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Synthetic Lethal and Biochemical Analyses of NAD and NADH Kinases in *Saccharomyces cerevisiae* Establish Separation of Cellular Functions*[S]

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Production of NADP and NADPH depends on activity of NAD and NADH kinases. Here we characterized all combinations of mutants in yeast NAD and NADH kinases to determine their physiological roles. We constructed a diploid strain heterozygous for disruption of *POS5*, encoding mitochondrial NADH kinase, *UTR1*, cytosolic NAD kinase, and *YEF1*, a *UTR1*-homologous gene we characterized as encoding a low specific activity cytosolic NAD kinase. *pos5 utr1* is a synthetic lethal combination rescued by plasmid-borne copies of the *POS5* or *UTR1* genes or by *YEF1* driven by the *ADH1* promoter. Respiratory-deficient and oxidative damage-sensitive defects in *pos5* mutants were not made more deleterious by *yef1* deletion, and a quantitative growth phenotype of *pos5* and its arginine auxotrophy were repaired by plasmid-borne *POS5* but not *UTR1* or *ADH1*-driven *YEF1*. *utr1* haploids have a slow growth phenotype on glucose not exacerbated by *yef1* deletion but reversed by either plasmid-borne *UTR1* or *ADH1*-driven *YEF1*. The defect in fermentative growth of *utr1* mutants renders *POS5* but not *POS5*-dependent mitochondrial genome maintenance essential because *rho− utr1* derivatives are viable. Purified Yef1 has similar nucleoside triphosphate specificity but substantially lower specific activity and less discrimination in favor of NAD *versus* NADH phosphorylation than Utr1. Low expression and low intrinsic NAD kinase activity of Yef1 and the lack of phenotype associated with *yef1* suggest that Utr1 and Pos5 are responsible for essentially all NAD/NADH kinase activity *in vivo*. The data are compatible with a model in which there is no exchange of NADP, NADPH, or cytoplasmic NAD/NADH kinase between nucleocytoplasmic and mitochondrial compartments, but the cytoplasm is exposed to mitochondrial NAD/NADH kinase during the transit of the molecule.

NADP is required by the pentose phosphate pathway, and NADPH is required for resistance to oxidative stresses. Deletion mutants in the gene encoding mitochondrial matrix NADH kinase, *POS5*, are sensitive to growth in hyperoxia, H_2O_2, and methyl viologen and are also respiratory-deficient (1, 2). Interestingly, transforming freshly prepared *pos5* mutants with a *POS5* plasmid restores resistance to reactive oxygen species and growth on nonfermentable carbon sources, but older *pos5* mutants lose the ability to have their respiratory defect restored by Pos5 expression. These data suggest that mitochondrial NADPH-dependent antioxidant reductases are required to protect mitochondria from reactive oxygen species damage and that this damage can lead to irreparable lesions to the mitochondrial genome (1, 2). In the cytosol, NADP is used by pentose phosphate pathway enzymes glucose-6-phosphate dehydrogenase Zwf1 and 6-phosphogluconate dehydrogenase Gnd1 and -2 to produce ribulose-5-phosphate and NADPH (3). The yeast glucose-6-phosphate dehydrogenase mutant *zwf1* is consequently sensitive to oxidizing agents that deplete cytosolic pools of reduced glutathione and thioredoxin, which depend on cytosolic NADPH (4, 5). In addition, the *zwf1* mutant requires sulfur to be supplied in organic form (6).

Beyond the requirement of Zwf1 and Gnd1 and -2 for NADP, cytosolic NADP is required by NADP-specific isocitrate dehydrogenase Ipd2, glutamate dehydrogenase Gdh1, and aldehyde dehydrogenase Ald6 (7). Thus, a cytosolic NAD kinase mutant would be expected to produce pleiotropic phenotypes consistent with deficiencies in multiple enzymes. In yeast, Utr1 was originally reported as a transcript of unknown function (8) and then shown to encode a component of ferrireductase (9) with NAD kinase activity (10). Although the *utr1* deletion mutant was recently shown to exhibit poor growth in low iron media (11) consistent with the function of Utr1 within ferrireductase (9), a more general phenotype has not been reported.

In many eukaryotes, NAD is synthesized from a *de novo* pathway from tryptophan (12, 13), an import pathway from nicotinic acid (14, 15), and a salvage pathway from nicotinamide riboside (16). NAD is not only a co-enzyme for enzymes such as inosine monophosphate dehydrogenase, which use NAD as a hydride acceptor, producing NADH (17), but also a substrate of NAD glycohydrolases. Although NAD glycohydrolases were once considered mysterious enzymes that cleave NAD to a nicotinamide and an ADP-ribosyl product for no obvious reason (18), we now understand that a class of protein lysine deacetylases related to yeast Sir2 (Sirtuins) (19–21) as well as cADP-

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[S] The on-line version of this article (available at http://www.jbc.org) contains a supplemental table showing oligonucleotides used in this study.

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ribose synthases and poly(ADP-ribose) polymerases (22) are NAD-dependent enzymes. Because these enzymes convert copious amounts of NAD to nicotinamide, organisms benefit from enzymes that salvage nicotinamide to either nicotinic acid or nicotinamide mononucleotide for reentry into NAD biosynthetic pathways. Phylogenetic analysis suggests that the former enzyme, nicotinamidase, found in fungi (23), and the latter enzyme, nicotinamide phosphoribosyltransferase, found in vertebrates (24), entered eukaryotic lineages via horizontal gene transfer.[2] The final steps in eukaryotic NAD synthesis are catalyzed either by glutamine-dependent NAD synthetase (26) for the *de novo* nicotinic acid import and nicotinic acid salvage pathways that go through nicotinic acid mononucleotide (NaMN) or by nicotinic acid mononuleotide/NMN adenylyltransferase (27) for the nicotinamide riboside kinase and nicotinamide phosphoribosyltransferase pathways that go through an NMN intermediate. In the nucleus and cytosol, NAD-dependent hydride transfer enzymes such as inosine monophosphate dehydrogenase, alcohol dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase reduce NAD to NADH.

NADH is imported into mitochondria by the voltage-dependent anion channel (porin isoform 1) (28, 29) and possibly by the translocase of the outer membrane complex (30). NAD is imported into mitochondria by the products of the *YIA6* (*NDT1*) and *YEA6* (*NDT2*) genes, which have essentially no transport activity on NADP or NADPH (31). Thus, unless NADP or NADPH is imported into mitochondria by a process that has not been described, mitochondrial NADP and NADPH should depend entirely on a mitochondrial NAD/NADH kinase activity. Similarly, unless NADP or NADPH can flow back from mitochondria to the cytosol, cytosolic NADP and NADPH are expected to depend on a cytosolic NAD/NADH kinase or mislocalization of the corresponding mitochondrial enzyme. Because two of three NAD/NADH kinase genes had been analyzed with respect to phenotypes and biochemical characteristics, we wished to perform a genetic and biochemical analysis of the remaining NAD/NADH kinase gene in yeast and to analyze phenotypes of double and triple mutants. Recently, Shi *et al.* (11) purified the third yeast NAD/NADH kinase, Yef1, and concluded, as we do, that Yef1 is a cytosolic NAD kinase. However, they claim to produce viable cells deleted for *pos5*, *utr1*, and *yef1* and suggested the existence of an additional NAD kinase unrelated to the sequence of NAD/NADH kinases (11). In contrast, we report that *pos5 utr1* is a synthetic lethal combination, with or without the deletion of *yef1*.

Our analysis confirms that *pos5* mutants have mitochondrial (*i.e.* respiratory) defects (1, 2) and establishes that Utr1 has cytoplasmic (*i.e.* fermentative) defects, indicating that neither enzyme is significantly mislocalized and that triphosphopyridine nucleotides do not exchange between nucleocytoplasmic and mitochondrial compartments. We also demonstrate that *pos5* and *utr1* produce a synthetic lethal combination. Moreover, our analysis shows that Yef1, encoded by the *YEL041W* gene, is a low specific activity NAD kinase. Although the deletion of the *yef1* gene did not exacerbate either the *pos5* or the *utr1* phenotypes, the *pos5 utr1* double mutant was synthetically lethal, indicating that there is not enough NADP produced by Yef1 to support the fermentation that must occur in *pos5* mutants. Finally, although the *pos5 utr1* mutant is inviable, we find that *rho*− *utr1* mutants are viable, suggesting that the viability of *utr1* mutants depends on cytosolic Pos5 NAD/NADH kinase activity prior to or during the transport of this molecule to the mitochondrion.

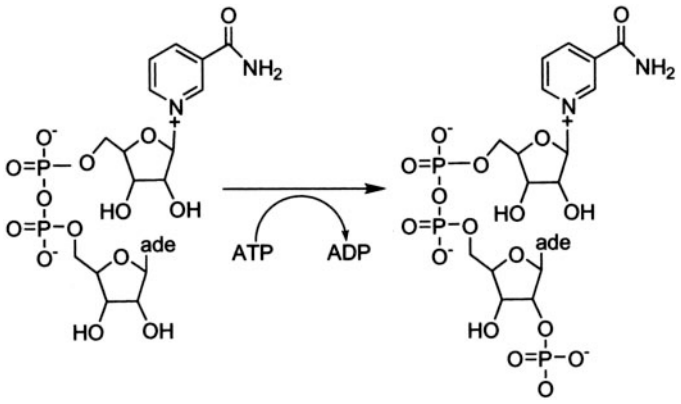

FIGURE 1. **Reaction scheme of NAD kinase.** NAD and NADH kinases catalyze phosphoryl transfer to the adenosine 2′ position of NAD and NADH, forming NADP and NADPH. Depicted is ATP as the phosphodonor and NAD as the phosphoacceptor.

EXPERIMENTAL PROCEDURES

Enzyme Expression and Purification—Expression plasmids were designed for purification of His-tagged versions of Utr1 and Yef1 from *Escherichia coli*. Primer sequences are provided in Supplementary Table 1. The *UTR1* open reading frame was amplified from *Saccharomyces cerevisiae* genomic DNA with primers 7125 and 7126 and cloned into vector pMR103 (32) using NcoI and BamHI restriction sites to create plasmid pB414. An N-terminal His tag was added by annealing oligonucleotides 7300 and 7301 and ligating to NcoI-digested plasmid pB414 to produce plasmid pB421. The *YEF1* coding sequence was amplified with primers 7318 and 7319, digested with NdeI and BglII, and cloned into vector pSGA04 (33) cleaved with NdeI and BamHI to create plasmid pB430. *E. coli* BL21 cells transformed with either plasmid pB421 or pB430 were used to express the His-tagged NAD kinases. Bacterial cells were lysed by sonication in Buffer A (20 mM sodium phosphate, pH 8, 100 mM NaCl, 10% glycerol, 1 mM β-mercaptoethanol, 10 mM imidazole and protease inhibitor mixture (Roche Applied Science)). Clarified lysates were loaded on cobalt chelate affinity columns. Columns were washed with 15 volumes of Buffer A, and enzymes were eluted with Buffer A supplemented with 100 mM imidazole. Purified enzymes were concentrated and dialyzed against Buffer A without imidazole.

Enzymatic Assays—To determine the specific activities of the Utr1 and Yef1 kinases, 100-μl pre-mixes containing 100 mM Tris-HCl, pH 7.1, 5 mM $MgCl_2$, 1 mM NTP or dNTP, and 1 mM NAD, NADH, or NaAD were incubated with either Utr1 or Yef1 enzyme (0–5 μg) for 20 min at 37 °C. EDTA (10 μl of 0.5 M) was added to stop the reactions, and NADP, NADPH, or NaADP products were separated and quantified by high pressure liquid chromatography using a strong anion exchange col-

[2] F. Gazzaniga and C. Brenner, submitted for publication.

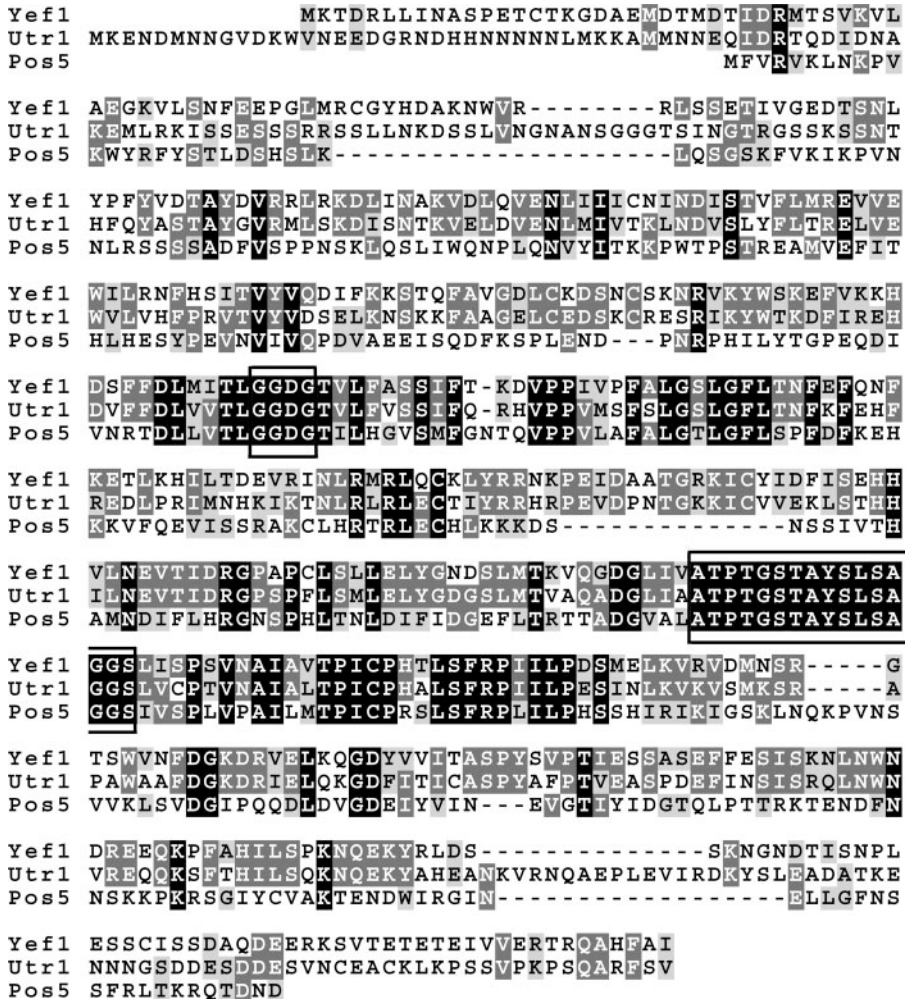

FIGURE 2. **Sequence Alignment of Yef1, Utr1, and Pos5.** *Black letters* on a *gray background* denote residues that are similar. *White letters* on a *gray background* denote positions at which two of three sequences are identical. *White letters* on a *black background* denote residues that are identical. Signature sequences for NAD/NADH kinases are *boxed* (39).

umn in a 10–750 mM gradient of sodium phosphate, pH 2.6. Mean specific activities of three separate experiments, expressed as μmol of NADP, NADPH, or NaADP min^{-1} mg^{-1}, were calculated from reactions in which no more than 10% of substrates were converted into products.

Yeast Strains—DNA fragments for disruption of *UTR1, POS5*, and *YEF1* genes were constructed as described (34). For *UTR1*, primers 7127 and 7128 were used to amplify the *HIS3* marker of plasmid pRS413 (35). Correct integration of *HIS3* at the *UTR1* locus was verified by PCR using primers 7125 and 7126. For *POS5*, primers 7343 and 7345 were used to amplify the Geneticin resistance marker of plasmid pRS401 (35). Correct integration was verified with primers 7342 and 7344. For *YEF1*, primers 7312 and 7313 were used to amplify the *TRP1* marker of plasmid pRS414 (35). Correct integration was verified with primers 7314 and 7315. Diploid yeast strain SEY6210.5 was subjected to three consecutive steps of gene replacement and verification using these disruption cassettes. The resulting strain, BY274, triply heterozygous for the deletion of *pos5*, *utr1*, and *yef1*, was incubated on sporulation medium. Following tetrad formation, spores were separated by micromanipulation, and the genotypes of the resulting haploid isolates were tested by growth on appropriate selective media (see Table 1). *UTR1, YEF1*, and *POS5* were amplified from the *S. cerevisiae* genome using primers 776 and 777, 778 and 779, and 780 and 781, respectively. The *UTR1* gene PCR product and plasmid pRS416 (35) were digested with EcoRI and SpeI and ligated to produce pB534. The *YEF1* open reading frame PCR product was similarly digested and ligated into p416ADH1 (36) to produce pB535. The *POS5* gene PCR product and pRS416 were digested with BamHI and XhoI and ligated to produce pB532. Diploid strain BY274 heterozygous for *pos5*, *utr1*, and *yef1* disruption was transformed with pB532, pB534, and pB535 and, after sporulation and tetrad dissection, haploids were isolated with each plasmid present in each combination of NAD/NADH kinase gene disruption. Strains were then plated on 5-fluororotic acid media to recover and characterize viable haploid genotypes. Media used to grow yeast cultures and protocols for transformations and tetrad dissection have been described (37).

Yeast Growth Assays—Triplicate growth curves of all viable haploid strains were obtained at 28 °C in YPD in 10 time points over 50 h. Triplicate growth curves of *utr1* and *pos5* strains transformed with pRS416 (empty vector), pB532, pB534, or pB535 were obtained at 28 °C in YPD in seven time points over 13 h. Plate assays of the *pos5* strain transformed with pRS416, pB532, pB534, or pB535 were completed on YPD, YP-glycerol, and synthetic dextrose media without arginine. Respiratory-deficient (*rho*−) yeast strains were obtained by passage on ethidium bromide media (38).

RESULTS AND DISCUSSION

The NAD/NADH kinase reaction with ATP as phosphoryl donor is schematized in Fig. 1. Sequence analysis of *YEF1* (*YEL041W*) indicates the presence of an NAD kinase signature (39) motif (residues 311–333) and a conserved GGDG motif (residues 190–193), which has been shown to be part of the ATP-binding site in highly divergent metabolite kinases (39–43). As shown in Fig. 2, at the level of primary sequence, Yef1 is more similar to the NAD kinase Utr1 than to NADH kinase Pos5. To compare their biochemical activities, we purified Utr1 and Yef1 proteins expressed in *E. coli* as N-terminally His-tagged fusions. Because yeast NAD kinase is a relatively unstable enzyme (44), our rapid, one-step purification pro-

TABLE 1
Specific activities of Utr1 and Yef1 (μmol of NADP, NADPH or NaADP min^{-1} mg^{-1})
ND, not determined.

	Utr1			Yef1		
	NAD phosphoacceptor	NADH phosphoacceptor	NaAD phosphoacceptor	NAD phosphoacceptor	NADH phosphoacceptor	NaAD phosphoacceptor
Phosphodonor						
ATP	51.5 ± 2.7	1.2 ± 0.05	0.4 ± 0.01	3.3 ± 0.12	1.2 ± 0.05	0.5 ± 0.02
CTP	7.3 ± 0.34	0.5 ± 0.04	ND	0.2 ± 0.04	0.5 ± 0.04	ND
GTP	5.1 ± 0.41	0.1 ± 0.01	ND	0.3 ± 0.02	0.1 ± 0.01	ND
dATP	20.0 ± 1.2	ND	ND	3.2 ± 0.24	ND	ND
dCTP	15.6 ± 0.78	ND	ND	0.3 ± 0.02	ND	ND
dGTP	3.9 ± 0.53	ND	ND	0.3 ± 0.04	ND	ND

duced higher specific activities than those previously reported (10, 45).

As shown in Table 1, Utr1 is an effective NAD kinase with ATP, dATP, and dCTP as phosphoryl donors and lesser activity with CTP, GTP, and dGTP as phosphoryl donors. Utr1 phosphorylates NADH with ~2% of the specific activity of its NAD kinase activity, preferring ATP as the phosphoryl donor for formation of NADPH. The maximal activity for Yef1 is with NAD and ATP, although it possesses ~33% of this activity with NADH and ATP. In terms of NAD-ATP kinase activity, Yef1 exhibits only about 6% of the specific activity of Utr1. However, with the low activity of Utr1 on NADH and the relative nonspecificity of Yef1 for phosphoacceptors, Yef1 has about as much NADH kinase specific activity as does Utr1.

To test whether either NAD kinase might have a role in formation of the calcium-mobilizing second messenger, NaADP (46), we examined Utr1 and Yef1 as ATP-dependent NaAD kinases (Table 1). The specific activity of Utr1 was 0.4 ± 0.01 μmol NaADP min^{-1} mg^{-1}, and that of Yef1 was 0.5 ± 0.02 μmol NaADP min^{-1} mg^{-1}. These values represent less than 1% of the specific activity of Utr1 as an ATP-dependent NAD kinase.

Enzyme activity *in vivo* is a function of enzyme localization, enzyme concentration, and intrinsic activity. Global analysis of protein expression indicates that Yef1 is expressed at a level of only about 300 molecules per cell, as compared with ~5000 molecules of Utr1 and Pos5 (47). Thus, with the important caveat that our recombinant preparations might have missed a factor that contributes to the activity of Utr1 or Yef1 *in vivo*, our data suggest that Yef1 contributes very slightly to total NAD and NADH kinase activities *in vivo*.

To determine the roles of NAD and NADH kinase genes in living cells, we created diploid yeast strain BY274 with confirmed heterozygous deletions of the *POS5*, *UTR1*,and *YEF1* genes. Following sporulation and dissection, the resulting haploids were analyzed. As shown in Table 2, of the eight possible genotypes that could potentially be recovered from the triheterozygous diploid, we recovered six genotypes 10–19 times. No strain was recovered with *utr1* and *pos5* deleted or with all three genes deleted. Synthetic lethality of *utr1* and *pos5* has not been reported previously. Because *UTR1* and *POS5* are not genetically linked, the nonrecovery of the double mutant is the first indication that either genetic loss renders the second gene to be essential.

To further characterize the viability and growth characteristics of NAD/NADH kinase deletion mutants, strain BY274 was

TABLE 2
Synthetic lethality of *utr1* and *pos5*

Genotype	Number of independent isolates
POS5 UTR1 YEF1	14
pos5 UTR1 YEF1	16
POS5 utr1 YEF1	12
POS5 UTR1 yef1	15
pos5 UTR1 yef1	10
POS5 utr1 yef1	19
pos5 utr1 YEF1	0
pos5 utr1 yef1	0

transformed with pB532 (*POS5*), pB534 (*UTR1*), or pB535 (*ADH1* promoter driving *YEF1*), and haploids containing each of these plasmids in the triple delete background were recovered. Although the *pos5 utr1* double mutant and the *pos5 utr1 yef1* triple mutant could not be recovered by simple dissection of strain BY274 or BY274 carrying the empty vector pRS416, we recovered both of these genotypes in the presence of each of the NAD/NADH kinase plasmids, pB532, pB534, and pB535. Although pB532 and pB534 transformants would be expected to confer a viable *utr1 yef1* phenotype and a *pos5 yef1* phenotype to the *pos5 utr1 yef1* chromosomal genotype, the viability of pB535 transformants indicates that overexpression of *YEF1* suppresses the lethal phenotype of *pos5 utr1*. As shown in Fig. 3, upon plating each of the transformants on 5-fluororotic acid medium, none of the strains could be recovered as plasmid-free *pos5 utr1 yef1* triple mutant strains. Taken together, Table 2 and Fig. 3 indicate that *pos5 utr1* and *pos5 utr1 yef1* are synthetically lethal mutant combinations that can be suppressed by overexpression of Yef1.

Pos5 has been characterized as a mitochondrial NADH kinase that phosphorylates NAD to a lesser extent (1, 2), whereas Utr1 has been characterized as a cytoplasmic NAD kinase that phosphorylates NADH to a lesser extent (10). Mutants within these genes display distinct phenotypes. Mutants lacking Pos5 exhibit a near normal rate of growth on glucose (Fig. 4, *A* and *C*) but are unable to grow on nonfermentable carbon sources, such as glycerol (Fig. 4*B*). Since NADPH is used as a cofactor by enzymes involved in the detoxification of peroxide, superoxide, and hydroxyl radicals that are generated in mitochondria (48–50), depletion of the mitochondrial NADPH pool would be expected to lead to an increased level of oxidative damage to mitochondrial proteins and DNA, consistent with the mitochondrial genome instability that results from *pos5* mutation (2). In contrast to the respiratory incompetence of *pos5* mutants, *utr1* mutants displayed wild-type growth on glycerol (Fig. 4*B*) and slow growth on glucose (Fig. 4, *A* and *C*).

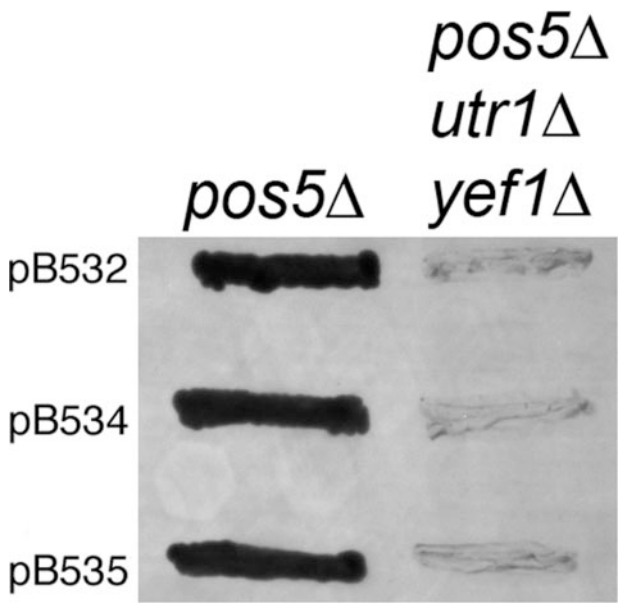

FIGURE 3. **Inviability of *pos5 utr1 yef1*.** Triheterozygous diploid strain BY274 was transformed with plasmids pB532, pB534, or pB535 containing *POS5*, *UTR1*, or *YEF1* (under the control of the *ADH1* promoter), respectively, and the *URA3* gene. Upon sporulation and dissection of the diploid transformants, *pos5* mutants (*left*) carrying each plasmid and *pos5 utr1 yef1* mutants (*right*) carrying each plasmid were streaked on 5-fluororotic acid medium to select for loss of the *URA3* plasmids. Although each plasmid carries a dispensable gene in the *pos5* strain, each plasmid carries an indispensable gene in the triple mutant.

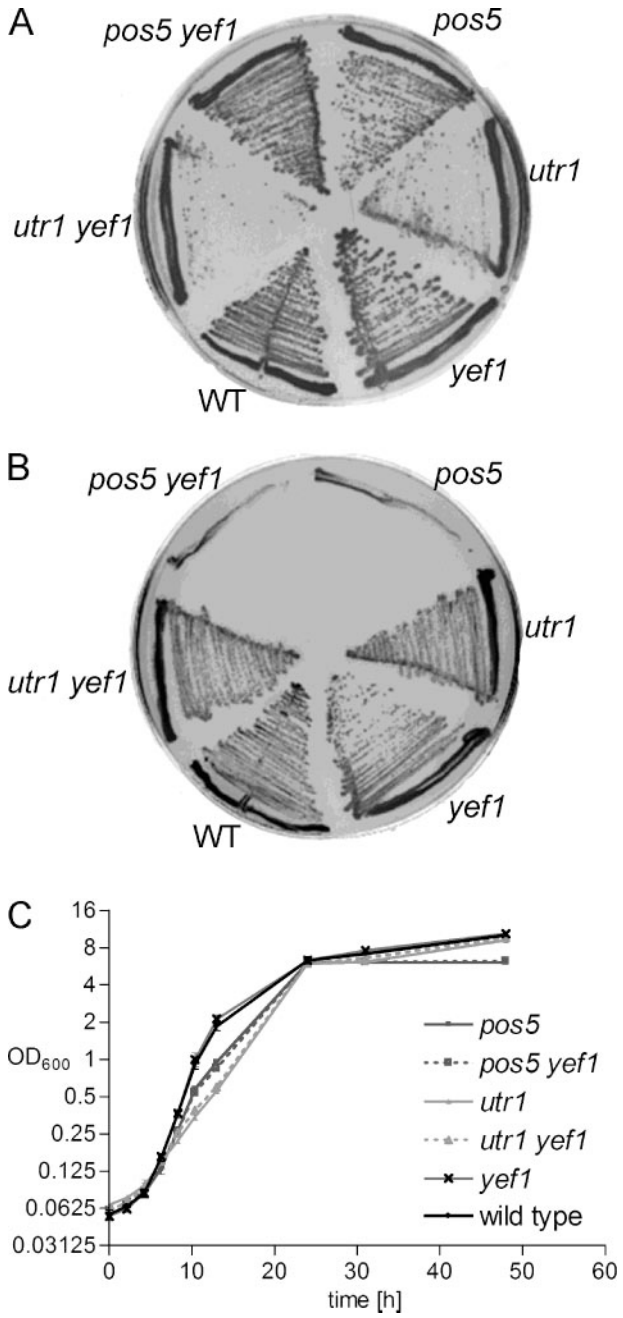

FIGURE 4. **Phenotypic analysis of the six viable NAD/NADH kinase genotypes.** Yeast strains with the indicated genotypes were grown on complete media containing glucose (*A*) or glycerol (*B*) as the sole carbon source. *pos5* mutants are respiratory-incompetent, whereas *utr1* mutants have a slow growth phenotype on fermentable carbon sources. *yef1* mutants are aphenotypic and not additive with either *pos5* or *utr1*. In YPD liquid cultures (*C*), the growth rate of an isogenic *yef1* mutant strain was indistinguishable from wild type (*WT*), whereas a modest slow growth phenotype for *pos5*, not made more deleterious by *yef1*, and the slow growth phenotype of *utr1*, not made more deleterious by *yef1*, were apparent. Average optical densities were plotted ± standard deviations, which in many cases were smaller than the plotting symbols.

As shown in Fig. 4, growth of *yef1* mutant strains on glucose and glycerol was indistinguishable from that of wild-type strains. Unlike mutants in glucose-6-phosphate dehydrogenase that are deficient in NADP, *yef1* mutants exhibited no increased sensitivity to methyl viologen and H_2O_2 (4, 5) and did not exhibit methionine and/or cysteine auxotrophy (6). The double mutants, *pos5 yef1* and *utr1 yef1*, did not demonstrate any additional growth defects on glucose or glycerol, relative to single *pos5* and *utr1* mutants. Similarly, *yef1* deletion did not enhance reactive oxygen sensitivity or auxotrophic requirements of *pos5* or *utr1* (data not shown). These results indicate that the Yef1 protein does not have a major role in production of triphosphopyridine nucleotides *in vivo*. Based on the synthetic lethality of *utr1* and *pos5*, it is apparent that Yef1 activity under its own promoter is insufficient to support the *UTR1* requirement of *pos5* mutants or the *POS5* requirement of *utr1* mutants.

These experiments established three facts that could be interpreted through at least two mutually exclusive mechanisms. First, a cell cannot live if it is devoid of the mitochondrial NAD/NADH kinase Pos5 and the cytosolic NAD/NADH kinase Utr1. Second, *ADH1*-driven Yef1 suppresses the Pos5 dependence of *utr1* mutants. Third, *ADH1*-driven Yef1 suppresses the Utr1 dependence of *pos5* mutants. The most distinctive explanations for these facts are that 1) the poorly fermenting *utr1* mutant is totally dependent on Pos5-dependent respiration or some specific mitochondrial function of Pos5 or 2) the poorly fermenting *utr1* mutant is dependent on at least transient cytosolic NADP/NADPH production by Pos5. Based on experiments with plasmid-borne copies of *POS5*, *UTR1*,and *YEF1* in *pos5* and *utr1* backgrounds and experiments in which *rho*− derivatives of *utr1* were prepared and characterized, we were able to eliminate the first mechanism and gather evidence for the second.

Plasmid pB535, encoding *YEF1* driven by the *ADH1* promoter, has the property of an overexpression-based suppressor of one phenotype of *pos5*, namely synthetic lethality with *utr1*.

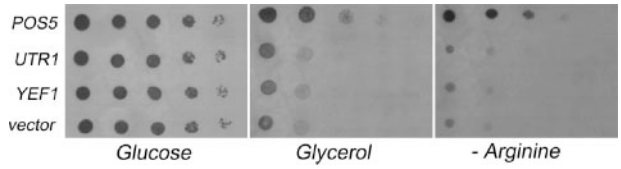

FIGURE 5. **Phenotypic analysis of *pos5*.** *pos5* yeast strains recovered by tetrad dissection of strain BY274, carrying plasmids pRS416, pB532, pB534, or pB535, were serially diluted on complete media containing glucose or glycerol as the carbon source and on glucose-containing −Arg media. Although *UTR1* and *ADH1*-driven *YEF1* plasmids suppress the lethality of *pos5 utr1*, only *POS5* expression repairs the mitochondrial phenotypes of *pos5*.

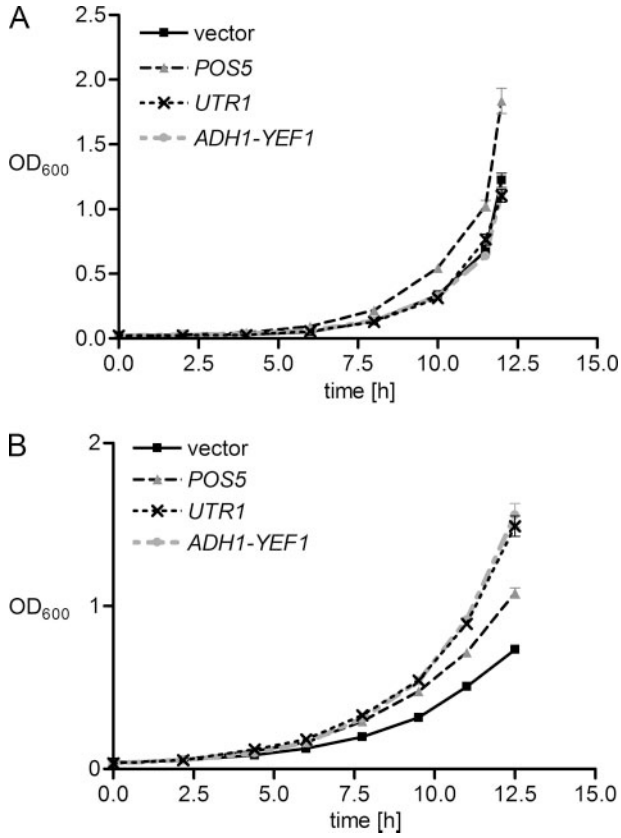

FIGURE 6. **Quantitative growth characteristics of *utr1* and *pos5* strains in glucose and analysis of plasmid-based complementation and suppression.** *A* and *B*, growth curves of a *pos5* haploid strain (*A*) and a *utr1* haploid strain (*B*) transformed with pRS416, pB532, pB534, or pB535. Although the quantitative growth phenotype of *pos5* is only complemented by plasmid-based expression of *POS5*, the slow growth phenotype of *utr1* is complemented by *UTR1*, fully suppressed by *ADH1*-driven *YEF1*, and partially suppressed by *POS5*. Average optical densities were plotted ± standard deviation, which in many cases were smaller than the plotting symbols.

To test whether plasmid-borne copies of *POS5*, *UTR1*, or *YEF1* would suppress specific mitochondrial phenotypes of *pos5*, we tested BY274-derived haploids of genotype *pos5 UTR1 YEF1* carrying pB532, pB534, and pB535 for growth on a nonfermentable carbon source and arginine prototrophy. As reported earlier (1), the *pos5* mutant is arginine auxotrophic, due to lack of NADPH needed for the NADPH-dependent step of arginine biosynthesis catalyzed by mitochondrial *N*-acetyl-γ-glutamylphosphate reductase (25). As shown in Fig. 5, the glycerol and arg$^-$ phenotypes of *pos5* were only rescued by *POS5* expression and not by plasmid-borne copies of *UTR1* or *YEF1*. Because the *ADH1*-driven *YEF1* construct allows a *pos5* deletion strain to survive *utr1* disruption but does not improve its respiratory

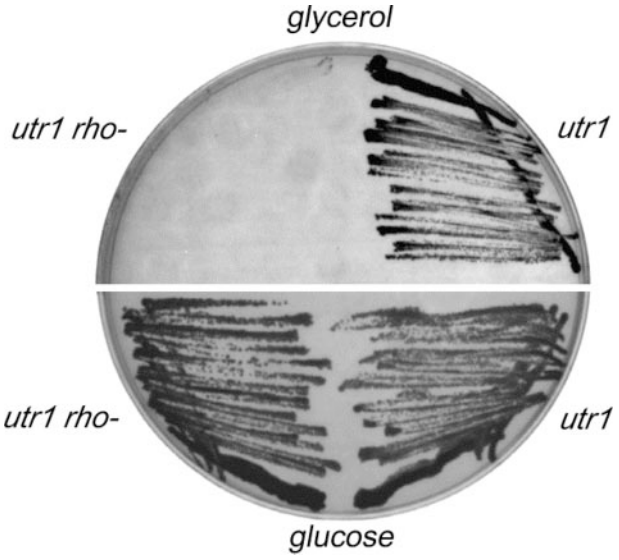

FIGURE 7. **utr1 is not synthetically lethal with lack of mitochondrial genome but with pos5 mutation.** A *rho*− derivative of a *utr1* haploid strain, obtained by passage on ethidium bromide media, is respiratory-incompetent yet viable. Because *utr1* is synthetically lethal with *pos5* but not with mitochondrial dysfunction, the *utr1* mutant must depend on at least transient exposure of Pos5 to the cytoplasm for viability.

or mitochondrial function, the mechanism of *ADH1*-driven *YEF1* as a suppressor of synthetic lethality with *utr1* appears to be production of cytoplasmic rather than mitochondrial NADP/NADPH.

As shown in Fig. 4, *pos5* mutants are incapable of growth on glycerol, whereas *utr1* mutants have a slow growth phenotype on glucose, and *pos5* mutants have a modest slow growth phenotype. To test whether the slow growth phenotypes of *pos5* or *utr1* might be suppressed by any of the NAD/NADH kinase genes, we compared the growth of *pos5* and *utr1* haploid isolates carrying empty vector pRS416 with those carrying plasmids pB532, pB534, and pB535. As shown in Fig. 6*A*, normal growth of a *pos5* strain can be restored by a plasmid-borne copy of *POS5* but not by either of the genes encoding cytosolic NAD/NADH kinases. In contrast, as shown in Fig. 6*B*, the slow growth phenotype of *utr1* can be fully reversed by plasmid-borne *UTR1* and by *ADH1*-driven *YEF1* and can be improved by plasmid expression of *POS5*. Thus, although expression of cytosolic NAD/NADH kinases have no effect on the phenotype of the mitochondrial NAD/NADH kinase mutant, expression of mitochondrial NAD/NADH kinase appears to reduce the phenotype of a deficiency in cytosolic NADP/NADPH.

Because *ADH1*-driven *YEF1* suppresses the *pos5* dependence on *UTR1* without restoring the mitochondrial function to *pos5*, we considered the possibility that *utr1* is not synthetically lethal with the respiratory defect of *pos5* but rather with the lack of at least a transient exposure of the Pos5 active site to the cytosol. As a test of this hypothesis, we set out to obtain *rho*− derivatives of *utr1* by growing cells overnight in YPD medium containing 10 μg/ml ethidium bromide and streaking the resulting cells on glucose medium (38). As shown in Fig. 7, such mutants were easily obtained and proved to be respiration-incompetent by failure to grow on glycerol medium. However, as judged by plate assays, the *rho*− *utr1*

mutants grown on glucose possessed the same growth rate as *rho+ utr1* mutants. Because there is not a respiratory component to the growth of *utr1* mutants but *utr1* mutants depend on *POS5*, we conclude that the viability of *utr1* mutants depends on cytosolic Pos5 enzymatic activity expressed during Pos5 transit to the mitochondria.

In conclusion, Utr1 is responsible for essentially all of the NAD/NADH kinase activity resident in the cytoplasm, whereas Pos5 is responsible for all mitochondrial NAD/NADH kinase activity and consequent mitochondrial genome maintenance. Yef1 can substitute for Utr1 when overexpressed. Because *utr1* is synthetically lethal with *pos5* but not with loss of respiration, the data indicate that transitory exposure of Pos5 to the cytoplasm is required for the viability of *utr1* mutants.

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Supplementary Table 1. Oligonucleotides used in this study.

776	CGCGAATTCATCAGCACATGACTGTC
777	CGCACTAGTGACAACCTGAAGTCTAGGTCC
778	GCGACTAGTATGAAAACTGATAGATTACTGATTAACG
779	CGCGAATTCTTAGATTGCAAAATGAGCCTGAC
780	CGCGGATCCGGAGTTTTTGACTGCGGTAATG
781	GCGCTCGAGTATGTCAGTTGAGATTTCTCTAGAC
7125	GGCGGATCCATATGAAGGAGAATGACATGAATAATGG
7126	GCCCTCGAGTTATACTGAAAACCTTGCTTGAG
7127	GGTAAATGAGGAAGATGGTCGAAATGATCATCATAACAACAATAATAACTAGATTGTACTGAGAGTGCAC
7128	CAAGCTTCGCAGTTTACACTCTCATCGTCGCTCTCATCATCGCTTCCGTTCTGTGCGGTATTTCACACCG
7300	CATGCACCACCACCACCACCACCACCACCT
7301	CATGAGGTGGTGGTGGTGGTGGTGGTGG
7312	GTGCAATCTCAATAAATCTGCTTACGTGACATTTTTTACTAAAAGAGAATAGATTGTACTGAGAGTGCAC
7313	TTAGATTGCAAAATGAGCCTGACGAGTCCGTTCAACAACTATTTCTGTTTCTGTGCGGTATTTCACACCG
7314	GTATTCATCTCACTTCTATAGCGTTGTG
7315	CCTTGACTACGGAAACGCAGGATGTG
7318	CGTACGCATATGAAAACTGATAGATTACTGATTAACGC
7319	CAGTGGAGATCTTAGATTGCAAAATGAGCCTGACGAG
7342	GTTCATCAGCAGATTTCGTGTCC
7343	CCACCAAATTCCAAATTACAATCTTTAATCTGGCAGAACCCTTTACAAAAGATTGTACTGAGAGTGCAC
7344	TTATCAGTCTGTCTCTTGGTCAGC
7345	CTAAAGCTAGAATTGAATCCTAAAAGTTCATTGATTCCTCTAATCCAGTCTGTGCGGTATTTCACACCG