Telomeric and rDNA Silencing in *Saccharomyces cerevisiae* Are Dependent on a Nuclear NAD$^+$ Salvage Pathway

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ABSTRACT

The Sir2 protein is an NAD$^+$-dependent protein deacetylase that is required for silencing at the silent mating-type loci, telomeres, and the ribosomal DNA (rDNA). Mutations in the NAD$^+$ salvage gene *NPT1* weaken all three forms of silencing and also cause a reduction in the intracellular NAD$^+$ level. We now show that mutation of a highly conserved histidine residue in Npt1p results in a silencing defect, indicating that Npt1p enzymatic activity is required for silencing. Deletion of another NAD$^+$ salvage pathway gene called *PNC1* caused a less severe silencing defect and did not significantly reduce the intracellular NAD$^+$ concentration. However, silencing in the absence of *PNC1* was completely dependent on the import of nicotinic acid from the growth medium. Deletion of a gene in the *de novo* NAD$^+$ synthesis pathway *BNA1* resulted in a significant rDNA silencing defect only on medium deficient in nicotinic acid, an NAD$^+$ precursor. By immunofluorescence microscopy, Myc-tagged Bna1p was localized throughout the whole cell in an asynchronously growing population. In contrast, Myc-tagged Npt1p was highly concentrated in the nucleus in ~40% of the cells, indicating that NAD$^+$ salvage occurs in the nucleus in a significant fraction of cells. We propose a model in which two components of the NAD$^+$ salvage pathway, Pnc1p and Npt1p, function together in recycling the nuclear nicotinamide generated by Sir2p deacetylase activity back into NAD$^+$.

TRANSCRIPTIONAL silencing in the budding yeast *Saccharomyces cerevisiae* occurs within the silent mating-type loci (*HML* and *HMR*; for review see Loo and Rine 1995), telomeres (Gottschling *et al.* 1990), and the ribosomal DNA (rDNA) locus (Bryk *et al.* 1997; Smith and Boeke 1997). These loci are believed to form a heterochromatin-like structure that somehow prevents RNA polymerases and/or various recombination enzymes from gaining access to the associated DNA (Gottschling 1992; Loo and Rine 1994; Fritze *et al.* 1997; Smith and Boeke 1997). Each form of silencing is fully dependent on the *SIR2* gene, which encodes an NAD$^+$-dependent histone/protein deacetylase with *in vitro* specificity for lysine 16 in the N-terminal tail of histone H4 and lysines 9 and 14 of histone H3 (Imai *et al.* 2000; Landry *et al.* 2000b; Smith *et al.* 2000). Mutations in *SIR2* that reduce histone deacetylation activity *in vitro* strongly weaken silencing *in vivo* (Imai *et al.* 2000), suggesting that one of the major functions of Sir2p in silencing is histone deacetylation. This is supported by a study showing that *SIR2* overexpression results in global histone hypoacetylation (Braunstein *et al.* 1993). However, the *in vivo* target(s) of Sir2p-mediated deacetylation in yeast have not been identified.

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Sir2p is the founding member of a highly conserved protein family with homologs identified in all three kingdoms of life (Brachmann *et al.* 1995; Derbyshire *et al.* 1996). In *S. cerevisiae* there are five family members, including Sir2p. The others, Hst1p, Hst2p, Hst3p, and Hst4p, have been implicated in silencing, targeted gene repression (Xie *et al.* 1999), and maintenance of genomic stability (Brachmann *et al.* 1995). There are also at least seven homologs in human cells (Brachmann *et al.* 1995; Afshar and Murnane 1999; Frye 1999). It is improbable that all the Sir2-like proteins will be involved in regulating chromatin structure. For example, the CobB protein in Salmonella has been implicated in the synthesis of vitamin B12 (Tsang and Escalante-Semerena 1998). However, for most homologs tested so far, including CobB, NAD$^+$-dependent HDAC activity has been identified (Imai *et al.* 2000; Landry *et al.* 2000b; Smith *et al.* 2000). It is therefore likely that nonhistone proteins are also *in vivo* targets for Sir2-like proteins, especially since an increasing number of nonhistone proteins are known to be acetylated (Kouzarides 2000; Sterner and Berger 2000).

The deacetylase activity of Sir2p not only requires NAD$^+$ for its activity, but also directly consumes NAD$^+$ (Landry *et al.* 2000a; Tanny and Moazed 2001). For every acetyl group removed from a lysine residue, one molecule of NAD$^+$ is hydrolyzed to form one molecule of nicotinamide (Nam; Tanny and Moazed 2001). The acetyl group is transferred from the lysine residue to the

ADP-ribose moiety of NAD^+ to produce one molecule of the unusual compound, *O*-acetyl-ADP-ribose (Tanner *et al.* 2000; Tanny and Moazed 2001). The reaction products are a mixture of 2′ and 3′ *O*-acetyl isomers (Sauve *et al.* 2001). Sir2p and its family members therefore have the potential for consuming a large amount of NAD^+.

The NAD^+ dependence of Sir2p raises the interesting possibility that cellular processes regulated by Sir2p, such as silencing and aging, are influenced by changes in cellular NAD^+ concentration. Indeed, mutations in the *NPT1* gene reduce cellular NAD^+ concentrations by approximately threefold and also cause a loss of rDNA and telomeric silencing (Smith *et al.* 2000). *NPT1* is also required for the extension of yeast cell life span when induced by caloric restriction (Lin *et al.* 2000). NAD^+ is produced by at least two pathways in all organisms, a *de novo* pathway and one or more salvage pathways (Penfound and Foster 1996). Npt1p is highly related to the PncB nicotinic acid phosphoribosyltransferase (NAPRTase) of *Salmonella typhimurium* that is responsible for carrying out the last step of the Preiss-Handler NAD^+ salvage pathway (Lalo *et al.* 1993; Rajavel *et al.* 1998). *In vitro*, it catalyzes the formation of nicotinic acid mononucleotide (NaMN) from nicotinic acid (NA) and 5-phosphoribosyl-1-pyrophosphate (PRPP; Rajavel *et al.* 1998).

From previous studies it was unclear whether the silencing and aging phenotypes associated with *npt1* mutations were directly caused by the reduction in NAD^+ concentration or something more complex (Lin *et al.* 2000; Smith *et al.* 2000). In this study, we therefore set out to more closely examine the role of Npt1p in silencing and to also test the relative contributions of the *de novo* and NAD^+ salvage pathways in regulating silencing. In the process we discovered that the *de novo* NAD^+ synthesis pathway was not essential for silencing, but that the salvage pathway in general was important. We also found that Npt1p is often highly concentrated within the nucleus, whereas the *de novo* NAD^+ synthesis protein, Bna1p, always appears evenly distributed throughout the cell, suggesting that the NAD^+ salvage pathway has an important nuclear function. Surprisingly, deletion of another salvage gene, *PNC1*, significantly weakened silencing without reducing the overall intracellular NAD^+ concentration. However, silencing in the absence of *PNC1* was completely dependent on nicotinic acid import from the growth medium. On the basis of these findings we present a model in which Pnc1p and Npt1p cooperate in the conversion of the nuclear nicotinamide generated by Sir2p back into NAD^+.

MATERIALS AND METHODS

Media and yeast strains: Yeast media were as previously described (Rose *et al.* 1990; Cost and Boeke 1996; Smith and Boeke 1997). All yeast growth was at 30°. SC medium contained 3.25 μM nicotinic acid (Difco 1998). When indicated, SC medium was supplemented with an additional 10 μM nicotinic acid. SC medium contained a limited concentration of adenine to allow development of a red colony color in the telomeric silencing assays.

NPT1, *BNA1*, and *TNA1* were deleted from several strains using one-step PCR-mediated gene replacement (Baudin *et al.* 1993; Lorenz *et al.* 1995; Smith *et al.* 1998). pRS400 (*kanMX4*) or pRS403 (*HIS3*) were used as templates for the PCR (Brachmann *et al.* 1998). DNA fragments for deleting *QPT1* and *PNC1* were produced by PCR amplification of *kanMX4* from genomic DNA isolated from strains that were already *qpt1*Δ*::kanMX4* or *pnc1*Δ*::kanMX4* (Winzeler *et al.* 1999). Three hundred base pairs of flanking DNA were included on either side of *kanMX4*. The heterozygous diploid JS664 was constructed by mating the *bna1*Δ*::kanMX4* strain JS663 to the *npt1-1* mutant JS646, which contains a *Tn3::LacZ::LEU2* transposon insertion in the C terminus of *NPT1* (Smith *et al.* 2000). The resulting diploid was sporulated and tetrads were dissected on YPD medium. Yeast strains and their genotypes are listed in Table 1.

Plasmid construction: Genomic clones of *NPT1* and *BNA1* were constructed by amplifying the genes from a highly purified *S. cerevisiae* genomic DNA preparation (Bachman *et al.* 2001), using Vent DNA polymerase (New England Biolabs, Beverly, MA). To facilitate cloning, *Xho*I sites were incorporated into each end of the PCR fragment. The resulting PCR fragments were ligated into the *Xho*I site of pRS414 (Christianson *et al.* 1992) to produce pJSS77 (*NPT1*) and pJOE22 (*BNA1*). The cloned *NPT1* gene consisted of −400 to +250 bp surrounding the open reading frame (ORF). The cloned *BNA1* gene consisted of −400 to +253 bp surrounding the ORF. To fuse the 5 × Myc tag in frame with *NPT1* or *BNA1*, we used the Stratagene (La Jolla, CA) QuikChange site-directed mutagenesis method to initially introduce an *Nco*I site immediately downstream of the start codon, thus changing the second codon. For *NPT1*, serine 2 was changed to an alanine. For *BNA1*, phenylalanine 2 was changed to valine. The 5 × Myc tag was isolated as an *Nco*I-*Nco*I fragment from pAS90 (kindly provided by Doug Koshland, via Bob Skibbens) and then ligated into the engineered N-terminal *Nco*I sites of *NPT1* and *BNA1*. The Myc-Npt1 vector was pJOE4, and the Myc-Bna1 vector was pJOE29. The 2μ plasmid versions were pJOE3 (2μ Myc-Npt1p) and pJOE28 (2μ Myc-Bna1p). The QuikChange method was also used to change His232 of Npt1p in pJSS77 to an asparagine residue in pJSS86. pTW1 was constructed by removing the *NPT1* C terminus (including His232) from pJOE4 by excising a *Not*I-*Nsi*I fragment and replacing it with the equivalent *Not*I-*Nsi*I fragment from pJSS86. Plasmid descriptions are in Table 2.

Multiple alignments: The entire open reading frames of Npt1-like proteins from *Drosophila melanogaster* (19%), *Caenorhabditis elegans* (15%), *Arabidopsis thaliana* (19%), *Homo sapiens* (18%), *Escherichia coli* (25%), *S. typhimurium* (25%), *Borrelia burgdorferi* (17%), *S. cerevisiae*, and *Archaeoglobus fulgidus* (9%) were aligned using ClustalW software and presented using Boxshade. The numbers in parentheses are percentage identity to the *S. cerevisiae* Npt1 protein. To identify the gene encoding for the human version of Npt1p, a BLAST search was carried out between the Drosophila protein sequence and the human expressed sequence tag (EST) database. Multiple ESTs were identified. The GenBank accession numbers were AI871064, AW081501, AA612667, AA069101, AA100897, and AI925574. A putative human ORF (with some gaps) was created and then compared to the working draft of the human genome using BLAST (Lander *et al.* 2001). The gene is present on chromosome 8 at nucleotide positions 146382485 to 146385926. The gap-free protein sequence provided is from I.M.A.G.E. clone 3957135.

Silencing assays: Two different rDNA silencing assays were

TABLE 1

Yeast strains

Strain	Genotype
JS306[a]	*MAT***a** *his3*Δ*200 leu2*Δ*1 met15*Δ*0 trp1*Δ*63 ura3-167 RDN1::Ty1-MET15, mURA3-HIS3*
JS740	JS306 pRS425
JS742	JS306 pSIR2μ
JS663	JS306 made *bna1*Δ*::kanMX4*
JJSY54	JS663 pJOE28
JS673	JS306 made *npt1*Δ*::kanMX4*
JS744	JS673 pRS425
JS746	JS673 pSIR2μ
JJSY51	JS673 pJOE3
JS804	JS306 made *pnc1*Δ*::kanMX4*
JS805	JS306 made *qpt1*Δ*::kanMX4*
JS860	*MAT***a** *his3*Δ*200 leu2*Δ*1 met15*Δ*0 trp1*Δ*63 ura3-167 sir2*Δ*::kanMX4 RDN1::Ty1-MET15, mURA3-HIS3* pRS425
JS646	*MAT*α *his3*Δ*200 leu2*Δ*1 met15*Δ*0 trp1*Δ*63 ura3-167 npt1-1*
JS664	*MAT***a***/MAT*α *his3*Δ*200/his3*Δ*200 leu2*Δ*1/leu2*Δ*1 met15*Δ*0/met15*Δ*0 trp1*Δ*63/trp1*Δ*63 ura3-167/ura3-167 bna1*Δ*::kanMX4/BNA1 NPT1/npt1-1 RDN1::Ty1-MET15, mURA3-HIS3/RDN1*
JS692[b]	*MAT***a** *ade2-101 his3*Δ*200 leu2*Δ*1 lys2-801 trp1*Δ*63 ura3-52 npt1*Δ*::kanMX4 TEL-VR::ADE2*
JS702	JS692 pRS414
JS703	JS692 pJSS77
JS704	JS692 pJSS86
YCB647[b]	*MAT***a** *his3*Δ*200 leu2*Δ*1 lys2*Δ*202 trp1*Δ*63 ura3-52 leu2*Δ*::TRP1 ADH4::URA3-TEL*
JJSY2	JS692 pJOE4
TWY2	JS692 pTW1
JS641	YCB647 made *npt1*Δ*::kanMX4*
JS807	YCB647 made *pnc1*Δ*::kanMX4*
JS809	YCB647 made *bna1*Δ*::kanMX4*
JS811	YCB647 made *qpt1*Δ*::kanMX4*
JS813	YCB647 made *tna1*Δ*::kanMX4*
JS861	YCB647 made *pnc1*Δ*::kanMX4 tna1*Δ*::HIS3*
BY4741[c]	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*
SY8[d]	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 bna1*Δ*::kanMX4*
SY10[d]	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 pnc1*Δ*::kanMX4*
SY15[d]	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 qpt1*Δ*::kanMX4*
SY16[d]	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 npt1*Δ*::kanMX4*

[a] Smith *et al.* (1999).
[b] Smith *et al.* (2000).
[c] Brachmann *et al.* (1998).
[d] Winzeler *et al.* (1999).

used (Smith *et al.* 1998). To assay for silencing of *mURA3* in the rDNA, strains were grown overnight on YPD or SC medium as patches. These patches were then replica plated to SC, SC-Ura, or modified lead acetate (MLA) plates. The rDNA silencing color assay for expression levels of the *MET15* gene was performed by streaking freshly grown cells onto Pb^{2+}-containing MLA medium (Cost and Boeke 1996) and allowing the plates to grow for 5 days at 30° before photos were taken under a Leica stereoscopic microscope.

Two different telomeric silencing (TPE) assays were used. To measure silencing of *URA3* adjacent to the left telomere of chromosome VII, cells were patched on YPD or selective SC media and grown for 1 day. The cells were then scraped off the plates and resuspended in sterile water. The cell mixtures were normalized to an A_{600} of 1.0 and then serially diluted in fivefold increments. Five microliters of each dilution was spotted onto SC medium, SC medium lacking uracil (SC-Ura), or SC with 1% of 5-fluoroorotic acid (5-FOA) added. Plates were incubated at 30° for 2 days before photography. Loss of silencing activates expression of *URA*3, preventing growth on 5-FOA. The colony color TPE assay is based on the *ADE2* reporter integrated next to the right telomere of chromosome V. Reporter strains are plated onto SC medium, which contains a limiting concentration of adenine. The cells turn red if they silence *ADE2*, but turn white if they do not silence *ADE2*. Switching between on and off states results in the characteristic sectored colonies (Gottschling *et al.* 1990).

NAD^+ concentration measurements: NAD^+ measurements were performed as previously described (Smith *et al.* 2000). Briefly, 500-ml yeast cultures were grown to an A_{600} of ~1.0 and then harvested by centrifugation. Cell pellets were extracted for 30 min with 5 ml of ice-cold 1 M formic acid (saturated with butanol). A total of 1.25 ml of 100% trichloroacetic acid (TCA) was 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 washed with 2.5 ml of 20% TCA and pelleted again. The supernatants were combined and used for the NAD^+ measurement. Acid extract (150 μl) 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, 150 μg/ml alcohol dehydrogenase (Sigma, St. Louis). Reactions were incubated at 30° for 20 min. The absorbance was then measured at 340 nm and compared to a standard curve.

TABLE 2

Plasmids

Plasmid	Description	Source
pRS425	2μ *LEU2* shuttle vector	Christianson *et al.* (1992)
pRS414	*CEN TRP1* shuttle vector	Christianson *et al.* (1992)
PRS424	2μ *TRP1* shuttle vector	Christianson *et al.* (1992)
pSIR2μ	pRS425 containing *SIR2*	Smith *et al.* (1998)
pJSS77	pRS414 containing *NPT1*	This study
pJOE3	pRS424 containing 5 × -Myc-tagged *NPT1*	This study
pJOE4	pRS414 containing 5 × -Myc-tagged *NPT1*	This study
pJOE22	pRS414 containing *BNA1*	This study
pJOE28	pRS424 containing 5 × -Myc-tagged *BNA1*	This study
PJOE29	PRS414 containing 5 × -Myc-tagged *BNA1*	This study
pJSS86	pRS414 containing H232N *npt1*	This study
pTW1	pRS414 containing 5 × Myc-H232N *npt1*	This study

Indirect immunofluorescence microscopy: A total of 10 ml of exponentially growing yeast cells (A_{600} of 0.5 to 1.0) was fixed for 90 min in 3.7% formaldehyde. Cells were recovered by centrifugation and washed three times in 1 ml SK buffer (1 M sorbitol, 50 mM KPO_4 pH 7.5) with a final resuspension in 900 μl SK buffer. Spheroplasts were prepared by adding 100 μl 10× Zymolyase cocktail (0.1% β-mercaptoethanol, 0.34 mg/ml Zymolyase-20) and incubating cells at 37° for 25 min. Cells were recovered by microcentrifugation, washed twice with 1 ml SK buffer, and resuspended in 250 μl SK buffer. Fixed spheroplasts were dropped onto 0.1% polylysine-treated 10-well glass slides and incubated in a humid chamber for 10 min. Slides were washed with SK buffer and then immersed in methanol (−20°) and acetone (−20°). Blocking solution (1× PBS, 0.1% BSA, 0.1% NaN_3, 10 mM $MgSO_4$, 10 mM $CaCl_2$) was placed in each well and incubated at room temperature for 15 min. The blocking solution was removed and the primary antibody (Roche α-c-myc, monoclonal 9E10) was added at a 1:1000 dilution and incubated in a humid chamber for 2 hr. SK buffer was used to wash each well eight times. Secondary antibody (Cy3-conjugated goat α-mouse IgG) was then added at a dilution of 1:1000 and slides were incubated for another 2 hr in a humid chamber. SK buffer was used to wash each well eight times. Mounting medium was Vectashield w/DAPI (H-1200; Vector Laboratories, Burlingame, CA). The slides were viewed and photographs were taken on a Nikon Eclipse E600 using the 100× objective.

RESULTS

Two NAD^+ synthesis pathways in yeast: To more clearly understand the role of NAD^+ in the regulation of silencing, we first wanted to test the validity of the current models for synthesizing NAD^+ in yeast. Eukaryotic NAD^+ synthesis is generally divided into two separate pathways, salvage and *de novo* (Figure 1A). The salvage pathway begins with the breakdown of NAD^+ into Nam and ADP-ribose. In the case of Sir2p-mediated deacetylase activity, the ADP-ribose byproduct is actually a mixture of 2′ and 3′ *O*-acetyl-ADP-ribose (Sauve *et al.* 2001). The nicotinamide is then deamidated by a nicotinamide deamidase to form NA and ammonia. The nicotinamide deamidase in *E. coli* is PncA (Frothingham *et al.* 1996). Through a BLAST search we identified *YGL037C* as the equivalent *S. cerevisiae* gene. This gene was recently assigned the name *PNC1* by the *Saccharomyces* Genome Database. Npt1p then converts NA to NaMN (Rajavel *et al.* 1998). At this point the salvage pathway merges with the *de novo* pathway to convert NaMN to NAD^+ through the sequential action of nicotinamide mononucleotide adenylyltransferases (*YLR328W* and *YGR010W*) and NAD^+ synthetase (*YHR074W*; Emanuelli *et al.* 1999). Nicotinic acid can also be imported into the cell through the action of the high affinity nicotinic acid plasma membrane permease encoded by *TNA1* (*YGR260W*; Llorente and Dujon 2000) and then presumably converted to NaMN by Npt1p (Figure 1A).

The *de novo* pathway in yeast begins with tryptophan, which through multiple enzymatic steps is converted to NaMN. In yeast, two of the *de novo* pathway genes are *BNA1* (*YJR025C*) and *QPT1* (*YFR047C*; Kucharczyk *et al.* 1998; Imai *et al.* 2000). Mutants for *bna1*, *qpt1*, *pnc1*, and *npt1* were all viable on rich YPD and SC media, indicating that they all have the capacity to make NAD^+ if provided with the proper nutrients. Strains that are deleted for *BNA1* are known to be nicotinic acid auxotrophs that are unable to grow on medium lacking nicotinic acid (Kucharczyk *et al.* 1998). Based on the proposed metabolic scheme in Figure 1A, only *de novo* pathway mutants should be nicotinic acid auxotrophs because they need nicotinic acid to produce NAD^+ through the salvage pathway. As predicted, the *bna1*Δ and *qpt1*Δ mutants were nicotinic acid auxotrophs, but the *npt1*Δ and *pnc1*Δ mutants were not (Figure 1B). We tested deletions of two other predicted *de novo* pathway genes, kynurenine 3-hydroxylase (*YBL098W*) and kynureninase (*YLR231C*), and as expected, these were also nicotinic acid auxotrophs (data not shown).

The metabolic scheme in Figure 1A also predicted that combining a *de novo* pathway gene mutation with a salvage pathway gene mutation would be lethal. To test this hypothesis, we crossed a *bna1*Δ*::kanMX4* strain with a strain carrying the *npt1-1* mutation (tagged with

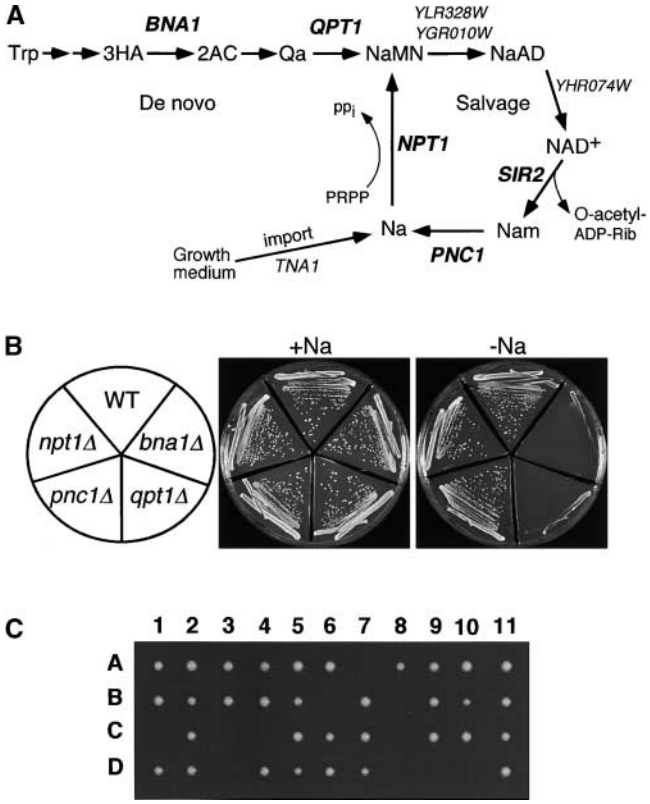

FIGURE 1.—Genetic analysis of NAD^+ synthesis in *S. cerevisiae*. (A) Schematic diagram outlining the *de novo* and salvage pathways for NAD^+ synthesis. The *de novo* pathway starts with tryptophan (Trp) and proceeds through four steps to 3-hydroxyanthranilic acid (3HA). Bna1p converts 3HA to 2-amino-3-carboxymuconic semialdehyde (2AC), and Qpt1p converts quinolinic acid (Qa) to nicotinic acid mononucleotide (NaMN). In the salvage pathway, the Nam produced by Sir2p is converted to NaMN by Pnc1p and Npt1p. Npt1p can also utilize nicotinic acid (Na) imported into the cell by the nicotinic acid permease (Tna1p). PRPP, 5-phosphoribosyl-1-pyrophosphate; NaAD, deamido NAD. (B) Nicotinic acid auxotrophy of *bna1*Δ and *qpt1*Δ mutants. WT (BY4741), *bna1*Δ (SY8), *qpt1*Δ (SY15), *pnc1*Δ (SY10), and *npt1*Δ (SY16) strains were streaked for single colonies on minimal medium either missing nicotinic acid (–Na) or containing 3.25 μM nicotinic acid (+Na). Colonies were grown for 3 days. (C) Synthetic lethality between *bna1*Δ and *npt1-1* mutations. Eleven tetrads of diploid JS664 were dissected into four individual spores (A, B, C, and D) on YPD medium. Phenotypes of the viable spores were consistent with synthetic lethality.

the *Tn3::lacZ::LEU2* insertion) and dissected tetrads from the resulting diploid strain. We observed 25% spore inviability, as predicted for two mutations lethal in combination (Figure 1C). The genotype of 16/18 dead spores was inferred to be *npt1-1 bna1*Δ*::kanMX4*. The double mutant spores arrested as four- to six-cell microcolonies. Moreover, out of 21 tetrads dissected, we never observed a single viable G418-resistant (*kanMX*-containing) Leu^+ spore clone. Synthetic lethality was also observed when the *bna1*Δ*::kanMX4* allele was combined with an *npt1*Δ*::kanMX4* allele (data not shown). Consistent with our findings, *npt1*Δ *qpt1*Δ double mutants were previously shown to have a severe slow growth phenotype (LIN *et al.* 2000). It is currently unclear why the *qpt1*Δ *npt1*Δ mutant combination was not completely lethal, but there could potentially be an unknown reaction that can weakly carry out the conversion of quinolinic acid to NaMN. We conclude that *NPT1* and *BNA1* represent genes in separate pathways required for NAD^+ synthesis, confirming our predicted view of NAD^+ synthesis in yeast and providing a framework for the following silencing experiments.

The NAD^+ salvage pathway is important for rDNA and telomeric silencing: Mutations in *NPT1* were already known to cause a loss of silencing at the rDNA and telomeres (SMITH *et al.* 2000). However, we did not know whether this was a general defect of the NAD^+ salvage pathway or an *npt1*Δ-specific phenotype. We also wanted to know whether the *de novo* NAD^+ synthesis pathway had any role in silencing. We therefore deleted *NPT1*, *PNC1*, *BNA1*, or *QPT1* from the rDNA silencing reporter strain JS306, which contained a *MET15* colony color reporter integrated into the rDNA nontranscribed spacer (SMITH *et al.* 1999). *MET15* is repressed by the rDNA, resulting in a tan colony color on rich medium containing Pb^{2+} (SMITH and BOEKE 1997). As expected (SMITH *et al.* 2000), deletion of *NPT1* caused a loss of silencing phenotype as indicated by a white colony color background and multiple dark brown sectors (Figure 2A). The *pnc1*Δ mutant had a similar loss of silencing phenotype, although it was less severe in that very few sectors were observed (Figure 2A). The entire NAD^+ salvage pathway is therefore important for rDNA silencing, not just *NPT1*. In contrast, the *bna1*Δ and *qpt1*Δ mutants produced a colony color similar to the wild-type (WT) strain, indicating that the *de novo* NAD^+ synthesis pathway is not required for rDNA silencing (Figure 2A). Since *npt1* mutants also dramatically weaken telomeric silencing, we tested whether the *pnc1*, *bna1*, or *qpt1* deletions affected TPE. As shown in Figure 2B, the *pnc1* deletion derepressed a telomeric *URA3* reporter gene by ~25-fold compared to WT, which was not as dramatic as the *npt1*Δ defect, but was similar to the intermediate defect observed with rDNA silencing. Deletions of *BNA1* or *QPT1* had no effect on TPE (Figure 2B).

The above results were surprising because elimination of the *de novo* NAD^+ synthesis pathway was expected to reduce intracellular NAD^+ levels and thus weaken silencing. We therefore measured NAD^+ in all four mutants grown in YPD medium. As expected, the *npt1*Δ mutant lowered the intracellular NAD^+ concentration by approximately threefold compared to the WT strain (Figure 2C). NAD^+ levels in the *bna1*Δ and *qpt1*Δ mutants were unchanged compared to the WT parent (Figure 2C), which was consistent with the lack of a silencing defect. Given its defects in rDNA and telomeric silencing, we expected the *pnc1*Δ mutant to have a reduced intracellular NAD^+ concentration. Surprisingly, we found that the mutant actually had a near-normal NAD^+ con-

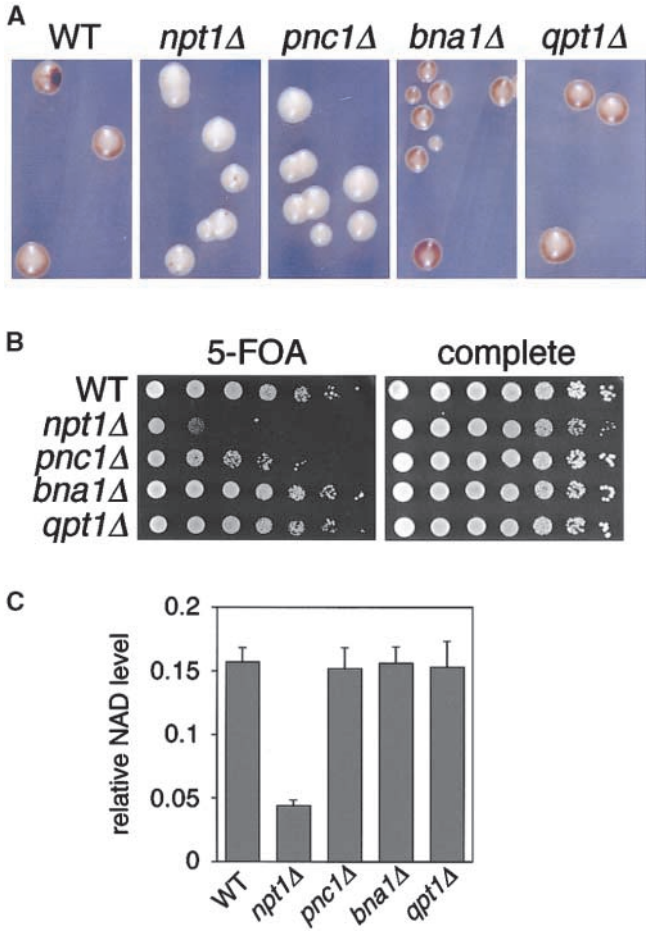

FIGURE 2.—Silencing phenotypes of *de novo* and salvage pathway mutants. WT (JS306), *npt1*Δ (JS673), *pnc1*Δ (JS804), *bna1*Δ (JS663), and *qpt1*Δ (JS805) strains were tested for rDNA silencing, telomeric silencing, and cellular NAD^+ concentration. (A) rDNA silencing phenotypes using a colony color assay. Lighter colony colors indicate a loss of rDNA silencing. (B) Telomeric silencing phenotypes using a telomeric *URA3* reporter gene. Loss of silencing is indicated by less growth on 5-FOA. (C) Relative intracellular NAD^+ concentrations. Values on the *y*-axis are the mean absorbance at 340 nm. Error bars indicate standard deviation from three independent experiments.

centration (Figure 2C). The phenotypic differences between *npt1*Δ and *pnc1*Δ mutants are addressed further below.

Regulation of rDNA silencing by nicotinic acid concentration: As described above, *de novo* pathway mutants were unable to grow in medium lacking nicotinic acid. The activity of Npt1p, which converts nicotinic acid to NaMN, allows these mutants to make NAD^+ from an exogenous source of nicotinic acid imported by Tna1p. We hypothesized that if nicotinic acid levels became limiting in a *bna1* mutant, Npt1p activity would become impaired due to reduced substrate concentration, possibly resulting in an rDNA silencing defect. To test this possibility, we first pregrew patches of WT (JS306) and *bna1*Δ (JS663) strains on either YPD, SC, or SC with excess nicotinic acid added (SC + NA). These patches were then replica plated to SC, SC-Uracil, or Pb^{2+} (MLA) media (Figure 3A). Compared to WT, silencing of the *mURA3* and *MET15* rDNA silencing reporters in the *bna1*Δ mutant was reduced when the strains were pregrown on SC medium (Figure 3A; more Ura^+ growth and lighter-colored patch, respectively). As expected, no difference was observed when the strains were pregrown on YPD medium prior to replica plating. This result suggested that there was a limiting nutrient in SC medium that was required for rDNA silencing. We surmised that this nutrient was nicotinic acid, which is present at 3.25 μM in normal SC medium. When pregrown on SC medium supplemented with an additional 10 μM nicotinic acid (13.25 μM final concentration), silencing was restored to the replica plated *bna1*Δ mutant (Figure 3A; note reduced Ura^+ growth and a darker-colored patch).

We next tested whether the silencing defect of a *bna1*Δ mutant on SC medium correlated with a reduction in NAD^+ concentration. Interestingly, the wild-type cells (JS306) grown on SC medium contained ~33% less NAD^+ compared to YPD (Figure 3B). There was also an additional 25% reduction in NAD^+ concentration in the *bna1*Δ mutant (JS663) compared to WT when grown on SC (Figure 3B), consistent with the loss of silencing observed in Figure 3A. When SC was supplemented with excess nicotinic acid, the NAD^+ concentration in the WT and *bna1*Δ strains increased to a level comparable to growth on YPD (Figure 3B). For the *npt1*Δ mutant (JS673), the NAD^+ concentration was low for all growth conditions. These results indicate that in the absence of the *de novo* NAD^+ synthesis pathway, rDNA silencing is highly sensitive to changes in environmental nicotinic acid concentrations.

***SIR2* overexpression can restore rDNA silencing to an *npt1*Δ mutant:** Sir2p is a limiting component of rDNA silencing, and its overexpression improves silencing (FRITZE *et al.* 1997; SMITH *et al.* 1998). We therefore hypothesized that *SIR2* overexpression would not strengthen rDNA silencing in an *npt1*Δ mutant due to the suboptimal intracellular NAD^+ concentration. WT and *npt1*Δ strains containing a high-copy *SIR2* vector were tested for increased rDNA silencing strength using a patch-replica-plating assay (Figure 4A). In this assay, increased silencing is measured by reduced Ura^+ growth. As expected from previous studies (SMITH *et al.* 1998), the *SIR2* high-copy vector strengthened silencing in the WT strain (Figure 4A, day 4). Surprisingly, high-copy *SIR2* almost completely restored rDNA silencing to the *npt1*Δ strain (Figure 4A). *SIR2* overexpression also restored rDNA silencing to a *bna1*Δ mutant under limiting nicotinic acid conditions (data not shown). How this happens is currently unclear, but it suggests that Sir2p could also have an additional NAD^+-independent function in rDNA silencing, perhaps a structural role. On the other hand, overexpression of *SIR2* could lower the threshold

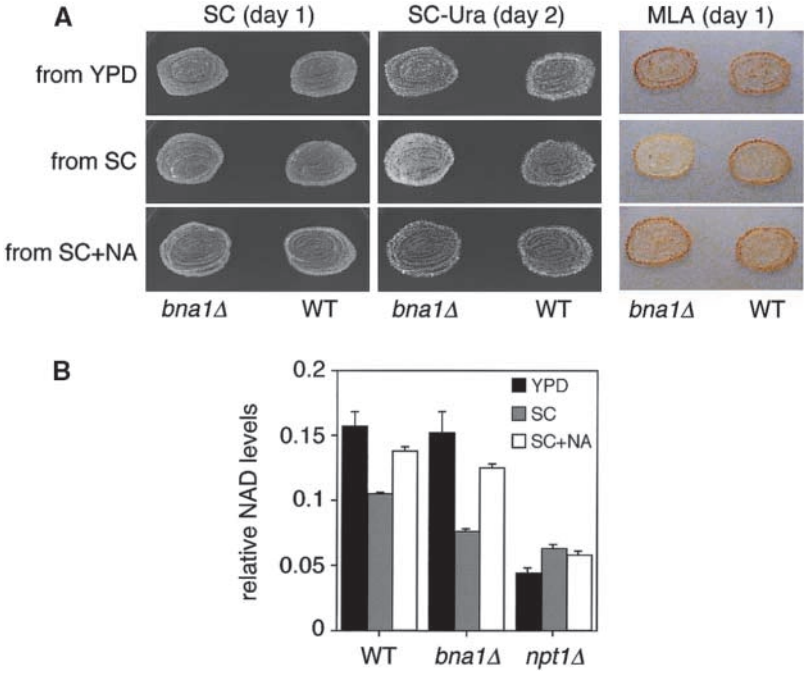

FIGURE 3.—rDNA silencing in a *bna1*Δ mutant is altered by variations in nicotinic acid concentration. (A) Silencing in JS306 (WT) and JS663 (*bna1*Δ) strains was measured qualitatively using a standard yeast patch assay. The media on which the strains were pregrown is indicated on the left side of the panel. After replica plating to the appropriate indicator medium (listed at the top of the panel), the patches were incubated for 1 or 2 days as noted. More Ura^+ growth and a lighter colony color on MLA medium indicate weaker rDNA silencing by the *bna1* mutant. (B) Relative intracellular NAD^+ levels. NAD^+ concentration was measured from strains JS306 (WT), JS663 (*bna1*Δ), and JS673 (*npt1*Δ). Each growth condition is indicated by a different shaded bar. The values are the average absorbances (340 nm). Error bars are the standard deviation from three experiments. SC, synthetic complete (3.25 μM nicotinic acid); SC + NA, synthetic complete supplemented with an additional 10 μM nicotinic acid.

concentration of NAD^+ required to effect silencing. In contrast, *NPT1* was required for the strengthening of telomeric silencing caused by *SIR3* overexpression (J. SMITH, unpublished data), suggesting that TPE is more sensitive than rDNA silencing to changes in NAD^+ concentration.

Since Sir2p consumes NAD^+ as part of its deacetylation reaction, it was possible that changes in *SIR2* dosage (such as in Figure 4A) could alter the intracellular NAD^+ concentration. To test this possibility, we measured NAD^+ levels in strains with varying *SIR2* copy number (Figure 4B). The NAD^+ concentration in strains that were deleted for *SIR2* or contained a high-copy *SIR2* plasmid was actually similar to the concentration from a normal $SIR2^+$ strain (Figure 4B). Therefore, mutations in *SIR2* do not significantly affect the overall intracellular NAD^+ concentration. This lack of a change could be partially due to compensatory changes in activity of the *de novo* and/or salvage NAD^+ synthesis pathways.

Npt1p enzymatic activity is required for silencing: NAPRTase activities have been described from bacteria, yeast, and humans. However, genes encoding these proteins have been described only for *S. typhimurium, S. cerevisiae,* and *Mycobacterium tuberculosis* (RAJAVEL *et al.* 1998). The PncB NAPRTase of Salmonella is known to utilize an unusual ATP-mediated energy-coupling mechanism to achieve a highly specific and efficient reaction. ATP increases the V_{max} by >10-fold and decreases K_m values by 200-fold (RAJAVEL *et al.* 1998). A phosphate group from ATP is covalently transferred to His219 during each catalytic cycle. It has been proposed that this is a regulatory mechanism to increase flux toward NAD^+ when cellular ATP levels are high or to render NaMN formation irreversible. A H219N mutation eliminates this energy coupling, but the basal kinetic properties of the enzyme in the absence of ATP are unchanged (RAJAVEL *et al.* 1998). Purified yeast Npt1p also displays similar ATP-modulated kinetics (RAJAVEL *et al.* 1998). In *S. cerevisiae*, the equivalent histidine residue is predicted to be His232 (RAJAVEL *et al.* 1998).

We wanted to know whether a high level of enzymatic activity by yeast Npt1p was required for silencing. The catalytic residues of NAPRTases have not been identified, but activity is severely impaired by knocking out the energy-coupling mechanism. BLAST searches for *NPT1*-like genes were conducted on the available public databases to determine whether the phosphorylated histidine residue of PncB was conserved across all species. *NPT1*-related genes were identified from all kingdoms. A multiple alignment of several proteins, including the putative human version, is shown in Figure 5. *S. cerevisiae* Npt1p was most closely related to the *E. coli* and Salmonella PncB proteins (~25% amino acid identity). For all family members, the conservation extended throughout the entire protein length. Importantly, one absolutely conserved block of homology included His232 of *S. cerevisiae*, suggesting that the energy-coupling mechanism is universal in NaPRTases.

To determine whether Npt1 enzymatic activity was critical for silencing in *S. cerevisiae*, we mutated the equivalent Histidine 232 residue in Npt1p to an asparagine. This mutant H232N *npt1* gene contained on a *CEN* plasmid was introduced into an *npt1*Δ TPE reporter strain. Silencing of a telomeric *ADE2* gene in this reporter strain results in a red colony color. We previously showed that deletion of *NPT1* caused this strain to have a white (derepressed) colony color (SMITH *et al.* 2000).

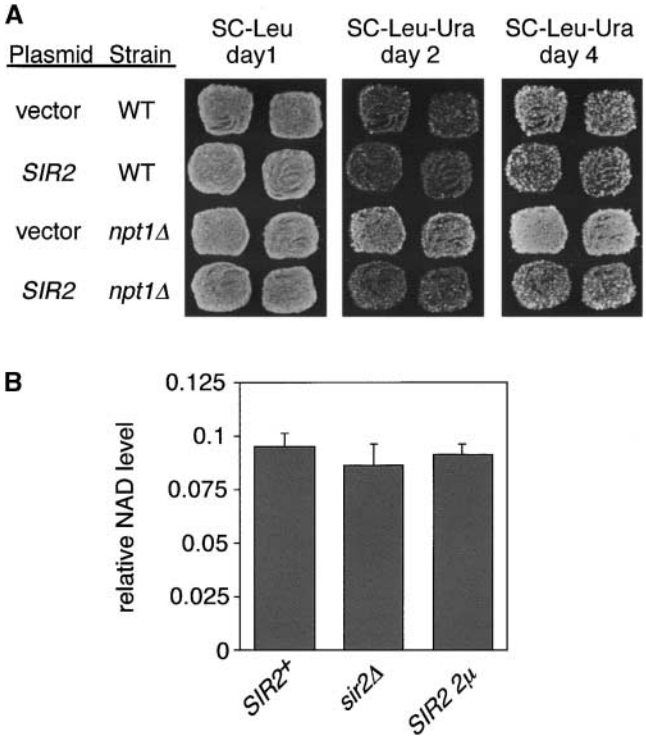

FIGURE 4.—*SIR2* overexpression restores rDNA silencing to an *npt1*Δ mutant. (A) The high-copy vector pRS425 or the high-copy *SIR2* plasmid pSIR 2μ were transformed into JS306 (WT) and JS673 (*npt1*Δ*::kanMX4*). Two independent transformants were patched onto SC-Leu media, grown for 1 day, and then replica plated to SC-Leu and SC-Leu-Ura media. Photos of the patches were taken after the indicated number of days. (B) Relative NAD^+ levels in *sir2*Δ and *SIR2* overexpressing strains grown in SC-Leu medium. The strains were JS740 (*SIR2*$^+$), JS860 (*sir2*Δ), and JS742 (*SIR2* 2μ). The values reported are the mean absorbances (340 nm). Error bars are the standard deviations.

Wild-type *NPT1* restored silencing but the H232N mutant (*npt1-3*) did not (Figure 6A). Western blotting using Myc-tagged versions of these *NPT1* alleles verified that the H232N (*npt1-3*) mutation did not affect steady-state protein levels (Figure 6B), compared to WT Myc-tagged Npt1p. These results indicate that a high level of Npt1p enzymatic activity is required for efficient silencing.

Npt1p is highly concentrated in the nucleus: Sir2p and its homologs are histone/protein deacetylases that consume one NAD^+ molecule for every lysine residue that they deacetylate (LANDRY *et al.* 2000a; TANNY and MOAZED 2001), suggesting a large demand for NAD^+ in the nucleus. Furthermore, this NAD^+ hydrolysis generates nicotinamide as one of the by-products (LANDRY *et al.* 2000a; TANNY and MOAZED 2001). We therefore wanted to determine whether the NAD^+ salvage pathway, which converts nicotinamide to NaMN, was compartmentalized in the nucleus. To test this possibility we analyzed yeast cells expressing 5 × Myc-tagged Npt1p or 5 × Myc-tagged Bna1p by indirect immunofluorescence microscopy using a Myc-specific primary antibody.

When the Myc-Npt1 or Myc-Bna1 constructs were integrated into the genome, the tagged proteins were undetectable by microscopy, but detectable by Western blotting (data not shown). However, when expressed from a *CEN* or 2μ vector, Myc-Npt1p was detectable and indeed concentrated in the nucleus in ~40% of the cells in which Myc-Npt1p was visible (Figure 7). In cells that did not have a nuclear concentration, Myc-Npt1p displayed a whole-cell localization pattern (Figure 7). There was no obvious indication of any subnuclear localization. In contrast, Myc-Bna1p expressed from a *CEN* or 2μ vector had a whole-cell localization pattern in close to 100% of the cells (Figure 7). Strong localization of Bna1p in the nucleus was never observed. For both Npt1p and Bna1p, the *CEN* and 2μ vectors produced similar localization patterns, but the staining intensity was much lower from the *CEN* vectors because of their lower copy number (data not shown). For clarity, the 2μ expression data are presented in Figure 7. These results strongly suggest that a large proportion of NAD^+ salvage in the cell takes place in the nucleus, which is consistent with the role it plays in modulating transcriptional silencing. The dual localization of Npt1p also suggests that Npt1p may shuttle between the nucleus and cytoplasm in a cell-cycle-regulated fashion.

Effects of nicotinic acid import on silencing: As described above, Npt1p is often concentrated in the nucleus where it must convert nicotinic acid to NaMN. Nicotinic acid can be produced by Pnc1p-mediated deamidation of nicotinamide or Tna1p (the nicotinic acid plasma membrane permease) can import it into the cell. As shown in Figure 2, the NAD^+ concentration in a *pnc1*Δ mutant was normal, but there was a partial silencing defect. We hypothesized that silencing was only partially defective in the *pnc1*Δ mutant because Npt1p could still utilize the environmentally imported nicotinic acid to produce NaMN (see Figure 1A). To test this hypothesis, we eliminated nicotinic acid import by deleting *TNA1* and then assayed for silencing of the telomeric *URA3* reporter gene. Deletion of *TNA1* caused a very slight silencing defect indicated by smaller colonies on FOA, but not a reduced colony number compared to the WT strain (Figure 8). However, silencing was almost completely eliminated when the *pnc1*Δ and *tna1*Δ mutations were combined (Figure 8; very little growth on the FOA plate). Interestingly, the silencing defect of the *pnc1*Δ *tna1*Δ mutant was even more dramatic than the defect caused by the *npt1*Δ mutation. Therefore, the import of nicotinic acid from the growth medium is required for telomeric silencing only when the NAD^+ salvage pathway is disrupted by deletion of *PNC1*.

DISCUSSION

Sir2p as an NAD^+ consumer in yeast: NAD^+ turnover is rapid in both bacteria and eukaryotic cells, with a

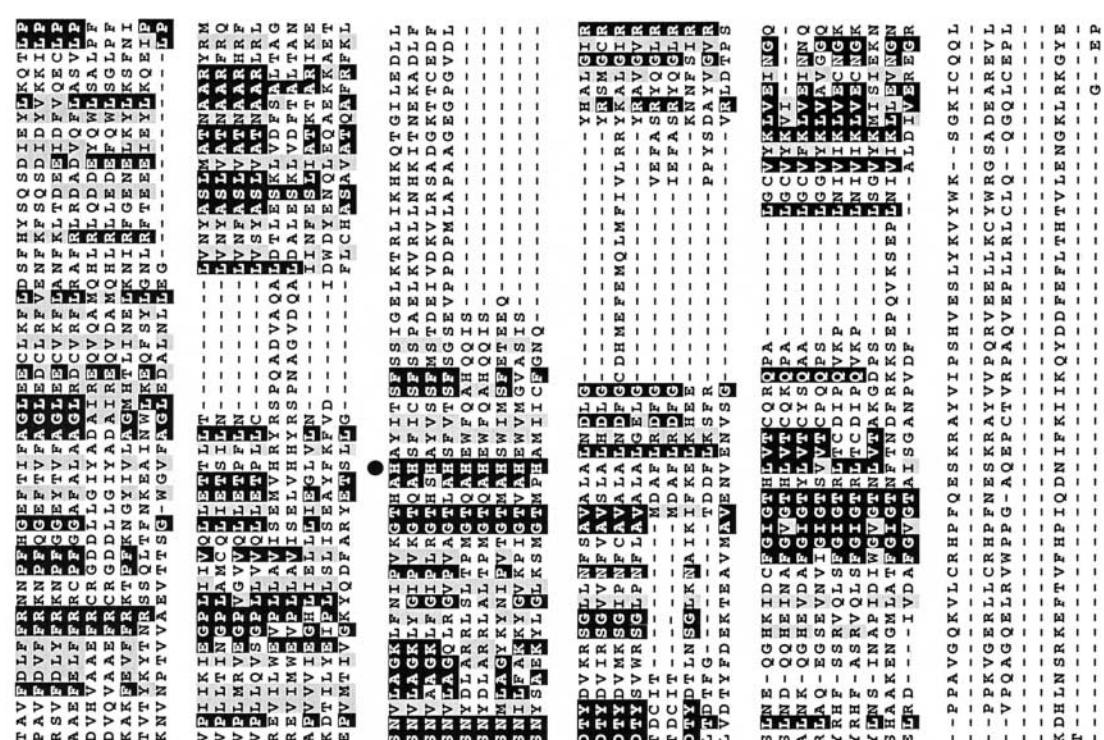

FIGURE 5.—Multiple alignment of proteins related to *S. cerevisiae* Npt1p. The species and amino acid numbering are indicated to the left. Black boxes represent amino acid identity in >50% of the species. Shaded boxes represent amino acid similarity in >50% of the species. The conserved histidine involved in energy coupling is indicated by a solid circle (His232 for *S. cerevisiae*).

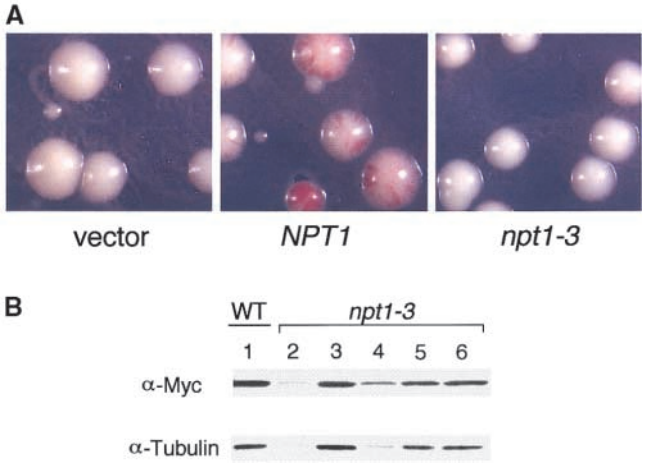

FIGURE 6.—Npt1p catalytic activity is required for silencing. (A) An H232N mutant of Npt1p (*npt1-3*) is defective for telomeric silencing. An *npt1*Δ mutant was transformed with *CEN* plasmids containing WT, *NPT1*, or *npt1-3*. (B) Western blot analysis of steady-state Npt1 protein levels. Myc-tagged Npt1 protein was detected from strain JJSY2 (lane 1). A Myc-tagged version of Npt1-3p was detected from five isolates of TWY1 (lanes 2–6). Parallel Millipore-P membranes were probed with α-Myc antibody or α-tubulin antibody.

half-life ranging from 25 to 90 min (MANSER *et al.* 1980; PENFOUND and FOSTER 1996). However, the mechanisms responsible for this high rate of NAD^+ consumption are not well understood. For example, although *E. coli* DNA ligase consumes NAD^+, substitution of the ligase gene with an ATP-dependent form has no effect on NAD^+ flux (PARK *et al.* 1989). In mammalian cells, poly(ADP) ribosylation reactions catalyzed by poly(ADP) ribose polymerase (PARP) can account for a large amount of NAD^+ consumption in the nucleus when cells undergo DNA damage (BERGER 1985). However, neither PARP activity nor a PARP-related gene has been identified in yeast. It is now known that Sir2p and its family members are NAD^+-dependent protein deacetylases that consume NAD^+ as part of their reaction mechanism (TANNER *et al.* 2000; TANNY and MOAZED 2001). Sir2p also has a weak NAD^+-dependent phosphoryl transferase activity that could potentially contribute to NAD^+ consumption (TANNY *et al.* 1999). In this study we have shown that deletion or overexpression of *SIR2* does not measurably change the intracellular NAD^+ concentration. There are four other Sir2 family members in yeast, and Hst2p actually contributes most of the NAD^+-dependent deacetylase activity from whole-cell extracts (SMITH *et al.* 2000). It may take changes in some or all of these genes to observe a change in NAD^+ concentration. It is still very possible that the Sir2p family members in other species also significantly contribute to NAD^+ turnover.

Nuclear localization of Npt1p: It is unclear how and from where Sir2p acquires its NAD^+ substrate/cofactor. Presumably, the histone deacetylase activity of Sir2p acts in the nucleus *in vivo*, although this has not yet been directly demonstrated. Since Sir2p generates one nicotinamide molecule for each acetyl group removed (LANDRY *et al.* 2000a), it would be very efficient for the cell to recycle this nicotinamide by-product back into NAD^+ near the sites of silencing. This would ensure a constant supply of NAD^+ for Sir2p in the nucleus. Indeed, we have shown that a Myc-tagged Npt1 protein is concentrated in the nucleus in a large percentage of cells.

Finding Npt1p in the nucleus raises the possibility that NAD^+ synthesis and NAD^+ salvage may be compartmentalized. We observed that a Myc-tagged Bna1 protein, which is part of the *de novo* NAD^+ synthesis pathway, was dispersed evenly throughout the cell. This suggests that a large proportion of the *de novo* synthesis pathway operates outside the nucleus, while in many cells the salvage pathway operates primarily inside the nucleus. After NaMN synthesis, the two pathways converge with the action of nicotinamide mononucleotide (NMN) adenylyltransferase, which catalyzes the essential formation of NAD^+ or deamido-NAD. Interestingly, this enzymatic activity is nuclear in vertebrate cells (HOGEBOOM and SCHNEIDER 1952). Furthermore, the yeast, chicken, and human enzymes have been shown to associate with

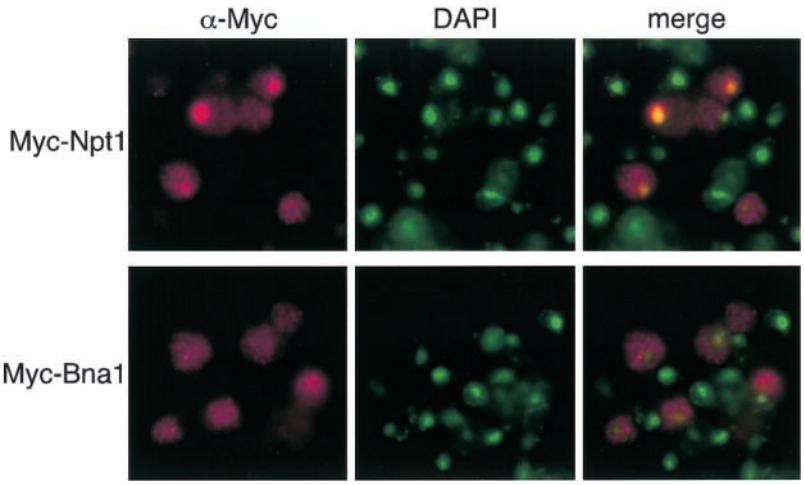

FIGURE 7.—Differential cellular localization of Npt1p and Bna1p. 5 × Myc-tagged Npt1p or 5 × Myc-tagged Bna1p were expressed from their own promoters off 2μ vectors in *npt1*Δ or *bna1*Δ strain backgrounds, respectively. Localization was observed using indirect immunofluorescence microscopy. Cy3 signal indicates the Myc-tagged protein (red) and DAPI staining indicates the location of the nucleus (green). In the merged image, yellow indicates co-localization. The Bna1p-specific strain was JJSy54 and the Npt1p-specific strain was JJSy51.

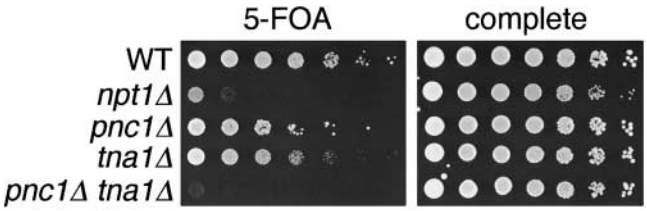

FIGURE 8.—The effect of nicotinic acid import on telomeric silencing. Loss of silencing is indicated by less colony growth on 5-FOA medium compared to growth on SC (complete) medium. Fivefold serial dilutions of cells were plated onto each plate. The strains were WT (YCB647), *npt1*Δ (JS641), *pnc1*Δ (JS807), *tna1*Δ (JS813), and *pnc1*Δ *tna1*Δ (JS861).

the nuclear matrix and/or chromatin (CANTAROW and STOLLAR 1977; BALDUCCI *et al.* 1992; MAGNI *et al.* 1997). The yeast enzyme is encoded by the *YLR328W* open reading frame (Figure 1A; EMANUELLI *et al.* 1999). Yeast also encodes a highly related, but uncharacterized gene called *YGR010W*, with 72% identity to *YLR328W* (EMANUELLI *et al.* 1999). Since *ylr328w* mutants are viable (WINZELER *et al.* 1999), the Ygr010w protein likely provides a redundant function. This class of enzyme was named as an NMN adenylyltransferase, but the bacterial and mammalian forms actually prefer NaMN as the substrate to produce deamido-NAD (MAGNI *et al.* 1997), the step immediately downstream of the Npt1-catalyzed reaction. Some older studies using enucleated human cells have reported that NAD^+ synthesis occurs almost exclusively in the nucleus (RECHSTEINER and CATANZARITE 1974).

If yeast NMN adenylyltransferase is located exclusively in the nucleus, then this suggests that one of the *de novo* NAD^+ synthesis enzymes makes an NAD^+ precursor molecule that can easily diffuse or be transported into the nucleus. Alternatively, one of the *de novo* synthesis enzymes could shuttle between the nucleus and the cytoplasm. It will be interesting to determine if and how such a cytoplasmic/nuclear transition occurs. Nuclear localization of Npt1p in 40% of asynchronously growing cells also raises the possibility that this process is cell cycle regulated. High levels of NAD^+ salvage in the nucleus may be required at certain phases of the cell cycle to help Sir2p establish and/or maintain silencing. At times when Npt1p is not concentrated in the nucleus, silent chromatin could become more susceptible to chromatin remodeling. For example, telomeric chromatin becomes accessible to transcriptional activators during the G_2/M phases of the cell cycle (APARICIO and GOTTSCHLING 1994), and Sir2p is released from the rDNA (nucleolus) at the end of mitosis (STRAIGHT *et al.* 1999). Perhaps nuclear localization of Npt1p is also lost during this time. Future work will address this possibility.

Nicotinic acid and rDNA silencing: Why does rDNA silencing in a *bna1*Δ mutant become sensitive to nicotinic acid concentration in the growth media? In a *bna1*Δ mutant, NAD^+ synthesis is completely dependent on the salvage pathway and an exogenous source of nicotinic acid. The cell internalizes nicotinic acid through action of the nicotinic acid transporter Tna1p (LLORENTE and DUJON 2000). The limiting concentration of nicotinic acid in the medium could drop the intracellular concentration below a critical value needed for efficient catalysis by Npt1p. For Salmonella PncB, the K_m for nicotinic acid is 1.5 μM (VINITSKY and GRUBMEYER 1993). The concentration of nicotinic acid in SC medium is 3.25 μM, which is approaching the K_m. Subsequent inhibition of Npt1p activity would reduce the cellular NAD^+ concentration, as we observed in Figure 3B, and result in weakened silencing. Alternatively, changes in the nicotinic acid concentration could also influence the expression of genes involved in rDNA silencing. Deletion of *SIR2* is known to elevate the expression of Bna1p (BERNSTEIN *et al.* 2000), and the expression of the nicotinic acid transporter Tna1p is downregulated by increasing nicotinic acid concentrations (LLORENTE and DUJON 2000). It is possible that other silencing-related genes could be affected.

What is the function of the NAD^+ salvage pathway in silencing? Npt1p and Pnc1p are both part of the NAD^+ salvage pathway. However, *pnc1*Δ and *npt1*Δ mutants do not have identical silencing phenotypes or the same effects on intracellular NAD^+ concentration. As shown in Figure 2, the *pnc1*Δ mutant has partial rDNA and telomeric silencing defects, but has a normal intracellular NAD^+ concentration. The *npt1*Δ mutant has a more severe silencing defect at the rDNA and telomeres that correlates well with the reduced intracellular NAD^+ concentration. This difference could be caused by differential usage of nicotinic acid pools (see below).

The role of nicotinamide deamidase (Pnc1p for *S. cerevisiae*) in the NAD^+ salvage pathway is to produce nicotinic acid. As a result, some nicotinic acid is derived intracellularly from NAD^+ hydrolysis and the rest is imported from the growth medium (environmental). Since Npt1p is responsible for converting both sources of nicotinic acid to NaMN, loss of Npt1p function causes a large reduction in NAD^+ concentration and severe silencing defects. In a *pnc1*Δ mutant, nicotinic acid is no longer produced intracellularly, but Npt1p can still convert the imported (environmental) nicotinic acid to NaMN. The result is no significant net change in the overall intracellular NAD^+ concentration. However, there is still a modest silencing defect. One possibility to explain this phenomenon is that the nicotinic acid generated by Sir2p and Pnc1p in the nucleus has a greater probability of conversion to Sir2p-utilized NAD^+ than does imported nicotinic acid. In support of this hypothesis, deletion of *TNA1* causes only a slight derepression of telomeric silencing (Figure 8), even though nicotinic acid import is eliminated. An alternative model is that Pnc1p and Npt1p cooperate to convert the nicotinamide specifically produced by Sir2p to NaMN. Nicotinamide is a non-

competitive inhibitor of the NAD^+- dependent histone deacetylase activity (Landry *et al.* 2000a). Therefore conversion of Sir2p-produced nicotinamide to nicotinic acid and NaMN could help facilitate the deacetylation activity of Sir2p by reducing product inhibition. Future work will be aimed at testing these models, which are not mutually exclusive. In both cases, Npt1p would be expected to be concentrated in the nucleus when silencing is being established and/or maintained.

The relationship between NAD^+, silencing, and aging: The Guarente lab has shown that yeast cell longevity is dependent on the *SIR2* gene (Kaeberlein *et al.* 1999). Part of this effect is due to the role of Sir2p in regulating rDNA recombination and silencing. Since Sir2p is an NAD^+-dependent histone deacetylase, changes in NAD^+ concentration could have profound effects on aging, similar to the effects on silencing. Indeed, as yeast cells age, the intracellular ATP content increases 2.6-fold and the NAD^+ content increases ~20% (Ashrafi *et al.* 2000). It has been proposed that the increased NAD^+ concentration may be a response designed to prevent genomic instability (Ashrafi *et al.* 2000).

NAD^+ has major roles in metabolic reactions and the maintenance of the cellular redox state. High metabolic activity corresponds to increased glycolysis (as an example) and therefore more NAD^+ is converted to NADH. It has been proposed that this would divert NAD^+ away from Sir2p, which uses NAD^+ for silencing (Guarente 2000). Sir2p has also been proposed to be a molecular sensor of NAD^+ levels that translates this information into the appropriate chromatin silencing (Guarente 2000; Smith *et al.* 2000). Npt1p is also involved in this process. The extension of yeast life span by caloric restriction requires *NPT1* (Lin *et al.* 2000). In contrast, *QPT1* of the *de novo* NAD^+ synthesis pathway was not required for the life-span extension (Lin *et al.* 2000). This is consistent with our data showing that *qpt1*Δ mutants do not affect rDNA or telomeric silencing. It will therefore be interesting to determine whether *PNC1* and/or *TNA1* also functions in extension of life span by caloric restriction.

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