (BITTERMAN *et al.* 2002; YEUNG *et al.* 2004; TSUCHIYA *et al.* 2006). For example, inhibition of yeast Sir2p with 5 mM NAM eliminates all three forms of silencing and shortens replicative life span (BITTERMAN *et al.* 2002). Overexpression of *PNC1* suppresses these silencing defects by converting the excessive NAM into nicotinic acid (GALLO *et al.* 2004). However, NAM is also a key intermediate of both the NAD⁺ and NR salvage pathways that could potentially have positive effects on sirtuin function by influencing NAD⁺ production. In this study, therefore, we have investigated the effects of inhibitory NAM concentrations in the growth medium on yeast NAD⁺ synthesis to gain a better understanding of the relevant pathways. We find that the exogenous NAM elevates the intracellular NAD⁺ concentration partly through the Preiss–Handler and NR salvage pathways. Interestingly, when *PNC1* is overexpressed to clear the excess NAM, the increased flux through the Preiss–Handler pathway triggers an epigenetic modification in the silencing of a *mURA3* reporter gene positioned at the rDNA, such that growth occurs on both synthetic complete (SC) –ura and SC +FOA, a phenotype that is very similar to telomeric silencing of

URA3. The results strongly suggest that NAM clearance by Pnc1 stabilizes the rDNA chromatin structure.

MATERIALS AND METHODS

Strains and plasmids: Yeast media were as previously described (SMITH and BOEKE 1997; SANDMEIER *et al.* 2002). Yeast extract–peptone–dextrose (YPD) and SC media (BURKE *et al.* 2000) were supplemented with NAM at the appropriate concentrations where indicated. Counterselection against *URA3* expression was carried out in SC medium containing 0.1% (w/v) 5-FOA (Toronto Research Chemicals). All yeast strains were grown at 30°. *BNA1*, *NPT1*, *NRK1*, *PNC1*, *TNA1*, *SIR2*, *SIR3*, and *SIR4* open reading frames (ORF) were deleted and replaced with *kanMX4* using a one-step PCR-mediated gene replacement protocol (LORENZ *et al.* 1995). All gene deletions were confirmed by PCR. The genotypes of strains used in this study are listed in Table 1. Plasmids pJOE30 and pJOE31 were constructed by PCR amplification of *PNC1*, including 451 bp upstream and 339 bp downstream of the ORF, from genomic DNA (GALLO *et al.* 2004). The PCR product was digested with *XhoI* and ligated into plasmid pRS424 (*TRP1*) or pRS425 (*LEU2*). Plasmids pJSS95-3 and pFR1 were constructed by PCR amplification of the yeast *PNC1* open reading frame from genomic DNA or *Escherichia coli pncA* open reading frame from genomic DNA with *HindIII* tails on the oligonucleotides. The resulting PCR products were digested with *HindIII* and ligated into pAAH5 at the *HindIII* site downstream of the *ADHI* promoter (AMMERER 1983). All plasmids used in this study are listed in Table 2.

Silencing assays: Strains were patched onto SC medium lacking leucine, tryptophan, or both (where indicated) and allowed to grow for ~15–20 hr. Cells were resuspended in sterile water, normalized to an OD₆₀₀ of 1.0, serially diluted in fivefold increments in a 96-well plate, and then 5 µl of each dilution spotted onto the appropriate SC agar plates. To assay for telomeric silencing, cells were spotted onto SC –leucine plates to measure overall growth while selecting for a *LEU2*-containing plasmid, and SC –leucine +FOA agar plates to detect repression of a *URA3* reporter gene positioned at the left arm telomere of chromosome VII as previously described (GOTTSCHLING *et al.* 1990; SMITH *et al.* 2000). To measure rDNA silencing, strains were spotted onto SC –leu plates to measure overall growth ability, and SC –leu –ura or SC –leu +FOA plates to detect silencing of the *mURA3* reporter gene positioned 50, 300, or 600 bp left of the rDNA array where indicated (BUCK *et al.* 2002). To measure *HMR* silencing, strains were spotted onto SC –leu to evaluate overall growth ability, and SC –leu –trp to detect silencing of the *TRP1* reporter integrated into *HMR* of strain background YLS59 (SUSSEL and SHORE 1991). NAM was added to the medium where indicated. Photos of SC, SC –leu, and SC –leu –trp plates were taken after 2 days growth and photos of all –ura and FOA plates were taken after 4 days growth.

Cellular NAD⁺ measurements: Determination of relative NAD⁺ levels in various strains was performed as previously described (SMITH *et al.* 2000). Two hundred fifty milliliters yeast cultures in SC medium with the indicated supplements were grown to an OD₆₀₀ of ~1.0 and then harvested by centrifugation. Cell pellets were extracted for 30 min with 2.5 ml of ice-cold 1 M formic acid (saturated with butanol). Six hundred twenty-five microliters of 100% trichloroacetic acid (TCA) were added and incubated on ice for 15 min. The mixture was centrifuged at 4000× *g* for 20 min, and the acid soluble supernatant (containing the NAD⁺) was saved. The pellet was reextracted with 1.25 ml of 20% TCA and pelleted again. The supernatants were combined and used for the NAD⁺ measure-

TABLE 1
Yeast strains

Strain	Genotype	Figures
YSB348 ^a	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDN1(50L)::mURA3-HIS3^b</i>	1B, 6B, 6C
CGY101	YSB348 pRS425	1C, 2A, 2C, 3A, 4C, 6A
CGY102	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDN1(300L)::mURA3-HIS3</i> pRS425	3A, 3B
CGY103	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDN1(600L)::mURA3-HIS3</i> pRS425	3A, 3B
CGY111	YSB348 pJOE31	1C, 2B, 2C, 3A, 3B, 4C, 6A
CGY112	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDN1(300L)::mURA3-HIS3</i> pJOE31	3A, 3B
CGY113	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDN1(600L)::mURA3-HIS3</i> pJOE31	3A, 3B
CGY129	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDN1(50L)::mURA3-HIS3 sir2Δ::kanMX4</i> pRS425	4C
CGY130	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDN1(50L)::mURA3-HIS3 sir2Δ::kanMX4</i> pJOE31	4C
CGY132	<i>MATα his3Δ200 leu2Δ1 ura3-167 trp1Δ::mURA3-HIS3</i> pRS425	3A, 3B
CGY133	<i>MATα his3Δ200 leu2Δ1 ura3-167 trp1Δ::mURA3-HIS3</i> pJOE31	3A, 3B
CGY145	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDN1(50L)::mURA3-HIS3 npt1Δ::kanMX4</i>	6B, 6C
CGY146	CGY145 pRS425	6A
CGY147	CGY145 pJOE31	6A
CGY164	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDN1(50L)::mURA3-HIS3 sir4Δ::kanMX4</i> pRS425	4C
CGY153	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDN1(50L)::mURA3-HIS3 bna1Δ::kanMX4</i>	6B
CGY165	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDN1(50L)::mURA3-HIS3 sir4Δ::kanMX4</i> pJOE31	4C
CGY166	CGY153 pRS425	6A
CGY167	CGY153 pJOE31	6A
DSY35 ^c	<i>MATα his3Δ200 leu2Δ0 lys2Δ202 trp1Δ63 ura3-52 ADH4::URA3-TEL</i> pRS425	4A
DSY37 ^c	<i>MATα his3Δ200 leu2Δ0 lys2Δ202 trp1Δ63 ura3-52 ADH4::URA3-TEL</i> pJOE31	4A
JS1153 ^c	<i>MATα his3Δ200 leu2Δ0 lys2Δ202 trp1Δ63 ura3-52 ADH4::URA3-TEL</i> sir2Δ::kanMX4 pRS425	4A
JS1154 ^c	<i>MATα his3Δ200 leu2Δ0 lys2Δ202 trp1Δ63 ura3-52 ADH4::URA3-TEL</i> sir2Δ::kanMX4 pJOE31	4A
JM98	<i>MATα his3Δ200 leu2Δ1 met15Δ0 ura3-167 RDN1(50L)::mURA3-HIS3 hst1Δ::kanMX4</i>	6D
JM212	<i>MATα his3Δ200 leu2Δ1 met15Δ0 ura3-167 RDN1(50L)::mURA3-HIS3 nrk1Δ::kanMX4 tna1Δ::kanMX4</i>	6B
JM234	<i>MATα his3Δ200 leu2Δ1 met15Δ0 ura3-167 RDN1(50L)::mURA3-HIS3 nrk1Δ::kanMX4 bna1Δ::kanMX4</i>	6B
JM236	<i>MATα his3Δ200 leu2Δ1 met15Δ0 ura3-167 RDN1(50L)::mURA3-HIS3 nrk1Δ::kanMX4 npt1Δ::kanMX4</i>	6B
JM238	JS932 pRS425	6A
JM240	JS932 pJOE31	6A
JM242	JS944 pRS425	6A
JM244	JS944 pJOE31	6A
JM254	JM236 pRS425	6A
JM256	JM236 pJOE31	6A
JM265 ^d	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hmrΔA::TRP1</i> pRS425	4B
JM267 ^d	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hmrΔA::TRP1</i> pJOE31	4B
JM269 ^d	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hmrΔA::TRP1 sir2::HIS3</i> pRS425	4B
JM271 ^d	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 hmrΔA::TRP1 sir2::HIS3</i> pJOE31	4B
JM311	YSB348 pFR1	2C
JM313	YSB348 pJSS95-3	2C
JM315	YSB348 pAAH5	2C
JS902	<i>MATα his3Δ200 leu2Δ1 ura3-167 pnc1Δ::kanMX4</i>	6B
JS932	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDN1(50L)::mURA3-HIS3 tna1Δ::kanMX4</i>	6B
JS944	YSB348 nrk1Δ::kanMX4	6B
JS1011	<i>MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1(50L)::mURA3-HIS3</i>	6D
JS1041	<i>MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1(50L)::mURA3-HIS3 sir2Δ::kanMX4</i>	6D

(continued)

TABLE 1
(Continued)

Strain	Genotype	Figures
JS1046	JS1041 pGLC463, pRS424	5
JS1047	JS1041 pGLC463, pJOE30	5
JS1048	JS1041 pGLC26, pRS424	5
JS1049	JS1041 pGLC26, pJOE30	5
JS1050	JS1041 pGLC65, pRS424	5
JS1051	JS1041 pGLC65, pJOE30	5
JS1056	JS1041 pGLC252, pRS424	5
JS1057	JS1041 pGLC252, pJOE30	5
JS1098	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDNI(50L)::mURA3-HIS3 sir3Δ::kanMX4</i> pRS425	4C
JS1099	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDNI(50L)::mURA3-HIS3 sir3Δ::kanMX4</i> pJOE31	4C
JS1100	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDNI(PRO⁺-61L)::mURA3-HIS3</i> pRS425	2D
JS1101	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDNI(PRO⁺-61L)::mURA3-HIS3</i> pJOE31	2D
JS1102	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDNI(proΔ-61L)::mURA3-HIS3</i> pRS425	2D
JS1103	<i>MATα his3Δ200 leu2Δ1 ura3-167 RDNI(proΔ-61L)::mURA3-HIS3</i> pJOE31	2D
JS1129	YSB348 pSB766	2B, 3B
JS1133	YSB348 pRS425, pRS424	3C
JS1134	YSB348 pSB766, pRS424	3C
JS1135	YSB348 pRS425, pJOE30	3C
JS1136	YSB348 pSB766, pJOE30	3C

^a Strain described in BUCK *et al.* (2002).

^b Other parental strains with *mURA3-HIS3* located in unique sequence flanking the rDNA locus were described in BUCK *et al.* (2002).

^c Parental strain JJSy143 was described in GALLO *et al.* (2004).

^d Parental strain YLS59 was described in SUSSEL and SHORE (1991).

ment. Acid extract (150 μ l) was added to a reaction buffer (1 ml final volume) containing 300 mM Tris-HCl, pH 9.7, 200 mM lysine-HCl, 0.2% ethanol, and 150 μ g/ml alcohol dehydrogenase (Sigma). Reactions were incubated at 30° for 20 min. The absorbance was then measured at 340 nm with a Shimadzu UV-1201S spectrophotometer and the cellular NAD⁺ concentration calculated as previously described (BELENKY *et al.* 2007).

RESULTS

Exogenous nicotinamide raises the intracellular NAD⁺ concentration: NAM that is produced by the

breakdown of NAD⁺ and NR, or is imported from the growth medium (Figure 1A, pathways 2 and 4), is converted to nicotinic acid in yeast cells by the nicotinamidase, Pnc1p. Since exogenous NAM is commonly used as a sirtuin inhibitor at high (0.5–25 mM) concentrations (BITTERMAN *et al.* 2002; YEUNG *et al.* 2004; TSUCHIYA *et al.* 2006), we were interested in determining the effects of NAM addition on the intracellular NAD⁺ concentration. In an earlier study, rDNA and telomeric silencing in a *PNC1* strain were inhibited by 5 mM NAM in the growth medium, but not 0.5 mM

TABLE 2
Plasmids used in the study

Plasmid	Description	Reference
pRS425	2 μ <i>LEU2</i> shuttle vector	CHRISTIANSON <i>et al.</i> (1992)
pRS424	2 μ <i>TRP1</i> shuttle vector	CHRISTIANSON <i>et al.</i> (1992)
pJOE30	<i>PNC1</i> in pRS424	GALLO <i>et al.</i> (2004)
pJOE31	<i>PNC1</i> in pRS424	This study
pSB766	<i>SIR2</i> in pRS425	BUCK <i>et al.</i> (2002)
pGLC463	<i>CEN LEU2</i> shuttle vector	CUPERUS <i>et al.</i> (2000)
pGLC26	<i>SIR2</i> in pGLC463	CUPERUS <i>et al.</i> (2000)
pGLC65	<i>sir2-81</i> in pGLC463	CUPERUS <i>et al.</i> (2000)
pGLC252	<i>sir2-424</i> in pGLC463	CUPERUS <i>et al.</i> (2000)
pAAH5	2 μ <i>LEU2</i> expression vector	AMMERER (1983)
pJSS95-3	Yeast <i>PNC1</i> ORF in pAAH5	This study
pFR1	<i>E. coli pncA</i> ORF in pAAH5	This study

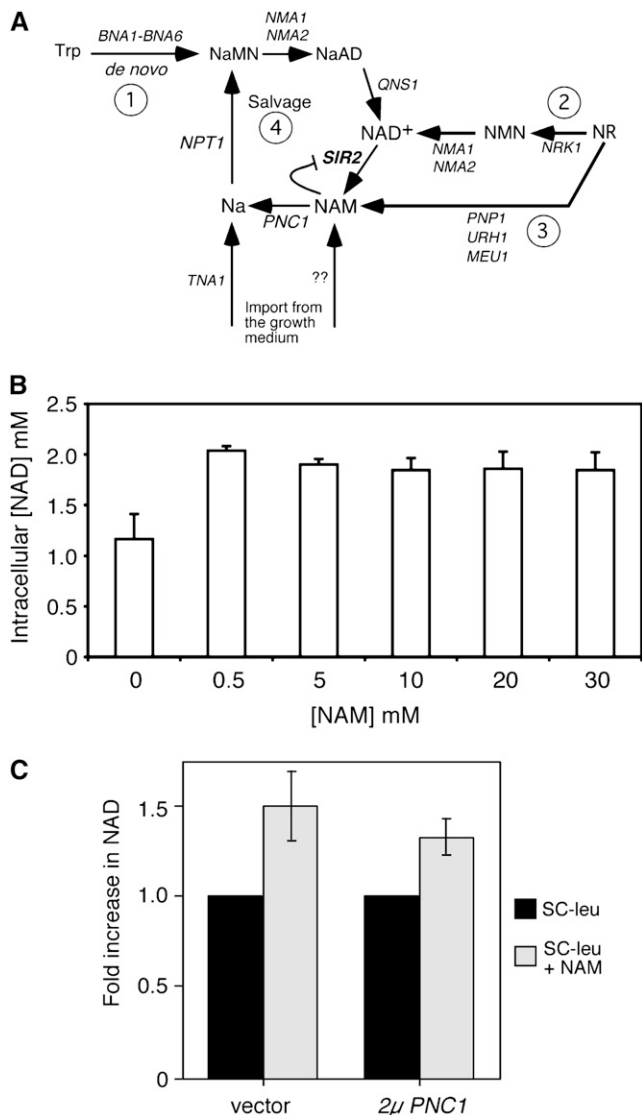


FIGURE 1.—Exogenous nicotinamide raises the intracellular NAD⁺ concentration. (A) Schematic of known NAD⁺ biosynthesis pathways in *S. cerevisiae*. (1) The *de novo* NAD⁺ synthesis pathway begins with tryptophan (Trp) and ends with the conversion of nicotinic acid adenine dinucleotide (NaAD) into NAD⁺ by the NAD synthetase, *QNS1*. (2) The nicotinamide riboside (NR) pathway begins with the import of exogenous NR and its phosphorylation by *Nrk1p* into nicotinamide mononucleotide (NMN). (3) NR can also be broken down into NAM by *Pnp1p*, *Urh1p*, and *Meu1p*. (4) The NAD⁺ salvage pathway begins with NAM produced by sirtuins or imported from the growth media and merges with the *de novo* pathway through the production of nicotinic acid mononucleotide (NaMN) by *NPT1*. Na, nicotinic acid. (B) Increase in cellular NAD⁺ concentrations caused by various concentrations of exogenous NAM in the growth medium with a WT strain (YSB348). (C) *PNC1* overexpression does not elevate the NAD⁺ level, even when clearing a high concentration of exogenous NAM (10 mM). The NAD⁺ level in the absence of nicotinamide was normalized to a value of 1.0 for each strain. Error bars represent the standard deviation from six independent experiments.

(GALLO *et al.* 2004). As shown in Figure 1B, NAM concentrations between 0.5 and 30 mM all caused a similar 30–50% increase in the intracellular NAD⁺ concentration. We hypothesized that overexpressing *PNC1* would increase flux through the salvage pathway at the higher NAM concentrations, leading to even higher NAD⁺. However, as shown in Figure 1C for 10 mM NAM, this was not the case. There was no greater increase in NAD⁺ levels for cells containing a high-copy *PNC1* plasmid compared to cells containing an empty plasmid. *Pnc1p* is therefore not a limiting factor for overall cellular NAD⁺ production from exogenous NAM at these concentrations.

Modifying the epigenetic properties of rDNA silencing: Having established that yeast can utilize exogenous NAM for NAD⁺ synthesis, we next tested whether the increased NAD⁺ levels affected Sir2p-mediated silencing activity. An increase in nuclear NAD⁺ concentration could potentially improve the histone deacetylase activity of Sir2p, leading to stronger silencing at the rDNA. To optimize the chances of detecting an increase in rDNA silencing, we utilized a reporter strain in which a modified *URA3* gene (*mURA3*) was stably integrated within unique chromosome XII sequence, 50 bp left (50L) of the tandem array (Figure 2A). Silencing at this position is easily monitored by poor growth on SC medium lacking uracil (BUCK *et al.* 2002) (Figure 2B, SC –leu –ura, 0 mM NAM). The level of *mURA3* repression that occurs with this reporter is usually not sufficient to support growth on counterselectable SC medium containing 0.1% 5-FOA, which is toxic for Ura⁺ cells (Figure 2B, SC –leu +FOA, vector). Strengthening silencing through the introduction of a 2µ *SIR2* plasmid suppressed colony formation even further on SC –leu –ura, and triggered robust colony growth on the FOA plate (Figure 2B, 2µ *SIR2*, 0 mM NAM). Therefore, a threshold of *mURA3* silencing has to be reached to develop an FOA-resistant colony with this stable reporter system, making it ideal for detecting improvements in rDNA silencing, and consequently Sir2p activity.

Enhancement of rDNA silencing (strong FOA-resistant growth) by the 2µ *SIR2* plasmid was blocked by NAM concentrations of 5 or 10 mM (Figure 2B), suggesting that any positive effect of the higher NAD⁺ levels on rDNA silencing would require clearance of the excess NAM. Introducing the 2µ *PNC1* plasmid had little effect on FOA-resistant growth with either 0 or 5 mM NAM in the media, but induced strong growth on FOA plates containing 10 mM NAM (Figure 2B, bottom row). Despite the strong growth on FOA, which was suggestive of improved silencing, the *PNC1* overexpression strain also grew well on SC –leu –ura plates containing 10 mM NAM. This phenotype closely resembled telomeric silencing of *URA3*, where growth occurs on both FOA and –ura media because there are two classes of cells in the population: one in which *URA3* is silenced and one in which it is not silenced (GOTTSCHLING *et al.* 1990).

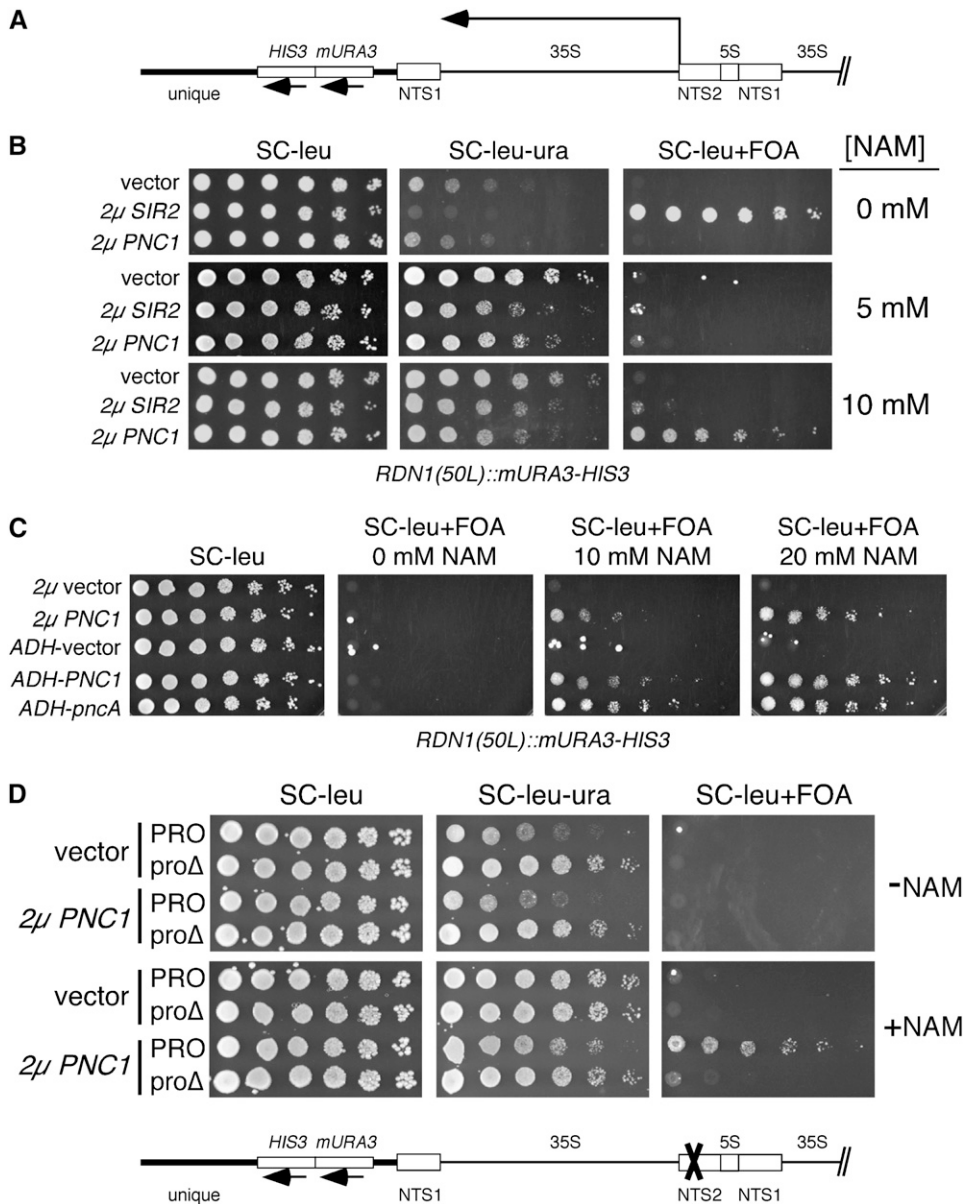


FIGURE 2.—Nicotinamide clearance alters the epigenetic properties of rDNA silencing. (A) Schematic of the centromere-proximal rDNA repeat on chromosome XII. The *mURA3-HIS3* silencing reporter cassette is integrated in unique sequence, 50 bp left (50L) of the rDNA NTS1 region. The directions of Pol I and Pol II transcription are indicated by horizontal black arrows. (B) rDNA silencing assay in which the 50L::*mURA3-HIS3* silencing reporter strain (YSB348) was transformed with an empty *LEU2* vector (pRS425) or a 2 μ *PNC1* vector (pJOE31). The concentration of NAM in the plates is indicated. (C) rDNA silencing assay showing the FOA^R phenotype in the presence of 0, 10, or 20 mM NAM. YSB348 was transformed with pRS425, pJOE31, an empty *ADH1*-promoter vector (pAAH5), or the same *ADH* vector expressing *PNC1* (pJSS95-3), or *E. coli pncA* (pFR1). (D) Requirement of modified rDNA silencing for RNA Pol I transcription. A control strain (PRO+; YSB505) and a strain deleted for the leftmost Pol I promoter (pro Δ ; YSB509) were transformed with the pRS425 vector or pJOE31 *PNC1* vector. The *mURA3-HIS3* reporter was located 61 bp left of the tandem array. An X in the schematic indicates the location of the promoter deletion. The addition of 10 mM nicotinamide is indicated as +NAM.

Ribosomal DNA silencing may, therefore, take on this telomere position effect (TPE)-like epigenetic characteristic under the *PNC1* overexpression/high NAM growth condition.

Since 10 mM NAM produced the strong FOA^R phenotype, but 5 mM NAM did not, we tested whether an even higher NAM concentration would improve the effect. Twenty millimolar NAM consistently produced growth on FOA that was stronger than with 10 mM (Figure 2C). However, concentrations of ≥ 30 mM produced nonspecific growth defects even when *PNC1* was overexpressed (data not shown). Overexpression of the *E. coli pncA* gene, a close homolog of yeast *PNC1*, was sufficient to produce the strong FOA^R phenotype in either 10 or 20 mM NAM (Figure 2C), suggesting that the nicotinamidase activity of the enzyme is the key trigger of silencing activity, not some other intrinsic

property of yeast Pnc1p. Despite causing an equivalent increase in NAD⁺ concentration, clearance of 0.5 or 5 mM NAM was not sufficient to induce the FOA^R silencing phenotype (Figure 2B and data not shown), suggesting that very high flux through the Preiss–Handler pathway is critical. Throughout the rest of the article we will refer to this 2 μ *PNC1*/10 mM NAM-induced FOA^R resistant phenotype as “modified rDNA silencing.”

Silencing of *mURA3* positioned in the unique sequence flanking the leftmost rDNA repeat was earlier shown to require transcription by Pol I, such that deleting the Pol I promoter of the leftmost repeat (Figure 2D, schematic) prevented silencing of the downstream *mURA3* reporter (BUCK *et al.* 2002). We used this promoter-deletion strain (pro Δ) to test whether modified rDNA silencing was also dependent on Pol I transcription. As shown in Figure 2D, without the addition of

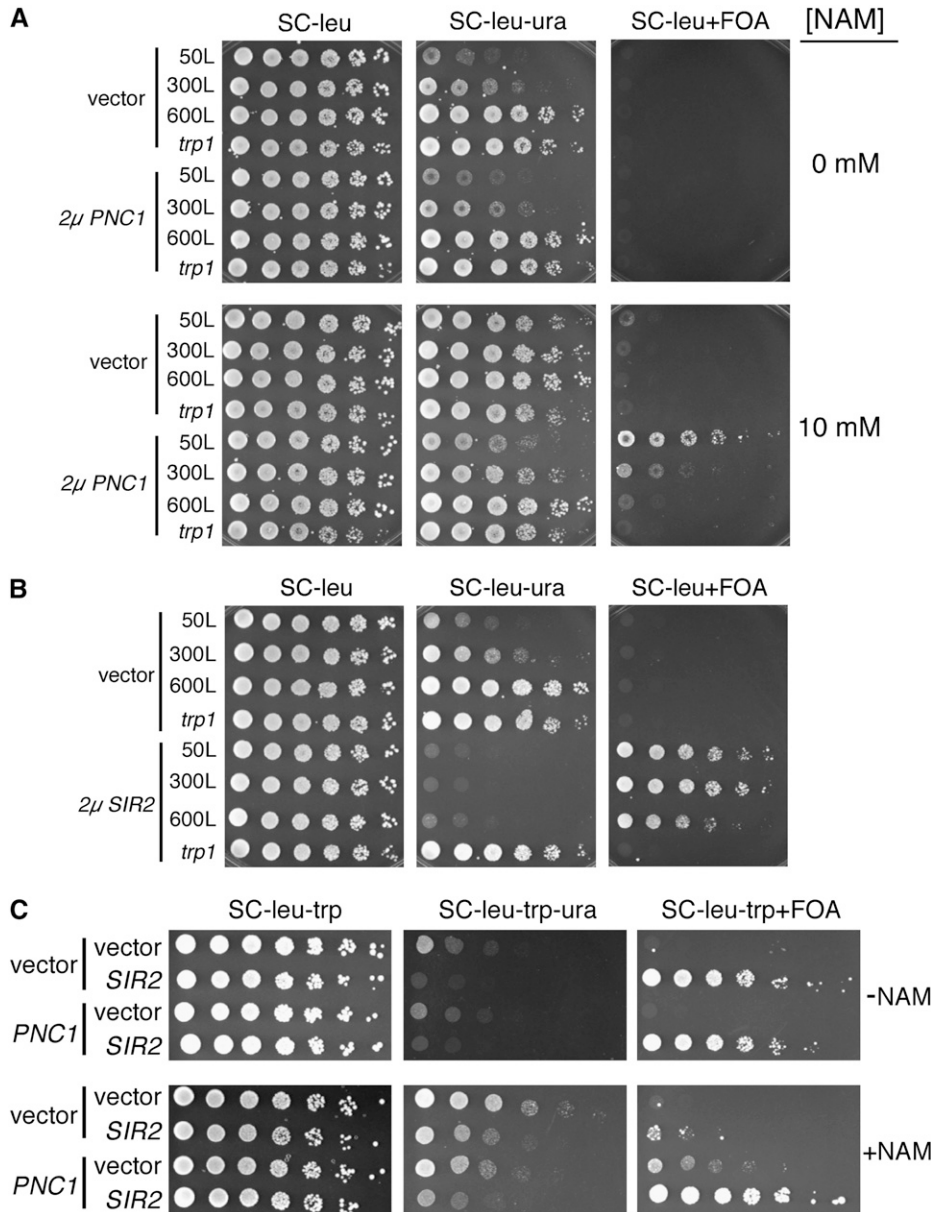


FIGURE 3.—Spreading of modified rDNA silencing. (A) rDNA silencing in which the *mURA3-HIS3* cassette was positioned 50 bp (50L), 300 bp (300L), or 600 bp (600L) away from the rDNA array or at the nonsilenced *TRP1* locus on chromosome IV. Plates contained 0 or 10 mM NAM, and the strains harbored either the empty pRS425 vector or the 2μ *PNC1* vector (pJOE31). (B) Effects of *SIR2* overexpression on rDNA silencing at the 50L, 300L, 600L, and *TRP1* locations. NAM was not added. (C) Effect of overexpressing *SIR2* (pSB766) and *PNC1* (pJOE30) at the same time on rDNA silencing in the absence or presence of 10 mM NAM. The *mURA3-HIS3* reporter was positioned at the 50L position.

NAM, silencing was lost in the promoter-deletion strain as expected (SC –leu –ura, vector/pro Δ). When NAM was added, FOA-resistant growth was observed only when *PNC1* was overexpressed in the control PRO⁺ strain, but not in the pro Δ strain (Figure 2D, bottom row). These results indicate that modified rDNA silencing retains the defining property of Pol I dependence.

Spreading of modified rDNA silencing: Typical rDNA silencing is also characterized by its ability to unidirectionally spread from the rDNA toward the centromere, although without *SIR2* overexpression, the spreading progresses only <600 bp. Overexpressing *SIR2* extends the spreading to at least 2000 bp beyond the rDNA (BUCK *et al.* 2002). Since modified rDNA silencing and *SIR2* overexpression both induced a strong FOA-resistant phenotype when *mURA3* was

positioned 50 bp (50L) from the rDNA (Figure 2), it was possible that modified rDNA silencing was also spreading beyond the 50L position. We therefore tested the effect of modified rDNA silencing of *mURA3* positioned 300 bp (300L) and 600 bp (600L) away from the rDNA (Figure 3A). The FOA-resistant phenotype was easily observed at the 300L position when *PNC1* was overexpressed in the presence of NAM, but was extremely weak at the 600L position. This was very different from the effect of overexpressing *SIR2* in the absence of NAM, which easily spread silencing to the 600L position (Figure 3B). Modified rDNA silencing, therefore, does not appear to spread from the rDNA any more efficiently than typical rDNA silencing. Importantly, the NAM/*PNC1* effect on *mURA3* was specific for the rDNA location because the *mURA3* expression was

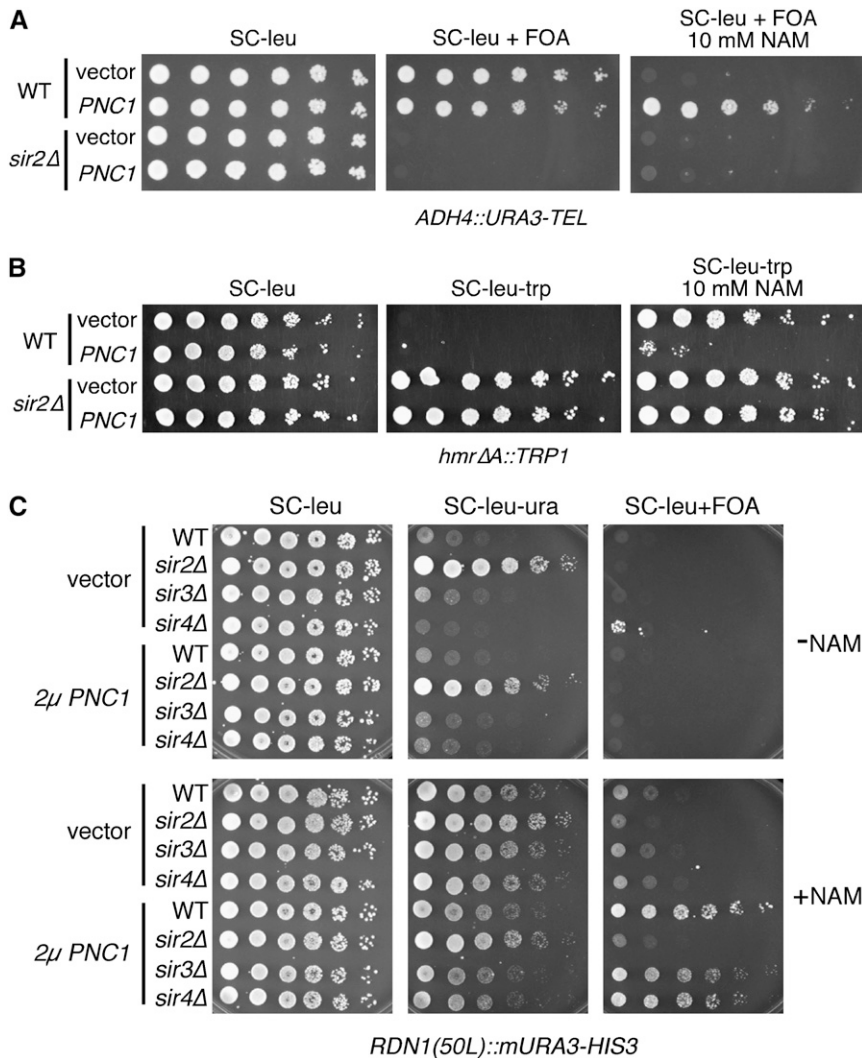


FIGURE 4.—Modified rDNA silencing does not disturb *HM* and telomeric silencing, but requires *SIR2*. (A and B) Telomeric and *HM* silencing are not significantly weakened or strengthened by 10 mM NAM coupled with *PNC1* overexpression. (A) Fivefold serial dilutions of WT and *sir2Δ* strains containing pRS425 or pJOE31. The *URA3* gene is silenced by an artificial telomere formed at the *ADH4* locus. Silencing is indicated by growth on 5-FOA. (B) Fivefold serial dilutions of strain YLS59 (WT) and MC119 (*sir2Δ*) containing either pRS425 vector or pJOE31 (*PNC1*). The *TRP1* gene is silenced at the *HMR* locus. Silencing is indicated by lack of growth on SC –leu –trp plates. (C) Effects of deleting *SIR2*, *SIR3*, or *SIR4* on rDNA silencing in the presence of 10 mM nicotinamide. The empty vector (pRS425) or *PNC1* overexpression vector (pJOE31) was transformed into rDNA reporter strains that were deleted for *SIR2* (YSB408), *SIR3* (CGY135), or *SIR4* (CGY152). Fivefold serial dilutions were spotted.

unaffected when it was positioned at the nonsilenced *TRP1* locus (Figure 3, A and B).

As mentioned above, growth on both FOA and –ura media is an epigenetic characteristic of *URA3* telomeric silencing. The similarities with modified rDNA silencing suggested that NAM clearance by Pnc1p may have stabilized the silent rDNA chromatin state without significantly increasing the percentage of these cells in the population. We hypothesized that increasing the *SIR2* dosage would stabilize the silenced phenotype even more and perhaps shift more cells in the population toward the silenced state. To test this possibility we overexpressed *PNC1* and *SIR2* at the same time in the presence of 10 mM NAM. As shown in Figure 3C, the addition of 2μ *SIR2* produced an even stronger FOA-resistant phenotype than 2μ *PNC1* alone. Importantly, it also reduced the proportion of cells that were able to grow on plates lacking uracil (SC –leu –trp –ura), which is consistent with more cells being in the silenced state.

Modified rDNA silencing does not occur at the expense of telomeric and *HMR* silencing: The telomeric and rDNA silenced domains compete for a limited pool

of Sir2p (SMITH *et al.* 1998). Therefore, we reasoned that the modified rDNA silencing phenotype could involve Sir2p mobilization from telomeres or the *HM* loci to the rDNA, resulting in weakened telomeric or *HM* silencing. To test this idea, we analyzed the effect of *PNC1* overexpression and 10 mM NAM on TPE and *HM* silencing (Figure 4, A and B). As expected, 10 mM NAM in the growth medium caused a loss of TPE when the empty vector was present, as indicated by a lack of growth on the SC –leu +FOA (10 mM NAM) plate (Figure 4A, top row). *PNC1* overexpression restored normal levels of silencing on the NAM plate in a *SIR2*-dependent manner (Figure 4A), indicating there was no significant loss of TPE under the same condition that induced FOA resistance for rDNA silencing. Similarly, *PNC1* overexpression almost completely restored silencing of a *TRP1* reporter gene integrated at the *HMR* locus in the presence of 10 mM NAM (Figure 4B, SC –leu –trp + 10 mM NAM plate). The modified rDNA silencing phenotype in this growth condition is therefore probably not caused by a large-scale redistribution of Sir2 from telomeres or the *HM* loci to the rDNA.

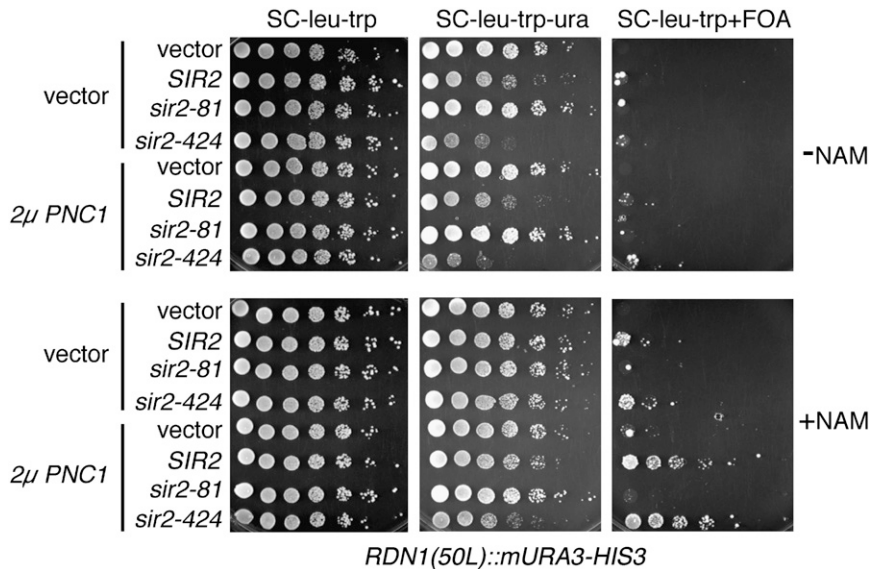


FIGURE 5.—Modified rDNA silencing is dependent on the RENT, but not the SIR complex. The effects of expressing a telomere/*HM* silencing-defective *sir2* mutation (*sir2-424*, class I) and rDNA silencing-defective mutation (*sir2-81*, class II) on rDNA silencing in the presence of 10 mM NAM and *PNC1* overexpression. The strain background was JS1041, which lacks the endogenous *SIR2* gene and has the *50L::mURA3-HIS3* reporter gene.

Modified rDNA silencing is dependent on Sir2p and RENT, but not the SIR complex: Another defining characteristic of typical rDNA silencing is dependence on Sir2p, but not the other Sir proteins (SMITH and BOEKE 1997). Since the modified rDNA silencing phenotype resembled telomeric silencing of *URA3*, it was feasible that the SIR complex was being utilized for rDNA silencing under this growth condition. To test this possibility, we deleted *SIR2*, *SIR3*, or *SIR4* from the *50L::mURA3* rDNA silencing reporter strain. As shown in Figure 4C, deletion of *SIR2* derepressed silencing of *mURA3* as measured by robust *Ura*⁺ growth either with or without 10 mM NAM added. As expected, deleting *SIR2* also prevented strong FOA resistance when *PNC1* was overexpressed in the presence of 10 mM NAM (Figure 4C, +NAM). However, deleting *SIR3* or *SIR4* had little effect. These mutations instead improved the repression phenotype on SC –leu –ura when NAM was cleared by *PNC1* overexpression, which was previously observed with typical rDNA silencing (SMITH and BOEKE 1997).

To determine whether the defect in modified rDNA silencing caused by the *sir2* deletion reflected a defect in RENT, we tested the effects of *sir2* point mutations that inhibited either the RENT or SIR complex. Low-copy *LEU2 CEN* plasmids expressing either wild-type *SIR2* or mutant versions of *sir2* that were specifically defective in either telomeric/*HM* silencing (class I mutant) or rDNA silencing (class II mutant) (CUPERUS *et al.* 2000) were transformed into a *sir2Δ* reporter strain. A *TRP1* 2 μ *PNC1* plasmid (pJOE30) or empty vector (pRS424) was then introduced such that the strains harbored two selectable plasmids on SC –leu –trp media. As shown in Figure 5 (–NAM, top row), the *sir2Δ* mutant was again defective for silencing when transformed with the empty vectors, as indicated by full growth on the SC –leu –trp –ura plate. The wild-type *CEN SIR2* plasmid restored some silencing on SC –leu –trp –ura, but not enough

to trigger FOA resistance. The class II mutant (*sir2-81*) did not strengthen silencing at all in the absence of NAM (Figure 5, –NAM), which was consistent with this type of *sir2* mutant being rDNA silencing defective (CUPERUS *et al.* 2000). The class I mutant (*sir2-424*) restored silencing even better than wild type (WT) (Figure 5, –NAM), most likely due to redistribution of telomeric/*HM* Sir2p to the nucleolus (CUPERUS *et al.* 2000), which also occurs in *sir4Δ* mutants (GOTTA *et al.* 1997; KENNEDY *et al.* 1997; SMITH *et al.* 1998; CUPERUS *et al.* 2000). When 10 mM NAM was added to the plates and *PNC1* was overexpressed, the WT *SIR2* and *sir2-424* (class I) mutant produced the FOA-resistant silencing phenotype (Figure 5, +NAM). No FOA resistance was observed with *sir2-81* (class II mutant). Combined with the *sir3Δ* and *sir4Δ* phenotypes (Figure 4C), these results demonstrate that the modified form of rDNA silencing is similar to typical rDNA silencing in that it utilizes RENT, but not the SIR complex. Despite the epigenetic similarities, modified rDNA silencing retains the key rDNA-specific properties that differentiate rDNA silencing from TPE and *HM* silencing.

Nicotinamide effects on rDNA silencing are mediated by the Preiss–Handler pathway: Efficient rDNA silencing was previously shown to be dependent on the Preiss–Handler pathway, of which the Npt1 protein is partially localized in the nucleus (ANDERSON *et al.* 2002; SANDMEIER *et al.* 2002). The FOA-resistant phenotype of modified rDNA silencing was shown in this study to be dependent on the clearance of high NAM concentrations by Pnc1, a key step of the salvage pathway (Figure 2). Therefore, we tested whether Npt1, or other salvage and biosynthesis enzymes, was required for development of the FOA-resistant phenotype. Deleting *BNA1*, *TNA1*, or *NRK1* caused little effect compared to WT (Figure 6A). In contrast, deleting *NPT1* completely eliminated growth on FOA in the presence of NAM,

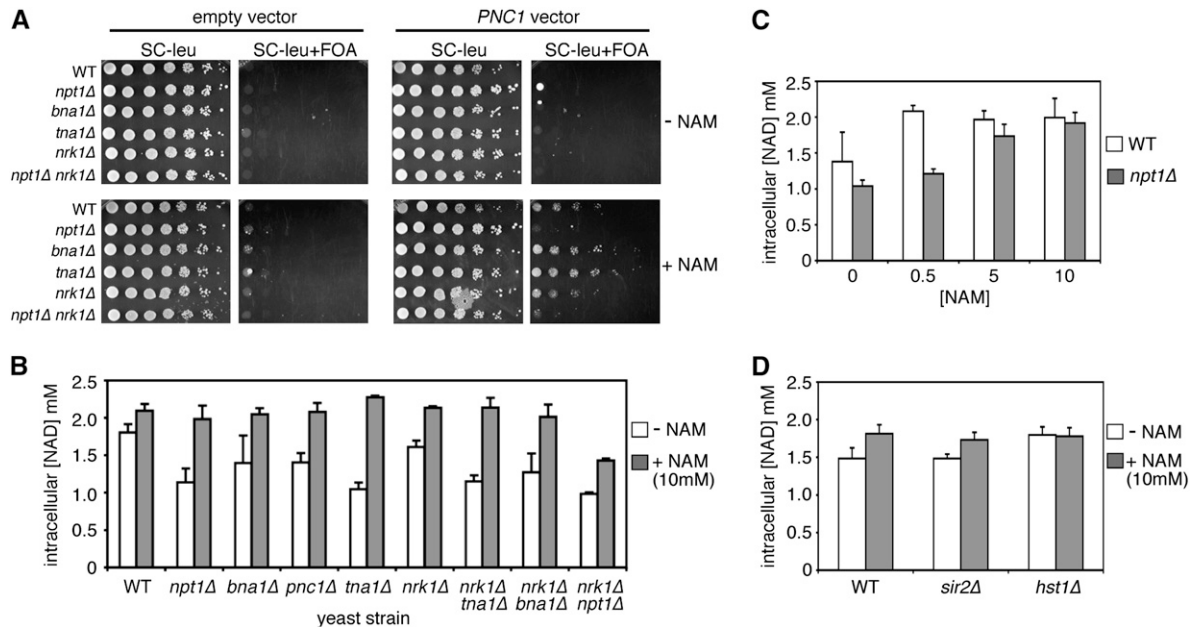


FIGURE 6.—Effects of NAD⁺ salvage and biosynthesis mutants on modified rDNA silencing and intracellular NAD⁺ concentrations. (A) Modified rDNA silencing in WT (YSB348), *npt1Δ* (CGY145), *bna1Δ* (CGY153), *tna1Δ* (JS932), *nrk1Δ* (JS944), and *npt1Δ nrk1Δ* (JM236) mutants. The reporter gene is *RDN1(50L)::mURA3-HIS3*. (B) NAD⁺ concentrations in the various NAD⁺ biosynthesis and salvage mutants when grown in the presence or absence of 10 mM NAM. (C) *NPT1* is required for increasing NAD⁺ levels when cells are grown in low concentrations (0.5 mM) of NAM. (D) Intracellular NAD⁺ concentrations in WT (JS1011), *sir2Δ* (JS1041), and *hst1Δ* (JM98) strains in the presence or absence of 10 mM NAM.

suggesting that the nicotinic acid produced by Pnc1p must pass through the Preiss–Handler pathway to induce the modified rDNA silencing phenotype.

Deleting *NPT1* was proposed to inhibit silencing by reducing the nuclear pool of NAD⁺ (ANDERSON *et al.* 2002; SANDMEIER *et al.* 2002). We therefore predicted that the absence of *NPT1* or *PNC1* would block the increase in NAD⁺ caused by the addition of 10 mM NAM. But surprisingly, these genes were not required for the elevation in NAD⁺ (Figure 6B). In fact, no single mutations in the NAD⁺ salvage/synthesis genes that we tested blocked the increase (Figure 6B). We hypothesized that Npt1p was utilizing a specific proportion of the excess NAM in the nucleus to impact silencing, but that the very high concentrations saturated the cellwide Preiss–Handler pathway. To test this hypothesis we examined whether *NPT1* was required for the increase in NAD⁺ caused by NAM concentrations <10 mM. As shown in Figure 6C, the lack of *NPT1* did block the elevation in NAD⁺ caused by 0.5 mM NAM, indicating that *NPT1* is capable of converting a relatively small proportion of external NAM into NAD⁺, but that higher concentrations overwhelm the system.

It was likely that another redundant pathway(s) was responsible for the rest of the NAM at higher concentrations. Deleting *NRK1* by itself did not block the increase in NAD⁺ (Figure 6B) and had little effect on modified rDNA silencing (Figure 6A). So we next tested whether there was any redundancy between *NRK1* and the other pathways in utilizing the high NAM concen-

trations. Interestingly, the *npt1Δ nrk1Δ* double mutant partially blocked the increase in NAD⁺ caused by the addition of 10 mM NAM (Figure 6B), suggesting that part of the effect is mediated by NR when the Preiss–Handler pathway is blocked. The remainder of the NAD⁺ increase could potentially come from the inhibition of Hst1 by NAM. Deleting the yeast *HST1* gene was previously shown to cause an increase in cellular NAD⁺ concentration through the activation of genes in the *de novo* NAD⁺ synthesis pathway (BEDALOV *et al.* 2003). Hst1 normally functions as a repressor of these genes (BEDALOV *et al.* 2003). As shown in Figure 6D, deletion of *HST1* did increase the NAD⁺ concentration under this growth condition, but the addition of 10 mM NAM did not cause an additional increase. Taken together these results suggest that there are redundant pathways (both direct and indirect) in which yeast cells utilize NAM to raise the intracellular NAD⁺ concentration. The results also support a model for Sir2p-dependent heterochromatin formation in the yeast rDNA, in which flux through the Preiss–Handler NAD⁺ salvage pathway (perhaps in the nucleus) leads to activation of the nuclear sirtuin, Sir2p.

DISCUSSION

The epigenetics of rDNA silencing: The rDNA is an unusual locus in that it is highly transcribed by RNA polymerase I, yet Pol II-transcribed genes that are

endogenous to the rDNA or artificially integrated into the rDNA are silenced in a *SIR2*- and Pol I-dependent manner (BRYK *et al.* 1997, 2002; FRITZE *et al.* 1997; SMITH and BOEKE 1997; CIOCI *et al.* 2003). Silencing at the *HM* loci and telomeres is stably maintained and inherited through a process that requires not only *SIR2*, but also *SIR3* and *SIR4*, which together encode the SIR complex (LOO and RINE 1995). Other factors involved in the maintenance include chromatin assembly factor (CAF) and PCNA (MONSON *et al.* 1997; ENOMOTO and BERMAN 1998; ZHANG *et al.* 2000). Silencing at these locations is characterized by the formation of two independent chromatin states, one repressive to transcription and the other not repressive. These two states can occasionally switch, especially with telomeric silencing and *HM* silencing when *SIR1* is deleted (PILLUS and RINE 1989; GOTTSCHLING *et al.* 1990).

It is not clear whether silencing in the rDNA involves two independent chromatin states or is the average of a mixture of varying degrees of repression (SMITH and BOEKE 1997). Whatever defines the silent state, it is not thought to be stably maintained from cell to cell due to the dynamic and stochastic nature of DNA replication and Pol I transcription within the tandem array, two processes that promote rDNA silencing (SMITH *et al.* 1999; BUCK *et al.* 2002; CIOCI *et al.* 2003). Only about one in five rDNA repeats are replicated in any given S phase (LINSKENS and HUBERMAN 1988; FANGMAN and BREWER 1991), and ~50% of the rDNA genes are transcribed by Pol I at any given cell cycle (DAMMANN *et al.* 1993). Moreover, the distribution of the active genes within the tandem array is stochastic (DAMMANN *et al.* 1995). Since the Pol I transcriptional status of a single rDNA repeat can influence the silencing of an adjacent reporter gene (BUCK *et al.* 2002), there is an ~50% chance that a given reporter gene will be silenced, which could change at each cell cycle. As a result, when silencing of a color-indicator reporter gene such as *MET15* is monitored, instead of colonies with large sectors of brown (silenced) and white (not silenced) cells, a tan color is observed, suggesting that the silent state is not stable enough to be inherited over multiple generations (SMITH and BOEKE 1997). A similar phenomenon is observed with silencing of a telomeric *ADE2* reporter gene. In a WT strain, large sectors of silenced (red) and nonsilenced (white) cells are typically observed. But when the maintenance of silencing is impaired by deletion of the *CAC1* gene, an overall pink colony color develops (MONSON *et al.* 1997).

If a *URA3* reporter is placed next to a telomere, there are cells in the population that are not silencing *URA3* and grow on SC –ura and cells that are silencing *URA3* that grow only on FOA plates. The result is the ability of the cell population to grow on both SC –ura and SC +FOA. Such a situation clearly does not exist in the typical rDNA silencing population. Silencing of *mURA3* when positioned next to the rDNA (the 50L position) is

characterized by poor growth on SC –ura plates (silencing), but no growth on SC +FOA plates. The combination of overexpressing *PNC1* with high NAM concentrations in the growth medium modified the epigenetic pattern of rDNA silencing, resulting in growth on both SC –ura and FOA plates. Since this *mURA3* reporter is not subjected to the high recombinational loss rates that plague reporters integrated within the rDNA array (data not shown), we propose that this growth condition sufficiently stabilizes the silent rDNA chromatin state in a subpopulation of cells to allow growth of a colony on FOA. At the same time, a large proportion of cells in the same population remained in a nonsilenced state, allowing them to grow on SC –ura plates. *SIR2* overexpression also induces strong silencing of the *mURA3* reporter gene, such that the cells can grow on FOA, but in this case, the population of cells that are not silencing *mURA3* is greatly reduced. This is reminiscent of telomeric silencing, in which the population of cells silencing *URA3* can be increased by overexpressing *SIR3* (RENAULD *et al.* 1993). *SIR2* overexpression was able to do the same thing with the *PNC1*/NAM-induced modified rDNA silencing, shifting the cell population toward the FOA-resistant phenotype (Figure 3C). Future work will utilize this system to investigate the inheritance properties of rDNA silencing.

Stabilizing rDNA silencing possibly by increasing flux through the Preiss–Handler NAD⁺ salvage pathway: The similarities between modified rDNA silencing and telomeric silencing of *URA3* initially suggested that the SIR complex was functioning in the rDNA. Sir3p had previously been shown to weakly interact with the rDNA (HOPPE *et al.* 2002), which we have also observed (C. GALLO and M. MATECIC, unpublished data). However, deleting *SIR3* or *SIR4* had little effect on the FOA-resistant phenotype. Additionally, only *sir2* mutations that affect the RENT complex (class II mutants), and not the SIR complex (class I) were deficient in modified rDNA silencing. Other key characteristics of typical rDNA silencing were retained, including the requirement for Pol I transcription and the ability to spread outward from the rDNA into adjacent unique sequence. These results strongly suggest that the combination of *PNC1* overexpression and high NAM concentrations alters the function of Sir2p within the RENT complex, although we cannot rule out the possibility that another unidentified repressive factor is also contributing.

Supplementing the yeast growth media with NAM raises the intracellular NAD⁺ concentration (this study), and *PNC1* overexpression prevents the exogenous NAM from inhibiting Sir2p (ANDERSON *et al.* 2003a; GALLO *et al.* 2004). We initially hypothesized that this combination of NAM clearance and high NAD⁺ concentration was triggering the FOA-resistant silencing phenotype. However, we did not observe the FOA-resistant phenotype at NAM concentrations of ≤5 mM,

even though the overall intracellular NAD⁺ levels remained elevated. Coupled with the absolute requirement of *NPT1*, these results instead suggest that the modified rDNA silencing phenotype may involve increased flux through the Preiss–Handler NAD⁺ salvage pathway, likely in the nucleus. Such a model for Sir2p activation via Npt1 and Pnc1 has also been proposed for typical rDNA silencing and the extension of replicative life span, in which increasing the copy number of *NPT1* or *PNC1*, or calorie-restriction growth conditions strengthens silencing and extends life span without measurably increasing the overall NAD⁺ concentration (ANDERSON *et al.* 2002, 2003a,b). It is expected that this modified rDNA silencing phenotype will be a useful tool for investigating nuclear NAD⁺ production in yeast.

How does exogenous nicotinamide raise the intracellular NAD⁺ concentration of yeast cells? The only known pathway in *S. cerevisiae* that can convert exogenous NAM into NAD⁺ is the Pnc1p/Npt1p-mediated NAD⁺ salvage pathway. This statement is supported by the finding that a *qns1Δ* mutant, which blocks both the *de novo* NAD⁺ synthesis and the salvage pathways (Figure 1A), is inviable when provided with exogenous NAM, but survives when given nicotinamide riboside (BIEGANOWSKI and BRENNER 2004). We have observed similar results with a *bnal1Δ npt1Δ* double mutant, which is inviable even in the presence of 10 or 20 mM NAM, but lives with 10 μM NR (data not shown). While not measurable due to its inviability, presumably there is very little NAD⁺ produced in the *bnal1Δ npt1Δ* mutant, implying that the amount of NAD⁺ produced by exogenous NAM outside of these pathways is not sufficient to maintain cell growth. Alternatively, the NAM-derived pool of NAD⁺ may not be utilized efficiently by essential NAD⁺-dependent processes such as glycolysis and the TCA cycle.

It was therefore surprising that the addition of 10 mM NAM to the growth medium elevated the intracellular NAD⁺ concentration by 30–50%, even when *NPT1* or *PNC1* was deleted (Figure 6B). At lower NAM concentrations, *NPT1* was fully required for the increase in NAD⁺ (Figure 6C), suggesting that the high NAM may induce one or more cryptic pathways. Yeast and most eukaryotic organisms lack a nicotinamide phosphoribosyltransferase enzyme (NAMPT), which in mammals converts NAM into nicotinamide mononucleotide (NMN) (REVOLLO *et al.* 2004). Since mammals lack a nicotinamidase homolog of Pnc1p, the NAMPT enzyme also likely serves to clear the inhibitory nicotinamide that would otherwise accumulate in the cell. In these organisms, the NMN is converted to NAD⁺ by the universally conserved nicotinamide mononucleotide adenyltransferases (encoded by *NMA1* and *NMA2* in yeast) (ANDERSON *et al.* 2002). NAMPT was reported as the rate-limiting step in NAD⁺ biosynthesis for mouse NIH3T3 cells because the addition of 5 mM NAM did not increase NAD⁺ levels, but overexpression of the

transferase gene did cause an increase (REVOLLO *et al.* 2004). However, an independent study showed that adding 5 or 10 mM NAM would raise the NAD⁺ level of normal human fibroblasts (KANG *et al.* 2006). In *Salmonella typhimurium*, the nicotinic acid phosphoribosyl transferase PncB (Npt1p in yeast) was identified as the limiting step of the Preiss–Handler pathway (IMSANDE 1964). Our data is consistent with Npt1p also being a limiting factor of the pathway in yeast, except at high NAM concentrations, where other cryptic pathways are involved, including one utilizing Nrk1p. NR is not a component of standard yeast growth media, but it was recently determined that the NR salvage enzymes as a group (Nrk1p, Pnp1p, and Urh1p) contribute to NAD⁺ biosynthesis even in the absence of supplemented NR, suggesting that NR is not only a vitamin, but also a metabolite (BELENKY *et al.* 2007).

Nicotinamide can also raise the NAD⁺ concentration through an indirect mechanism by inhibiting Hst1. However, this mechanism is unlikely to play a role in formation of the modified rDNA silencing phenotype because *PNC1* overexpression prevents exogenous NAM from inhibiting Hst1 (GALLO *et al.* 2004). Given the importance of NAD⁺ as a key energy currency for the cell, it makes sense that there would be multiple pathways for the cell to respond to nicotinamide, which is both an NAD⁺ precursor and inhibitor of NAD⁺ consuming enzymes such as the sirtuins. However, there also appears to be a limit to the amount of NAD⁺ that the cell can synthesize in response to NAM. Despite the multiple mutants and NAM concentrations utilized in this study, the maximum NAD⁺ concentration we observed was always close to 2 mM and likely reflects a high degree of homeostasis for this critical cellular cofactor.

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