Characterization of the *PNT1* Pentamidine Resistance Gene of *Saccharomyces cerevisiae*

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The *Saccharomyces cerevisiae PNT1* gene was isolated and characterized. When present in high copy number in *S. cerevisiae*, *PNT1* confers resistance to the anti-*Pneumocystis carinii* drug pentamidine. The *PNT1* gene encodes a previously uncharacterized polypeptide of 409 amino acids. The predicted gene product is a very basic (pI 9.9) polypeptide with one potential membrane-associated region. *PNT1* is located on chromosome XVR of *S. cerevisiae*. It is transcribed at a very low level. Overexpression of the gene increases resistance to the cytostatic and mitochondrial DNA-damaging effects of pentamidine and related cationic compounds. Disruption of the gene leads to slightly increased levels of susceptibility to pentamidine and some related compounds.

Pentamidine isethionate [1,5-bis(*p*-amidinophenoxy)-pentane bis(2-hydroxyethanesulfonate salt)] has been used since the 1930s to treat protozoal infections such as leishmaniasis and trypanosomiasis (27). Since the onset of the AIDS epidemic in the 1980s its major application has been as a therapeutic agent for the treatment and prevention of *Pneumocystis carinii* pneumonia in AIDS patients (26). Approximately 80% of AIDS patients suffer from *Pneumocystis* pneumonia, and 20% ultimately die from this disease. Unfortunately, pentamidine is not an ideal therapeutic drug; it is somewhat toxic and is not completely effective (13). Although potential mechanisms of action for pentamidine have been suggested (6, 15, 19), the mechanism has not been definitively established. In addition, the bases for the drug's side effects are unknown. Finally, although it has not yet been established that resistance to pentamidine is arising in *P. carinii*, prophylactic treatment of a large population should select for such resistance. Resistance may be responsible for "breakthrough" cases in which patients fail to respond to pentamidine therapy (26, 28). Moreover, resistance to pentamidine has been widely reported in trypanosomes (5, 7). This study focuses on identifying the cellular components that are important to pentamidine action and on characterizing potential resistance mechanisms in order to provide rational bases for improved drug therapy.

We used *Saccharomyces cerevisiae* as a model system for *P. carinii* (15) for several reasons. First, considerable molecular and morphological data indicate that *P. carinii* is a fungus (25), with no relative being clearly closer phylogenetically than *S. cerevisiae* (3). Second, although pentamidine has been thought to be primarily an antiprotozoal drug (and *P. carinii* has been thought to be a protozoan), we have determined that *S. cerevisiae* (10, 15) and many other fungi (14a) are susceptible to pentamidine. Third, the metabolic reactions of *P. carinii* are susceptible to pentamidine in vitro at concentrations similar to those inhibitory to *S. cerevisiae* (4, 15), although the limited replication by *P. carinii* in vitro prohibits measurement of viability by conventional means (23). Finally, *P. carinii* is an intractable organism. Although it can be cultured in animal systems and it can be sustained in vitro, genetic approaches are impossible and performance of biochemical or metabolic ex-

periments is difficult with the organism. We hypothesize that pentamidine may inhibit homologous targets in *S. cerevisiae* and *P. carinii* and that the resistance mechanisms in these fungi may also be similar. Therefore, one can use the metabolic, biochemical, and genetic approaches available in *S. cerevisiae* to examine the mechanisms of pentamidine action that cannot be examined in *P. carinii* (12, 23). The approach applied in the present study was to select and characterize genes that can confer resistance to pentamidine when they are overexpressed. Although these genes are part of the genomes of normal, susceptible *S. cerevisiae*, overexpression of these genes confers drug resistance. This approach has been used repeatedly to identify drug targets and to delineate the cellular processes affected by drugs in *S. cerevisiae* (18).

Pentamidine has several effects on *S. cerevisiae* cells (15). Pentamidine inhibits growth on nonfermentable carbon sources at low concentrations. Pentamidine also induces petite mutations and inhibits respiration at higher concentrations. These effects all suggest that the mitochondrion may be the cellular target of pentamidine, although they do not identify the individual cellular components involved in pentamidine toxicity or in potential resistance mechanisms. Study of the mechanisms of resistance will identify the cellular components and processes involved in drug susceptibility in normal cells and will also identify resistance mechanisms that may be broadly conserved in fungi, including *P. carinii*.

MATERIALS AND METHODS

Media and reagents. Pentamidine isethionate (Sigma, St. Louis, Mo.) was dissolved in sterile water at 25 mg/ml and was stored at −20°C. All other test compounds were purchased from Sigma or Aldrich (Milwaukee, Wis.) and were dissolved in water or ethanol. Solidified media contained 2% agar. Media were sterilized by autoclaving. Pentamidine and the other test compounds were added after the medium had cooled to 55°C. Standard media for yeast culture were YPD (1% yeast extract, 2% peptone, 2% glucose) or YPG (1% yeast extract, 2% peptone, 2% glycerol). Strains bearing plasmids were cultured in Yeast Nitrogen Base (Difco) with appropriate supplements and either 2% glucose (YNBD) or 2% glycerol (YNBG) as the carbon source and ammonium as the nitrogen source.

Strains and plasmids. Yeast strains MGD-535-46D (*MATα trp1-289 ura3-52 leu2-3 leu2-112 his3Δ1 cyh*^r) and MGD-535-

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13D (*MATa trp1-289 ura3-52 leu2-3 leu2-112 ade2-112 arg4*) were obtained from B. Rymond (University of Kentucky). Strain SI21R (*MATa ura3-52 leu2-3 leu2-112 MEL1 ade1 GAL4*) was obtained from M. Nagiec (University of Kentucky), who also supplied a genomic yeast DNA library from strain SI21R (16). All strains were pentamidine susceptible. The library originally contained 27,000 members. The average insert size was 8 kb; inserts were derived by partial *Sau*3A digestion of genomic DNA. These fragments were cloned into the *Bam*HI site of the *S. cerevisiae* multicopy vector YEp434.

Plasmids pRS425, pRS426, pRS416, and pRS406 (22) were provided by P. Hieter (Johns Hopkins University). Plasmid YEplac195 (8) was obtained from M. Mendenhall (University of Kentucky). Plasmid pBSKS$^+$ was from Stratagene.

Measurement of drug resistance. Pentamidine resistance was assayed qualitatively by growth on solid media containing pentamidine. The primary assay medium was YNBG with supplements appropriate for the strain to be tested. Supplements (vitamins, amino acids, etc.) were used at standard concentrations (9). When present, the concentration of pentamidine was 10 μg of the isethionate form per ml. Plates were incubated for 7 days at 30°C. Under these conditions pentamidine-susceptible cells form very small colonies that are readily distinguishable from the large colonies formed by resistant strains. The toxicities of drugs other than pentamidine were tested in a similar manner on YNBG or YPG. A series of three to five concentrations in twofold dilutions were tested for each drug.

Measurement of respiration. Respiration was measured by determination of oxygen consumption (15). An overnight culture of *S. cerevisiae* was grown in YNBD to maintain selection for plasmids, as appropriate. This culture was diluted with YPG and was incubated for 4 h at 30°C. The O$_2$ consumption of the stirred cell suspension was measured with an oxygen electrode (Yellow Springs Instruments) and polarograph (George Johnson, Baltimore, Md.). The relative rates of oxygen consumption by standardized cell suspensions with and without pentamidine treatment were compared.

Genetic and molecular genetic methods. Standard recombinant DNA methods for the manipulation of *Escherichia coli*, manipulation of plasmids, and nucleic acid hybridizations were used (20). Probes for nucleic acid hybridizations were prepared by random priming of isolated DNA fragments.

The sequence of the *PNT1* gene was determined by dideoxy sequencing of both strands with multiple overlaps. The majority of the sequence was obtained from nested deletions generated by the Erase-a-Base (Promega) exonuclease method. Additional sequences from subclones and specific primers were also used.

Standard yeast genetic manipulations and culture were used (9). *S. cerevisiae* was transformed by the polyethylene glycol method (21). Total yeast RNA was isolated by standard methods (9).

Measurement of petite mutation frequencies. To measure the frequencies with which test compounds induced petite mutations, yeast cultures with 10^6 cells per ml of YPD were incubated with various concentrations of the compounds for 24 h. Then samples from the cultures were plated onto YPG plates and plates containing YP with 0.7% glucose. Petite mutants formed tiny (YPG) or small (YPD) colonies, which did not grow when transferred to glycerol medium.

Nucleotide sequence accession number. The DNA sequence has been submitted to GenBank under accession number U15217.
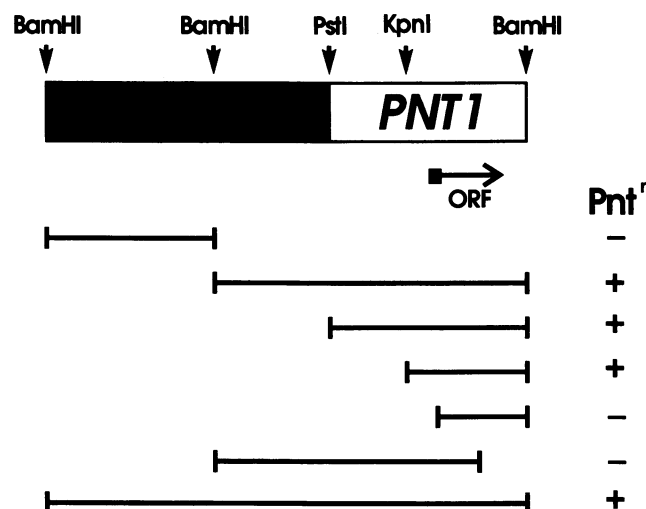

FIG. 1. Restriction and functional map of pGLK11. The figure correlates the restriction map of the insert in pGLK11 to the pentamidine resistance phenotype conferred by subclones. +, ability to confer resistance to 20 μg of pentamidine per ml. The location of the open reading frame (ORF) for Pnt1p is indicated. The region between the *Kpn*I and distal *Bam*HI sites was sequenced.

RESULTS

Isolation of *PNT1* pentamidine resistance gene. Pentamidine-susceptible *S. cerevisiae* MGD-535-46D was transformed with a library of genomic yeast DNA in the multicopy vector YEp434. Eleven pentamidine-resistant clones were identified from 45,000 independent transformants. Plasmids from the resistant *S. cerevisiae* clones were transfected into *E. coli*. Only two isolates, which were derived from separate transformation experiments, consistently conferred high-level pentamidine resistance. Restriction mapping and DNA hybridization analysis indicated that these two plasmids were identical. One isolate, pGLK11, was chosen for further analysis.

Functional map of *PNT1* gene on pGLK11. The *PNT1* gene was mapped by constructing shortened subclones of pGLK11 in the YEplac195 and pRS426 vectors. Initial transformation experiments indicated that the 3.5-kb *Bam*HI-*Pst*I segment conferred resistance. We determined the DNA sequence of this fragment. The fragment contained three open reading frames longer than 200 bp. The longest open reading frame (1,230 bp) presumably corresponded to the open reading frame of the *PNT1* gene. We analyzed the functions of additional fragments from this region (Fig. 1). The 2.1-kb *Bam*HI-*Kpn*I fragment, containing the single long open reading frame, appeared to encode the *PNT1* resistance gene. Subclones extending either upstream or downstream from the open reading frame, but bearing only portions of this reading frame, did not confer resistance (Fig. 1). The shortest subclone tested was truncated at an *Xho*I site 4 bases distal to the presumed initiation codon.

Sequence of *PNT1* gene. The sequence of the 2.1 kb of genomic DNA encoding the *PNT1* gene and the amino acid sequence of its presumed polypeptide product, Pnt1p, are presented in Fig. 2. Features of the sequence are described in the Discussion section.

Expression of *PNT1* gene. The *PNT1* gene was expressed at very low levels in normal *S. cerevisiae* (Fig. 3). Comparison of Northern (RNA) blots probed with the *ADE3* (data not shown) and *PNT1* genes suggested that *PNT1* is expressed at

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GGTACCGAAGATGTCAGTGATGCAAGATCCGCTATTACGTCTGCACAGGAATTACTCGATTCTAAATAATCT    72
ATAGATAACCTTTTTCTTACCTTTTCGCATTTATTTTGATGCAGGTTTTTATACTTTATTATGTACAAACCT   144
GTTGCGGATGAATAATAAATCTCGCTAGAAAATGCACGGAATATTATATGCAAAAGTAGAAAAGTGGAAGAC   216
GGCTGTTGATACAGTGACGAGATATAAGAACATTTTACTCTATATTTAAAAAGAACTTTATTAATCTATTTA   288
ATTGAGTTAACACCTTTAATGCCATCGATATGGATTCTCGAGTTGCACTGGTACGAAAATACATAGCACCAT   360
                               M  D  S  R  V  A  L  V  R  K  Y  I  A  P

CGGTGATAAAAAGCGATTCTATTCAATTGCACGGTTTAGTGAAGGCACCACTATTTAAAGCGTTGAATAGCC   432
S  V  I  K  S  D  S  I  Q  L  H  G  L  V  K  A  P  L  F  K  A  L  N  S

GTTACAAATTAGGTTCACTACAGATAGTACAGGATGTCGATTGGAATGCGAAGACAACACCATCCGATTCTC   504
R  Y  K  L  G  S  L  Q  I  V  Q  D  V  D  W  N  A  K  T  T  P  S  D  S

CAGAGCCTCTGGCCGCTACTCTAAACTCTAATCGATCCCTACCAATGACTAAGTTCCCCAAGCAGGAGATCT   576
P  E  P  L  A  A  T  L  N  S  N  R  S  L  P  M  T  K  F  P  K  Q  E  I

TGGAACAGGTAAAATTGGATACTAAGGTTGGCAAATGGAGGAAATTCATGACAGGTTGGTTTAGAATTGGAT   648
L  E  Q  V  K  L  D  T  K  V  G  K  W  R  K  F  M  T  G  W  F  R  I  G

TGTATCTTTTAAAAAGCTATAAAACAGGAATTCAGAACACGTTAAAAGTATTCTGGGATACTCGAAACGAAG   720
L  Y  L  L  K  S  Y  K  T  G  I  Q  N  T  L  K  V  F  W  D  T  R  N  E

AGCAGAAATTCAGCATAAAAAACGGTGCATTGGCTAACTTAGTACGGGAAATAGAAATGCATGAAATCAATA   792
E  Q  K  F  S  I  K  N  G  A  L  A  N  L  V  R  E  I  E  M  H  E  I  N

CAAGACTATCGTCTTCATCATTGCCTACAAGTTCTTCAGCAAAAGCCCCATTAAGGCCACTTTCAATCAATA   864
T  R  L  S  S  S  S  L  P  T  S  S  S  A  K  A  P  L  R  P  L  S  I  N

GGAAAACACTGGTGGAACTGATCAGACGAGATCAAATCTGGAAATTACCTGTATTTTTCACCTTAGTATTCA   936
R  K  T  L  V  E  L  I  R  R  D  Q  I  W  K  L  P  V  F  F  T  L  V  F

TATTTGAGGAGGTAAGTGTATTGATCTTTACCTTTTTTCCACGTGTCTGTCCGTACAATTGTTTAACTCCTG  1008
I  F  E  E  V  S  V  L  I  F  T  F  F  P  R  V  C  P  Y  N  C  L  T  P

GTGGATACAAGAAGCTTTCCAACTCATATATCAAGGGAACAACAAGCACACAAGGTAACTACGGTCTGGGTC  1080
G  G  Y  K  K  L  S  N  S  Y  I  K  G  T  T  S  T  Q  G  N  Y  G  L  G

CCTTAGAGTTTACTAAGCAAGGTACAATTAAGTATGAGCCACCATATGCAGTTCCTATTGAGAATTTGTATA  1152
P  L  E  F  T  K  Q  G  T  I  K  Y  E  P  P  Y  A  V  P  I  E  N  L  Y

ATTTTCTGACAAGCTTTCCTCAAAGTATGATATCAAACTGGAAACTGTATATTTATAAAAAGTTAAAATTAC  1224
N  F  L  T  S  F  P  Q  S  M  I  S  N  W  K  L  Y  I  Y  K  K  L  K  L

AGAAGCTACTTTGCAATGAGATAGAAAAAATTTACCAGTATCTGTTTATCGATGACTGGTTGCTGTTGCAGA  1296
Q  K  L  L  C  N  E  I  E  K  I  Y  Q  Y  L  F  I  D  D  W  L  L  L  Q

GTATACTCAACACTGATGTTGAGAAAACTAAAATCGCGCTAAGTGATAGAGAATTGGTTAACTGTATACTTG  1368
S  I  L  N  T  D  V  E  K  T  K  I  A  L  S  D  R  E  L  V  N  C  I  L

AAAGAAAACTTTATCACATGGGCGATGACTTGAATGAGATGGTTAATGACACGTTAGGGAAAGAAATCTTAC  1440
E  R  K  L  Y  H  M  G  D  D  L  N  E  M  V  N  D  T  L  G  K  E  I  L

TGAAAAGATTGTTTCTTTATTGGACATTAAGATATAATGACACTATATCCTTAAACGGAAAGCATACATTCA  1512
L  K  R  L  F  L  Y  W  T  L  R  Y  N  D  T  I  S  L  N  G  K  H  T  F

GTGAGAAATGGGGTGTCAACATATATCATTGTTAAAATATAATTCAGAATTGGTAGCTACGAAAGATATTCA  1584
S  E  K  W  G  V  N  I  Y  H  C  *

ATAGATGTAAATATTCATGACATTATCTATGCTGTACATATTGTTTAAAACAAAAACTCTTTTAAACTTTTC  1656
TAATTGCAAATTCAAACAATAATTCAGTGATGTTTCTTGCTTTTGGAGCAAAGGGCAATGTTACTCTATATT  1728
GTATGTATAATATGATAGATACTCTTCCGTATATATAGTATATAGTTTTCGTCAAGCGTTTCAATTGGAAAG  1800
TGAAAGAAAAAAATAAGAAAAAATAGAAAATACAATTAAATTAATCGAAAAAAGAAGTGAAAATAATTGAGT  1872
AGTTCGTATTAAACCTTGTAGGTCTCTAAGTGAGCATTTTCCTCCCTAACCAATGTATGGTGATGGCCAGGC  1944
GCTCTAATAACTTTATTTTTTGAATCCATGGTACAGGCAGCAATGGCGGCAAACCATTCATCATTAAAAATA  2016
TCATCTAAGGTTGCTCTTTTCTTTGGATCC
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FIG. 2. Nucleotide and deduced amino acid sequences of *PNT1*. The putative TATAA box, the TCGA initiation site, and the polyadenylation signals (TATAA and TARYTA) are underlined. Numbering is from the *Kpn*I site to the *Bam*HI site at the end of PGLK11.
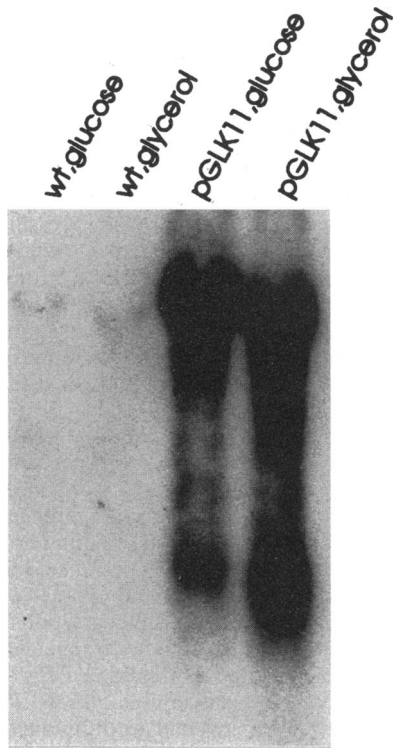
FIG. 3. Expression of the *PNT1* gene. A Northern blot of total *S. cerevisiae* RNA was probed with the *Bam*HI-*Kpn*I fragment of the *PNT1* gene. RNA was isolated from cells grown as indicated above each lane. Equal amounts of RNA (on the basis of ethidium bromide staining of rRNA and hybridization with the *ADE3* probe [data not shown]) were loaded. The upper transcript, which is barely visible in the wild type (wt), is 1,800 bases; the smaller transcript that is visible in the transformed cells is 1,000 bases.
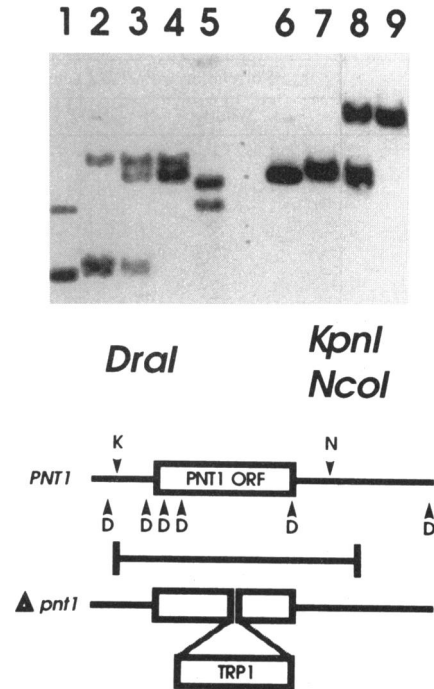

FIG. 4. Structure of the *PNT1* locus in wild-type and disrupted *S. cerevisiae*. A Southern blot of DNA digested with *Dra*I or *Kpn*I and *Nco*I as indicated and probed with the *Kpn*I-*Bam*HI fragment from *PNT1* is shown. DNA samples were from *PNT1* clone pGLK1101 (lanes 1 and 6), SI21R (lanes 2), MGD-535-46D (lanes 7), diploid of MGD-353-13D and MGD-535-46D Δ*pnt1* (lanes 3 and 8), MGD-535-46D Δ*pnt1* haploid (lanes 4 and 9), and Δ*pnt1* clone pGLK1108 (lane 5). The drawings below the blot indicate the presumed structures of the loci in wild-type and disrupted *S. cerevisiae*. The bar indicates the probe used for hybridization. Several small *Dra*I fragments (D) were not visible on this autoradiogram. ORF, open reading frame.

levels lower than those at which *ADE3*, which represents approximately 0.001% of the mRNA, is expressed (24). Expression of *PNT1* mRNA from the multicopy vector was significantly enhanced relative to expression from the chromosomal copy. Expression was similar in glycerol- and glucose-containing media, indicating that the insusceptibility of *S. cerevisiae* to pentamidine in glucose medium is not due to the enhanced expression of *PNT1* under these conditions.

Disruption of *PNT1* gene. The *PNT1* gene was disrupted by replacement of the chromosomal copy with a DNA construct in which the *TRP1* gene had been substituted for the *Hind*III-*Hind*III piece between bases 1020 and 1163. This construct was made by restriction digestion and ligation and resulted in a replacement of 143 bp of *PNT1* sequences with a 1-kb insertion. This insertion leaves intact the amino-terminal half of the reading frame. Replacement was done in both haploid and diploid *S. cerevisiae*. Southern blot mapping of the genomic DNAs of the replacement strains confirmed that the expected disruptions had occurred (Fig. 4).

Haploids in which the *PNT1* gene contained the large *TRP1* insertion were viable, indicating that the normal *PNT1* gene serves no function essential for growth on glucose-rich medium. Haploid Δ*pnt1* disruptants were able to mate and sporulate and grew normally at low (18°C) and high (38°C) temperatures and at high osmotic strength (500 mM KCl). The Δ*pnt1* strains as well as wild-type controls grew on glycerol and glucose. We observed no morphological abnormalities or altered properties associated with the Δ*pnt1* disruption. The effects on pentamidine susceptibility are described below.

Chromosomal location of *PNT1* gene. Southern blots of intact yeast chromosomes separated on contour-clamped homogeneous electric field gels were probed with the cloned *PNT1* gene. This probe detected the second largest of 13 separated chromosome bands, representing linkage groups VII, XII, and XV (data not shown). The *PNT1* probe hybridized to λ clone 2025 of the original Riles-Olson library (17); the clone is not linked to any contiguous region in the genome map derived from these clones (data not shown) (17a). We also examined the segregation of the *TRP1* marker integrated at the *PNT1* locus. The 30 tetrads analyzed exhibited the following recombination frequencies: 29% between *HIS3* and the *TRP1* marker, 27% between *ADE2* and *HIS3*, and 48% between *ADE2* and the *TRP1* marker. This linkage assigns the *PNT1* gene to chromosome XVR, consistent with the contour-clamped homogeneous electric field gel analysis. The *PNT1* gene seems to be located in the sparsely mapped region 25 centimorgans distal to *HIS3*, near the *GCD1* gene. Nevertheless, the sequence of *PNT1* is clearly distinct from that of *GCD1*. Southern blot hybridization indicated the presence of only one *PNT1* gene. Hybridization at reduced stringency (data not shown) revealed no distant homologs.

Effects of *PNT1* gene on pentamidine susceptibility. The resistance of cell growth to pentamidine appeared to be closely related to the copy number of the *PNT1* gene (Table 1). The

TABLE 1. Correlation of cell growth on YPG-pentamidine plates with *PNT1* gene copy number

Pentamidine concn (µg/ml)	Correlation with *PNT1* gene status[a]			
	Δpnt1	Wild type	*CEN-PNT1*	*2µ-PNT1*
0	+	+	+	+
0.5	+	+	+	+
1.0	+	+	+	+
2.5	±	+	+	+
5.0	−	±	+	+
10.0	−	−	±	+
25	−	−	−	+
50	−	−	−	±

[a] +, cell growth comparable to that of the control; ±, strongly reduced cell growth; −, no cell growth. The status of the *PNT1* gene is indicated as either *Δpnt1* (disrupted), wild type (single copy), *CEN* (present on a centromere-based plasmid in low copy number), or 2µm (present on a plasmid with a 2µm origin; presumed copy number greater than 20).

Δpnt1 disruptant strain was susceptible to 2.5 µg of pentamidine per ml, wild-type cells were resistant to 5 µg of pentamidine per ml, and the strain with *PNT1* on a low-copy-number *CEN* plasmid was resistant to 10 µg/ml; transformants bearing multicopy (2µm origin) plasmids with *PNT1* were susceptible only to 50 µg/ml or more. These strains showed the same relative pentamidine susceptibilities and resistance at 18 and 38°C as those at 30°C (data not shown). The independence of susceptibility from temperature suggested that susceptibility and resistance to pentamidine are not functions of cell growth rate.

Pentamidine at concentrations of 100 µg/ml inhibits oxygen uptake by wild-type *S. cerevisiae* by approximately 50% (15). Similar concentrations of pentamidine also inhibited respiration by cells overexpressing *PNT1* (data not shown). Respiration by *Δpnt1* cells did not appear to be more susceptible to inhibition by pentamidine.

In addition to growth inhibition and the inhibition of respiration, pentamidine induces mutations to a petite phenotype in *S. cerevisiae* (15). We infer from the phenotype and the frequency of these mutations that they are mutations of the mitochondrial genome. The *PNT1* gene also affected the frequency with which pentamidine induced petite mutations (Table 2). Strains disrupted at *PNT1* (*Δpnt1*) were more susceptible than wild-type cells to petite mutation induction at low concentrations of pentamidine. The frequency with which

TABLE 2. Dependence of *pet* mutant induction on *PNT1* copy number

Treatment	Frequency of *pet* mutant induction (%) in organisms with the following *PNT1* gene status[a]:			
	Δpnt1	Wild type	*CEN-PNT1*	2µm-*PNT1*
None	11	10	9	16
Pentamidine				
10 µg/ml	44	15	16	11
100 µg/ml	93	81	71	23
Ethidium bromide				
0.5 µg/ml	76	70	68	42
1 µg/ml	98	88	95	80

[a] The columns *CEN-PNT1* and 2µm-*PNT1* represent anticipated overexpression because of increased copy number from plasmids of 1 to 5 times and more than 20 times, respectively.

petite mutations were induced by pentamidine was inversely proportional to the *PNT1* copy number; overexpression of *PNT1* protected cells from the mutagenic effects of pentamidine.

Effects of *PNT1* gene on resistance to other inhibitors and mutagens. The *PNT1* gene affected the interaction of *S. cerevisiae* with inhibitory compounds other than pentamidine, but it did not confer a typical pleiotropic drug resistance phenotype. In this regard, strains overexpressing *PNT1* and *Δpnt1* strains were indistinguishable from wild-type cells in their susceptibilities to carbonyl cyanide *m*-chlorophenylhydrazone, chloramphenicol, cycloheximide, 4',6-diamidino-2-phenylindole (DAPI), diuron, flavone, hygromycin, nalidixic acid, novobiocin, and spiromycin. Nevertheless, strains overexpressing *PNT1* were consistently twofold more resistant to growth inhibition by ethidium bromide.

Antimycin A, chloramphenicol, flavone, Hoechst 33258, nalidixic acid, oligomycin, and valinomycin did not induce elevated frequencies of petite mutations at concentrations up to 100 µg/ml. Treatment with 20 µg of diamidine (Berenil) per ml, 1.5 µg of DAPI per ml, or 2 µg of ethidium bromide per ml for 24 h resulted in more than 90% petite colonies in all cell backgrounds (wild-type, *Δpnt1*, and *PNT1*-overexpressing strains [data not shown]). Lower concentrations of ethidium bromide induced petite mutations at frequencies that were inversely proportional to the expression of *PNT1* (Table 2). The *Δpnt1* cells were more susceptible and the *PNT1*-overexpresser strains were more resistant to the induction of petite mutants by this drug. Coincubation with concentrations of antimycin A sufficient to inhibit respiration reduced the percentage of petite mutants induced by 1 µg of ethidium bromide per ml or 100 µg of pentamidine per ml (data not shown). However, antimycin A increased the inhibitory effect of pentamidine on respiration in wild-type *S. cerevisiae* (data not shown).

DISCUSSION

PNT1 gene structure and expression. The *S. cerevisiae PNT1* gene does not appear to have been characterized previously. The gene is on chromosome XVR, approximately 25 centimorgans distal to the *HIS3* locus. The *PNT1* gene encodes an open reading frame of 409 amino acids. A potential TATAA sequence is located 79 bases upstream from the presumed ATG initiation codon. A TCGA transcription initiation site lies just 5 bases upstream from the ATG initiation codon. Nucleotide sequences associated with general amino acid control (TGACTC) or nitrogen control (GATAA) were not present in the 5' upstream region. The open reading frame region contained no matches to consensus intron splice sites, which suggests that the mRNA is not spliced. A TARYTA polyadenylation signal (14) is found 20 bases 3' to the open reading frame, and presumably, this signal is near the end of the mature *PNT1* transcript. The *PNT1* transcript was 1.8 kb, which is slightly longer than the 1.5 kb expected for a polyadenylated mRNA spanning the open reading frame and initiating and terminating at the consensus signals. The low level of gene expression is consistent with the low codon bias of the gene: −0.002 by the codon bias index of Bennetzen and Hall (2). The origin and significance of the smaller transcript (1 kb) have not been determined. This transcript was observed only in cells bearing the plasmid overexpressing *PNT1*.

The function of the polypeptide encoded by *PNT1* was not clear from its presumed amino acid sequence. The sequence of this polypeptide had no convincing similarity to sequences within current databases that were detected by FASTDB

(Intelligenetics), BLASTP, or TBLASTN (1). The best match in the BLOCKS database was to a family of anion transporters, although this match did not pass suggested significance tests (11). No protein functional motifs described in KeyBank were detected in the open reading frame by the Quest program (Intelligenetics).

The Pnt1p polypeptide had a predicted pI of 9.9, although two segments (residues 49 to 93 and 308 to 372) are acidic. Several secondary structure programs predicted three large α-helical regions in the carboxyl terminus and one highly hydrophobic helical region in the center of the polypeptide (residues 196 to 222) which appears to be a potential membrane-associated helix. However, the overall secondary structure lacks the multiple transmembrane domains of typical membrane transport proteins. The sequence of the amino terminus does not resemble typical mitochondrial import or secretory signal sequences, although such sequences are not always clearly distinct or located in the amino terminus. The polypeptide contains five Cys residues in the carboxyl-terminal half of the polypeptide, suggesting the possibility of a C-terminal domain stabilized by disulfide bonds. Nevertheless, we are not able to infer from the sequence either a cellular location or a biochemical function for Pnt1p.

Role of *PNT1* gene in resistance to pentamidine and other inhibitors. The *PNT1* gene affected the inhibition of growth and the induction of petite mutants by pentamidine, but it did not significantly affect the inhibition of respiration by pentamidine. This is consistent with the hypothesis that the primary inhibitory effect of pentamidine is not the inhibition of respiration (15). The overexpression or disruption of *PNT1* had no effect on the toxicities of a wide variety of drugs (including inhibitors of respiration, mitochondrial and cytoplasmic protein synthesis, or topoisomerase II). *PNT1* is therefore not a typical pleiotropic drug resistance gene of *S. cerevisiae*. Although chromosome XVR contains a cluster of *PDR* genes between *ADE2* and *HIS3*, this region is distinct from the *PNT1* locus and the sequence of *PNT1* is distinct from those of known *PDR* genes. The overexpression of *PNT1* induced slight cross-resistance to other positively charged, DNA-interacting compounds (Berenil, ethidium bromide). Although cross-resistance was much lower than the resistance to pentamidine (2- versus 10-fold), the mechanisms of resistance may be similar.

Implications for pentamidine mechanism of action and pentamidine therapy. Because the insertion of *TRP1* within the *PNT1* gene did not inhibit growth, the primary growth-inhibitory effect of pentamidine in *S. cerevisiae* cannot be inactivation of the *PNT1* gene product. Nevertheless, both growth inhibition and petite mutant induction were affected by *PNT1* expression, so we infer that *PNT1* affects a general process involved in pentamidine toxicity, such as enhanced drug efflux, increased detoxification, or increased target number. Other molecular approaches (such as immunolocalization of Pnt1p) will now allow the identification of the organelles and other cellular components involved in pentamidine toxicity and metabolism. The characterization of the *PNT1* gene begins a molecular description of these components and the mechanisms of resistance to pentamidine in fungi.

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