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Genetics

NIP1, a gene required for nuclear transport in yeast

(*Saccharomyces cerevisiae*/nuclear import/mitochondrial import/cytochrome *c*)

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ABSTRACT **Cytochrome *c* with a nuclear localization signal added at the N terminus was mistargeted to the nucleus, resulting in a yeast strain deficient in mitochondrial cytochrome *c*. Reversion of this strain allowed the isolation of temperature-conditional mutants defective in nuclear transport, as demonstrated with one of these mutants, *nip1-1*, that was shown to be defective in nuclear accumulation of a LacZ protein containing a nuclear localization signal of the yeast ribosomal protein L29. The *NIP1*$^+$ gene was cloned and shown to encode a 93,143-Da protein. Furthermore, an epitope-labeled NIP1 protein migrated in SDS/polyacrylamide gels with a mass of ≈100,000 Da and was shown by immunofluorescence to localize mainly in the cytoplasm. *NIP1*$^+$ was shown to be an essential gene by gene disruption experiments. Intriguingly, NIP1 has a serine-rich acidic N-terminal region that is similar in this regard to the N-terminal region of a previously described nuclear localization signal-binding protein, NSR1.**

Scores of gene products are likely to be involved in the nucleocytoplasmic trafficking of macromolecules. The 125-MDa nuclear pore complex (NPC) alone may contain >50 distinct polypeptides (ref. 1; G. Blobel and S. Rout, personal communication). Although significant progress has been made with higher eukaryotes on the cell biology, biochemistry, and structure of the transport apparatus (2), the use of genetics to elucidate the process of nuclear transport is still in its infancy (3).

We describe here a genetic selection with the yeast *Saccharomyces cerevisiae* for the isolation of mutants that are defective in the nuclear import of proteins. The selection is based on the fact that yeast cells do not utilize lactate as a sole carbon source if mitochondrial cytochrome *c* (cyto *c*) levels fall below 10% of the normal level. Also, because the N-terminal region of yeast iso-1-cyto *c* is not required for mitochondrial import (4), most alterations can be made in this region without significantly affecting the import or function of the protein. We have altered the N-terminal region to correspond to the nuclear localization signal (NLS) of the simian virus 40 (SV40) large T antigen. The T-antigen NLS has been shown to target proteins efficiently to yeast nuclei (refs. 5 and 6; Z.G., unpublished results). Because the N-terminal NLS apparently is functionally dominant over the native mitochondrial import signal, which is located internally in the protein, NLS-cyto *c* is misdirected to the nucleus, causi a >20-fold reduction in mitochondrial cyto *c* and conseque. ly diminished growth on lactate medium.

Our strategy was to isolate temperature-conditional r u-tants with defects in nuclear transport that resulted in hig ier mitochondrial levels of NLS-cyto *c*. These strains sh ould show both elevated holo-cyto *c* levels and improved growth on lactate medium, although they should not grow at restrictive temperatures because they are conditional for nuclear transport, an essential cellular process. Mutants were selected for increased growth at 30°C on lactate medium and subsequently screened for temperature-conditional growth at 37°C on glucose medium. Among these should be mutants with defects in nuclear targeting and translocation. Here, we describe the isolation and characterization of the *nip1-1* mutant and the cloning and sequence of the *NIP1*$^+$ gene.‖

METHODS

Nomenclature. *CYC1*$^+$ and *CYC1* denote, respectively, the wild-type allele and the chromosomal locus encoding yeast iso-1-cyto *c*. The *cyc1-31* allele causes complete deficiency because of a nonsense/frameshift mutation corresponding to amino acid position 4 (7). The *cyc1-1004* allele, denoted *cyc1-NLS* in this paper, contains <5% of the normal holo-iso-1-cyto *c* because the N-terminal region contains the NLS of the SV40 large T antigen; the protein encoded by *cyc1-NLS* is denoted NLS-cyto *c*. Holo-NLS-cyto *c* denotes the form of NLS-cyto *c* that contains the heme group required for function and for its spectral properties; whereas apo-NLS-cyto *c* denotes the form of NLS-cyto *c* that lacks the heme group. *NIP1*$^+$ and *nip1-1* denote, respectively, the wild-type allele and a mutant allele; whereas *NIP1-HA* denotes the functional allele of *NIP1* that contains an epitope from the influenza hemagglutinin protein (HA). NLS-β-gal denotes the *Escherichia coli* LacZ protein [β-galactosidase (β-gal)] with the NLS of the yeast ribosomal protein L29 (6).

Yeast Strains and Plasmids. The major yeast strains used in this study were B-7528 (*MATa cyc1-31 cyc7-67 ura3-52 lys5-10*), B-8106 (*MATa cyc1-NLS cyc7-67 ura3-52 lys5-10*), B-8302 (*MATa cyc1-NLS nip1-1 cyc7-67 ura3-52 lys5-10*), B-8303 [*MATa cyc1-NLS nip1-1 cyc7-67 ura3-52 lys5-10* (pNLS-lacZ)], and B-8305 [*MATa cyc1-NLS cyc7-67 ura3-52 lys5-10* (pNIP1-HA)]. These strains completely lack iso-2-cytochrome *c* because of the *cyc7-67* deletion.

The major plasmids used were YCp50, a centromere-carrying (*CEN*) shuttle vector (8); pNLS-lacZ, denoted pNLS-ElZ by Underwood and Fried (6), containing the NLS-lacZ gene; pNIP1, constructed by insertion of a 3.6-kilobase (kb) *Hin*dIII fragment, encompassing the *NIP1*$^+$ gene, at the *Hin*dIII site of YCp50; and pNIP1-HA, derived from pNIP1 and containing the *NIP1-HA* gene.

Cyto *c* Levels. Relative amounts of cyto *c* were determined by low-temperature (−196°C) spectroscopic examination of intact yeast (9) grown under derepressed conditions (10), and by comparisons to strains having known amounts of iso-1-cyto *c*.

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Abbreviations: NPC, nuclear pore complex; NLS, nuclear localization signal; cyto *c*, cytochrome *c*; HA, hemagglutinin; SV40, simian virus 40; β-gal, β-galactosidase.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. L02899).

Construction of Altered Genes. The *cyc1-NLS* mutation was constructed by transforming strain B-7528 directly with oligonucleotide OL89.204 (5'-TTCACACACTAAATTAATGCCGAAGAAAAAAAGGAAGGTTGAAGATCCGAAAGGTGCTAC-3'), using the method of Moerschell *et al.* (7). This method allows the recovery of *cyc1* alleles that produce even less than 1% of the normal level or activity of iso-1-cyto *c*. The sequence was verified by PCR amplification and DNA sequencing. pNIP1-HA was constructed by ligating OL91.201 (5'-TGTACCCATACGACGTCCCAGACTACGCTG-3') and its complementary oligonucleotide OL91.202 into pNIP1 cleaved at the unique *Pvu* II site, resulting in the insertion of a 9-amino acid epitope (YPYDVPDYA; ref. 11) at amino acid position 598.

Genetic Analysis and Molecular Techniques. Previously described methods and media were used for testing and genetic analysis of general yeast mutations (12, 13) and of *cyc* mutations specific for the iso-1-cyto *c* system (10, 14). Standard YPD medium, denoted glucose medium in this paper, and synthetic media used for growing and testing yeast strains have been described (13). Oligonucleotides were prepared as described (7).

Cloning and Sequencing of the *NIP1*$^+$ Gene. *NIP1*$^+$ was cloned by first complementing the *ura3-52* marker in strain B-8302 with a YCp50 single-copy genomic bank (8). The Ura$^+$ transformants were suspended in sterile water and streaked on glucose plates, which were incubated at 37°C. Plasmids complementing *nip1-1* in the presumptive *NIP1*$^+$ transformants were transferred from yeast to *E. coli* and analyzed with restriction endonucleases. After a number of plasmids with various deletions were examined, a 3.6-kb *Hin*dIII restriction fragment was shown to complement the *nip1-1* defect.

NIP1$^+$ was sequenced by first inserting the 1.6-kb *Hin*dIII–*Sal* I fragment and the 2.0-kb *Hin*dIII–*Sal* I fragment into M13 mp18 and mp19 vectors (15). A series of unidirectional nested deletions was made with exonuclease III (16). DNA sequencing was performed as described (17). Sequences were analyzed with the University of Wisconsin Genetics Computer Group program (18).

Immunofluorescence Microscopy. Cells in culture medium were fixed with 4% formaldehyde at room temperature for 2 hr. Spheroplasts were prepared with zymolyase and glusulase and immobilized on polylysine-coated slides (6). Antibody incubations were performed at room temperature for at least 2 hr. For NLS-β-gal localization, cells containing pNLS-lacZ were incubated at 37°C for 2 hr before induction at 37°C in medium containing 2% galactose. β-gal was induced to approximately the same level in normal (1-hr induction) and *nip1-1* mutant (2-hr induction) cells as determined by enzyme activity (19). Mouse anti-β-gal (Promega), used at 1:200, was stained with rhodamine-conjugated goat anti-mouse IgG at a dilution of 1:400. NIP1-HA was labeled with mouse anti-HA monoclonal antibody 12CA5 (11) at 1:100 and localized with rhodamine-conjugated goat anti-mouse IgG at 1:200. Nuclei were stained with 4',6-diamidino-2-phenylindole (1 μg/ml).

Immunoblot Analysis. Total yeast protein (20) was electrophoresed in SDS/10% polyacrylamide gels and transferred electrophoretically to nitrocellulose filters, which were then blocked with PBS (225 mM NaCl/10 mM Na_2HPO_4, pH 7.5) containing 2% Carnation nonfat dry milk. After a 3-hr incubation at room temperature incubation with primary antibody 12CA5, the filters were washed with PBS/0.05% Tween 20 and developed with alkaline phosphatase-conjugated anti-mouse IgG (Bio-Rad).

RESULTS

Expression of NLS-cyto *c* Protein in Yeast. The N-terminal region of iso-1-cyto *c* was changed from T^{1}EFKAGSAKKG^{11} to P^{-1}KKKRKVEDPKG^{11} by direct oligonucleotide transformation of the yeast strain B-7528 to incorporate the NLS of SV40 large T antigen (underlined) (21). The N-terminal proline is assigned position −1 because the fusion protein is one residue longer than the wild type. The resulting *cyc1-NLS* strain B-8106 contained <5% of the normal level of holo-cyto *c* (Table 1). Because the N-terminal region of cyto *c* is dispensable for function and mitochondrial import (4), the simplest explanation for the extreme deficiency of the *cyc1-NLS* strain is that apo-NLS-cyto *c* is misdirected toward the nucleus before heme attachment, which occurs within the mitochondria (22). Cyto *c* covalently crosslinked with synthetic peptide NLSs is imported into tissue culture nuclei by a receptor-mediated mechanism (23), and, in yeast, a NLS-cyto c_1 gene fusion product is imported into nuclei (24).

Isolation of *nip* Mutations. The impaired growth on lactate medium of the *cyc1-NLS* strain (Table 1) permits the isolation of revertants with restored levels of holo-cyto *c*. The restoration can occur by two types of distinct mutations: cis mutations at the *CYC1* locus that destroy the NLS on the *cyc1-NLS* allele, and trans mutations that either somehow impair nuclear localization or enhance mitochondrial import. Further, some of the trans mutations are expected to exhibit temperature-sensitive growth on glucose medium.

The *cyc1-NLS* strain B-8106 was treated with UV light and plated on lactate medium at 30°C. Revertant colonies were subcloned on glucose medium and subsequently tested for growth on glucose medium at 30°C and 37°C. Subclones exhibiting temperature-conditional growth were then examined for holo-cyto *c* content at permissive and restrictive temperatures. Desired mutants, as illustrated by the *cyc1-NLS nip1-1* strain in Table 1, had enhanced holo-cyto *c* levels and conditional growth on glucose medium at higher temperatures. A total of 6 candidates having the desired characteristics were uncovered after examination of 200 revertants. Only one of these, B-8302, was chosen for further analysis. The analysis of the meiotic progeny from heterozygous B-8302 crosses revealed a single-gene mutation, *nip1-1*, that both caused temperature-sensitive growth on glucose medium and enhanced the holo-cyto *c* levels (Table 1). Genetic analysis also revealed that the degree of the *nip1-1* defect was modified by a single gene in some heterozygous crosses and that *nip1-1* was unlinked to *cyc1-NLS* (data not shown).

Characteristics of *nip1-1*. *cyc1-NLS* cells containing the *nip1-1* allele grew better than the host strain on lactate medium at 30°C because holo-cyto *c* levels were elevated (Table 1). Because holo-NLS-cyto *c* levels increased in *nip1-1* cells at 33°C and 35°C, the temperature-conditional growth on glucose medium was probably due directly to a defect in nuclear transport that allowed more apo-NLS-cyto *c* to bypass the nucleus and enter mitochondria (see below).

Cellular Localization and Molecular Mass of the NIP1 Polypeptide. The cloned *NIP1*$^+$ gene (see below) was epitope-labeled at the unique *Pvu* II site by inserting a synthetic oligonucleotide encoding a 9-amino acid HA sequence recog-

Table 1. Phenotypes of strains with the *cyc1-NLS* and *nip1-1* mutations

Pertinent genotype	Growth			Holo-cyto *c* level, %		
	Lactate (30°C)	Glucose 30°C	Glucose 37°C	30°C	33°C	35°C
CYC1$^+$	+	+	+	100	100	100
cyc1-31	0	+	+	0	0	0
cyc1-NLS	0	+	+	<5	<5	<5
cyc1-NLS nip1-1	±	±	0	15	20	25

The *cyc1-31* strain completely lacks iso-1-cyto *c*. All of these strains lack iso-2-cyto *c* because of the *cyc7-67* deletion.

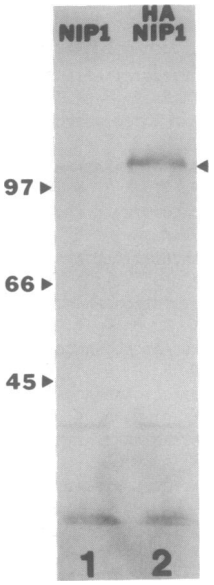

FIG. 1. Immunoblot analysis. Lane 1, *NIP1* strain; lane 2, *NIP1-HA* strain. Markers at left are in kilodaltons. The NIP1-HA protein (arrowhead) migrated with a mass of about 100 kDa.

nized by the mouse monoclonal antibody 12CA5 (11). The epitope-labeled NIP1 protein was able to fully complement the *nip1-1* lesion, indicating that it was functional. On immunoblots, 12CA5 recognized an ≈100-kDa polypeptide that was absent from cells lacking the HA sequence (Fig. 1). By immunofluorescence, the epitope-labeled NIP1 localized predominantly to the cytoplasm (Fig. 2), although it is possible that a minor fraction was associated with the nucleus.

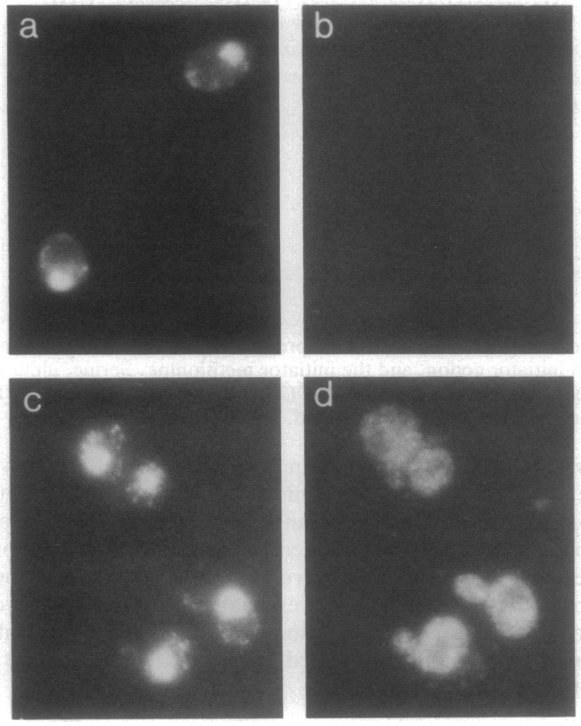

FIG. 2. Localization of NIP1-HA epitope fusion in cytoplasm. *NIP1* strain (*a* and *b*) and *NIP1-HA* strain (*c* and *d*) were incubated either with mouse anti-HA epitope antibody (12CA5) followed by rhodamine-conjugated goat anti-mouse IgG, to localize NIP1-HA epitope fusion (*b* and *d*), or with 4',6-diamidino-2-phenylindole to localize nuclei by staining DNA (*a* and *c*). (×4300.)

Defective Nuclear Import in *nip1-1* Cells. We examined the effect of the *nip1-1* lesion on the nuclear import of an inducible, nonessential karyophile that contained a native yeast NLS instead of a mammalian NLS. The *E. coli lacZ* gene fused to the NLS of ribosomal protein L29 produces a protein (NLS-β-gal) that accumulated in nuclei of *NIP1* yeast (6) (Fig. 3*A*). In contrast, the import of NLS-β-gal was defective in *cyc1-NLS nip1-1* (Fig. 3*B*). Depending on the focal plane, almost all mutant cells displayed perinuclear NLS-β-gal staining. Rarely, a wild-type cell displayed a β-gal staining phenotype that might be interpreted as partially perinuclear. To achieve similar levels of NLS-β-gal activity in wild-type and mutant cells, it was necessary to induce expression for 1 hr in the wild type and 2 hr in the mutant. We could not determine by this experiment whether NLS-β-gal was binding to or simply concentrating in the vicinity of the nuclear envelope. After longer induction at 37°C (up to 6 hr), NLS-β-gal stained much of the cell, including the entire nucleus (data not presented). Relating to this, the growth of

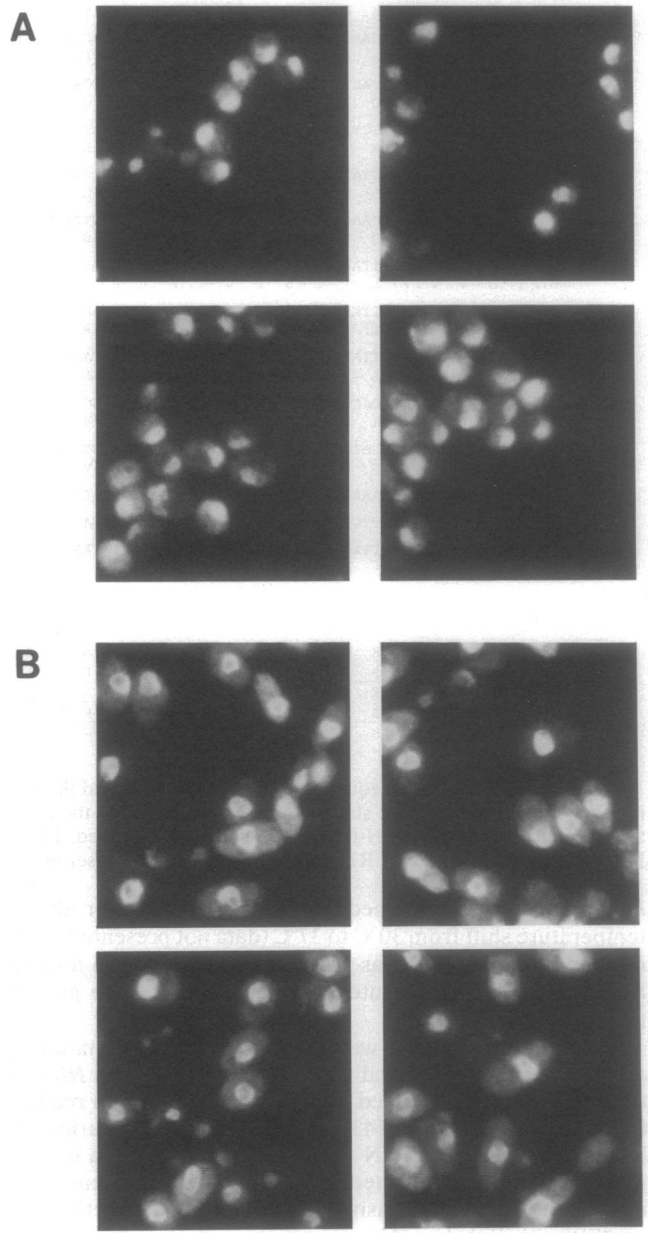

FIG. 3. Immunofluorescence localization of L29 NLS-β-gal in *NIP1*$^+$ cells induced for 1 hr (*A*) and *nip1-1* cells induced for 2 hr (*B*). (×3200.)

A

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-190 GCTTTTTTTTTCGCATTTTTTTCGCCGTTGAAAAATTTTTCAATGAGAAAAAGAGCTTTGAAAGGAATGAAAAATTGACTACAGGTTCGCAGTTGCACTTGTTAGTAGAGTAGCATAGAA
 -70 GGAAAAAAAGGAGAACAAGGCACATATTTGCAATCTTCACACAATAGCCTGCCAAGCCCGAGATCTACGAAAATGTCCCGTTTCTTTTCGTCTAATTACGAATACGATGTAGCCAGTTCT
   1                                                                                                   M  S  R  F  F  S  S  N  Y  E  Y  D  V  A  S  S
  50 TCATCCGAAGAAGATCTTTTATCTTCGTCTGAAGAAGATTTGTTAAGCTCTTCCTCCTCTGAGTCTGAATTGGACCAAGAATCTGACGACTCCTTTTTCAATGAAAGTGAAAGTGAAAGT
  17 S  S  E  E  D  L  L  S  S  S  E  E  D  L  L  S  S  S  S  S  E  S  E  L  D  Q  E  S  D  D  S  F  F  N  E  S  E  S  E  S
 170 GAAGCTGATGTAGACTCCGATGATTCTGATGCAAAGCCTTATGGTCCTGACTGGTTCAAGAAATCTGAGTTCAGAAAACAAGGTGGAGGTTCAAATAAATTTTTGAAAAGCTCTAACTAT
  57 E  A  D  V  D  S  D  D  S  D  A  K  P  Y  G  P  D  W  F  K  K  S  E  F  R  K  Q  G  G  G  S  N  K  F  L  K  S  S  N  Y
 290 GATTCCAGTGATGAAGAATCCGATGAAGAAGATGGCAAGAAGGATGTCAAGTCTGCCAAAGAAAAACTATTGGATGAAATGCAAGACGTTTATAATAAGATCTCTCAAGCTGAGAACTCA
  97 D  S  S  D  E  E  S  D  E  E  D  G  K  K  D  V  K  S  A  K  E  K  L  L  D  E  M  Q  D  V  Y  N  K  I  S  Q  A  E  N  S
 410 GATGACTGGTTGACTATTTCTAATGAGTTTGATTTGATCTCGCGTCTCTTAGTTAGGGCTCAACAACAAAACTGGGGGACTCCAAATATTTTCATCAAGGTTGTTGCCCAAGTGGAGGAC
 137 D  D  W  L  T  I  S  N  E  F  D  L  I  S  R  L  L  V  R  A  Q  Q  Q  N  W  G  T  P  N  I  F  I  K  V  V  A  Q  V  E  D
 530 GCTGTGAATAATACACAACAAGCTGATTTGAAGAATAAAGCTGTTGCAAGAGCTTATAACACTACAAAGCAAAGAGTCAAGAAAGTTTCTAGAGAAAATGAAGACTCAATGGCTAAATTC
 177 A  V  N  N  T  Q  Q  A  D  L  K  N  K  A  V  A  R  A  Y  N  T  T  K  Q  R  V  K  K  V  S  R  E  N  E  D  S  M  A  K  F
 650 AGAAATGATCCTGAATCATTTGATAAGGAACCAACCGCAGATTTGGATATTTCTGCTAATGGATTCACAATTTCTTCGTCTCAAGGCAATGACCAAGCGGTACAAGAAGATTTCTTCACT
 217 R  N  D  P  E  S  F  D  K  E  P  T  A  D  L  D  I  S  A  N  G  F  T  I  S  S  S  Q  G  N  D  Q  A  V  Q  E  D  F  F  T
 770 AGATTACAAACAATAATTGACTCAAGAGGTAAGAAGACTGTCAATCAACAATCCTTGATTTCTACTTTGGAGGAGTTATTAACTGTAGCTGAAAAACCATATGAATTCATAATGGCTTAT
 257 R  L  Q  T  I  I  D  S  R  G  K  K  T  V  N  Q  Q  S  L  I  S  T  L  E  E  L  L  T  V  A  E  K  P  Y  E  F  I  M  A  Y
 890 TTGACTTTGATTCCATCAAGATTCGATGCCTCAGCTAACCTATCTTACCAACCAATTGATCAATGGAAATCTTCATTCAACGATATTAGTAAATTATTGTCTATTTTAGACCAGACAATT
 297 L  T  L  I  P  S  R  F  D  A  S  A  N  L  S  Y  Q  P  I  D  Q  W  K  S  S  F  N  D  I  S  K  L  L  S  I  L  D  Q  T  I
1010 GACACCTACCAAGTTAATGAATTTGCTGATCCAATCGATTTCATTGAAGATGAACCTAAAGAAGATTCTGATGGTGTCAAGAGGATTCTGGGTTCCATTTTCTCATTTGTTGAAAGATTA
 337 D  T  Y  Q  V  N  E  F  A  D  P  I  D  F  I  E  D  E  P  K  E  D  S  D  G  V  K  R  I  L  G  S  I  F  S  F  V  E  R  L
1130 GATGACGAATTCATGAAATCCCTGTTAAACATCGATCCTCATTCCAGTGATTATTTGATCCGTTTAAGGGATGAACAATCAATCTATAATTTGATCCTAAGAACTCAATTGTACTTTGAA
 377 D  D  E  F  M  K  S  L  L  N  I  D  P  H  S  S  D  Y  L  I  R  L  R  D  E  Q  S  I  Y  N  L  I  L  R  T  Q  L  Y  F  E
1250 GCGACTTTGAAAGATGAACACGACCTAGAAAGAGCATTGACACGTCCATTCGTCAAGAGATTGGATCATATCTACTATAAATCCGAAAATTTGATAAAAATTATGGAAACTGCTGCTTGG
 417 A  T  L  K  D  E  H  D  L  E  R  A  L  T  R  P  F  V  K  R  L  D  H  I  Y  Y  K  S  E  N  L  I  K  I  M  E  T  A  A  W
1370 AATATCATACCTGCTCAATTCAAATCTAAATTTACTTCAAAAGACCAGCTCGATTCTGCTGATTATGTCGACAATTTAATAGACGGATTATCGACAATCTTATCCAAGCAAAACAACATT
 457 N  I  I  P  A  Q  F  K  S  K  F  T  S  K  D  Q  L  D  S  A  D  Y  V  D  N  L  I  D  G  L  S  T  I  L  S  K  Q  N  N  I
1490 GCTGTTCAAAAACGTGCTATTTTATACAACATTTACTACACTGCATTAAACAAAGATTTCCAAACTGCTAAAGATATGTTACTAACTTCCCAAGTTCAAACAAATATCAACCAATTCGAT
 497 A  V  Q  K  R  A  I  L  Y  N  I  Y  Y  T  A  L  N  K  D  F  Q  T  A  K  D  M  L  L  T  S  Q  V  Q  T  N  I  N  Q  F  D
1610 TCATCCCTACAAATTTTATTCAACAGGGTTGTTGTTCAATTGGGTCTATCCGCCTTTAAATTATGTTTGATTGAAGAATGTCATCAAATTTTGAATGATCTTCTGTCAAGTTCTCACTTA
 537 S  S  L  Q  I  L  F  N  R  V  V  V  Q  L  G  L  S  A  F  K  L  C  L  I  E  E  C  H  Q  I  L  N  D  L  L  S  S  S  H  L
1730 AGAGAAATTTTGGGCCAACATTCCCTACACAGAATATCTCTCAATTCTAGTAACAATGCTTCAGCTGATGAGCGTGCTAGACAATGTTTGCCATATCACCAACACATCAATCTCGATTTA
 577 R  E  I  L  G  Q  H  S  L  H  R  I  S  L  N  S  S  N  N  A  S  A  D  E  R  A  R  Q  C  L  P  Y  H  Q  H  I  N  L  D  L
1850 ATCGATGTCGTCTTCTTAACATGTTCCTTATTGATCGAAATTCCAAGAATGACTGCCTTCTATTCCGGTATTAACGTCAACAGAATTCCTTACTCTCCAAAATCCATTCGTCGTTCCTTA
 617 I  D  V  V  F  L  T  C  S  L  L  I  E  I  P  R  M  T  A  F  Y  S  G  I  N  V  N  R  I  P  Y  S  P  K  S  I  R  R  S  L
1970 GAACATTACGACAAGTTAAGTTTCCAAGGTCCACCAGAAACTTTAAGAGATTATGTCTTGTTTGCTGCCAAATCAATGCAAAAAGGTAACTGGAGAGACTCTGTTAAATACTTAAGAGAA
 657 E  H  Y  D  K  L  S  F  Q  G  P  P  E  T  L  R  D  Y  V  L  F  A  A  K  S  M  Q  K  G  N  W  R  D  S  V  K  Y  L  R  E
2090 ATAAAATCTTGGGCTTTATTACCAAACATGGAAACGGTGTTGAATAGTTTAACGGAAAGAGTACAAGTTGAATCTTTGAAGACTTATTTCTTTTCTTTCAAGAGGTTCTATTCAAGTTTT
 697 I  K  S  W  A  L  L  P  N  M  E  T  V  L  N  S  L  T  E  R  V  Q  V  E  S  L  K  T  Y  F  F  S  F  K  R  F  Y  S  S  F
2210 TCTGTTGCTAAACTAGCCGAATTATTTGATCTTCCAGAAAATAAGGTGGTTGAAGTTTTGCAATCTGTTATCGCAGAATTGGAAATCCCAGCCAAATTAAACGACGAGAAGACCATCTTT
 737 S  V  A  K  L  A  E  L  F  D  L  P  E  N  K  V  V  E  V  L  Q  S  V  I  A  E  L  E  I  P  A  K  L  N  D  E  K  T  I  F
2330 GTTGTCGAAAAGGGTGATGAAATTACTAAATTGGAAGAAGCAATGGTAAAATTGAACAAAGAATATAAAATCGCTAAAGAACGTCTTAACCCACCATCAAATCGTCGTTGATCAATAAAA
 777 V  V  E  K  G  D  E  I  T  K  L  E  E  A  M  V  K  L  N  K  E  Y  K  I  A  K  E  R  L  N  P  P  S  N  R  R  End
2450 TCAAAAACTACTTCGAAAGCATTCTTAACAAAAAATCATTGTAGATAGAAGAATAACATCAGCAACCAAAAAATATAGAAGGAAACTAAGCAGGAGAGTATAAACCATATTCACTTTTGC
2570 TTTTTATTCTTGATATTGCTCATCTTTTTTTTTTTCGATTCTTTTAGCGATCTCTTTATTTTTAGTTTTAGGGTTTGAATCTAATCGTCTTCACTGTACATTACACATAAGCAAATATAT
2690 ATATAGAAAAAAAACTTACCGTAAACACTCTTTATAATATAATACAAACTATTAAACTTTAAGAAAAGTATGATAT
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B

```
M  A  K  T  T  K  V  K  G  N  K  K  E  V  K  A  S  K  Q  A  S  K  Q  A  K  E  E  K  A  K  A  V  S  S  S  S  S  E  S  S
S  S  S  S  S  S  S  E  S  E  S  E  S  E  S  E  S  E  S  S  S  S  S  S  S  S  S  D  S  E  S  S  S  S  S  S  S  D  S  E  S
E  A  E  T  K  K  E  E  S  K  D  S  S  S  S  S  S  D  S  S  S  D  E  E  E  E  E  E  K  E  E  T  K  K  E  E  S  K  E  S
S  S  S  D  S  S  S  S  S  S  D  S  E  S  E  K  E  E  S  N  D  K  K  R  K  S  E  D  A  E  E  E  E  D  E  D  E  E  S
S  N  K  K  Q  K  N  E  E  T  E  E  P  A  -  -  -
```

FIG. 4. (*A*) Nucleotide sequence of the *NIP1*$^+$ gene and the deduced amino acid sequence of the NIP1 protein. Nucleotide and amino acid numbers, in the left margin, start with, respectively, A of the ATG translation initiator codon, and the initiator methionine. Serine, glutamic, and aspartic residues in the N-terminal region are underlined. The putative 3'-end-forming signal TATATAT (25), is doubly underlined. (*B*) The N-terminal region of the NSR1 protein (26, 27), with the serine, glutamic, and aspartic residues underlined.

nip1-1 cells in glucose medium stopped within 2 hr after a temperature shift from 30°C to 37°C (data not presented). The doubling time at 30°C was 5 hr. Thus, the block in nuclear transport was not absolute even at nonpermissive growth temperatures.

The *NIP1* Gene. *NIP1* was cloned by complementation of a *nip1-1* strain at 37°C and was isolated on a 3.6-kb *Hin*dIII fragment, which contained an 813-amino acid open reading frame that encoded a 93,143-Da protein (Fig. 4*A*). Curiously, NIP1 contains an acidic N terminus rich in serine, a feature that is strongly represented in NSR1, a yeast protein originally identified on the basis of its binding to synthetic NLS peptides (26, 27) (Fig. 4*B*).

***NIP1*$^+$ Is an Essential Gene.** An 1175-base-pair *Xba* I–*Pvu* II fragment comprising nucleotides 619–1793 of *NIP1* was deleted and replaced with the selectable marker *URA3*. The *ura3-52/ura3-52* diploid strain ZY10 was transformed with the DNA fragment containing this construct, denoted *nip1*::*URA3*. Stable Ura$^+$ transformants were selected and shown to have no growth defect. Nine tetrads from a sporulated heterozygous strain were analyzed. In all cases, only two spores from each tetrad were viable, and all of them were Ura$^-$. Microscopic examination revealed that the nonviable spores germinated and divided once or twice to produce no more than four cells.

DISCUSSION

We have described the genetic selection, characterization, and molecular cloning of a gene, *NIP1*, that is required for efficient nuclear transport in yeast. For the selection procedure, we assumed that a SV40 T-antigen NLS-cyto *c* fusion

protein would be mostly targeted to the nucleus even though the apo form of cyto *c* is unstable in the cytoplasm (20). Consistent with this assumption, this strain is partially deficient in mitochondrial holo-cyto *c* (about 5% the wild-type level) and exhibits diminished growth on lactate medium. The *nip1-1* mutation, which causes an increase in mitochondrial holo-cyto *c* levels, was selected for growth on lactate medium and was pursued because of its temperature-sensitive growth on glucose medium.

The localization of NLS-β-gal in *nip1-1* cells revealed a defect in nuclear transport. The defect, which is expressed best at 37°C, is characterized by the transient perinuclear localization of NLS-β-gal (Fig. 3). Nuclear import has been divided into two stages: (*i*) initial NLS-mediated targeting to the nuclear envelope/NPC and (*ii*) ATP-dependent translocation across the nuclear envelope (28, 29). When translocation is prevented by ATP depletion, by chilling, or by inhibitors of NPC function, karyophiles accumulate at the nuclear envelope (23, 28, 29). Perinuclear localization is, therefore, expected to be a natural intermediate along the import pathway. This fact may explain why a few wild-type cells displayed vague NLS-β-gal perinuclear staining at early times following induction. An interpretation of the *nip1-1* phenotype is that these cells can efficiently target nuclear proteins to the nuclear envelope but are partially defective at a subsequent step. However, it has yet to be determined whether the *nip1-1* allele encodes a defective component of the nuclear transport apparatus or, alternatively, encodes a gene that controls other processes, such as nuclear envelope assembly (24), that could indirectly affect protein import. Another class of mutations that could produce conditional increases in mitochondrial cyto *c* are those that increase the mitochondrial import of NLS-cyto *c*. Although such a mutation was found in a similar selection (3), we can rule out this possibility for *nip1-1* because in this strain it is the nuclear transport of the NLS-β-gal protein that is clearly defective. If the *nip1-1* defect were in mitochondrial import, then the nuclear import of NLS-β-gal would be unaffected.

The 813-amino acid sequence of NIP1 reveals no obvious membrane-spanning domains or apparent endoplasmic reticulum targeting signals, so it is likely that NIP1 is not a membrane protein. By immunofluorescence, the NIP1-HA protein localized predominantly to the cytoplasm. We do not know whether or not the NIP1 protein is also associated with the nuclear envelope, where a role in nuclear transport is easily understood. Also, we cannot rule out the remote possibility that the insertion of the epitope caused much of the NIP1-HA to mislocalize in the cell, even though *NIP1-HA*$^+$ gene fully complements the *nip1-1* mutation. Nevertheless, the disruption experiments clearly revealed that *NIP1*$^+$ was essential.

In higher eukaryotes, both cytoplasmic and nuclear envelope-associated proteins have roles in nuclear transport (2, 3, 30). Curiously, the N-terminal regions of both NIP1 and NSR1, a previously described yeast protein, are rich in serine, aspartate, and glutamate, and both N-terminal regions contain potential casein kinase phosphorylation sites. NSR1 was identified in nuclear envelope extracts on the basis of its *in vitro* binding to NLS peptides (26, 27). Aside from the similar N-terminal region, NIP1 and NSR1 are dissimilar at the sequence level and, while NIP1 is mostly cytoplasmic, NSR1 is probably a nucleolar, RNA-binding protein (27). Because both NIP1 and NSR1 were discovered in efforts to identify nuclear transport factors, we must consider the possibility that both NSR1 and NIP1 function in the trafficking of NLS-containing proteins via their serine-rich acidic N termini.

The NLS-cyto *c* selection scheme we have introduced here has significant advantages over a previously described scheme that used a NLS-cyto c_1 fusion (24). The N-terminal region of cyto *c* can be modified with various sequences without significantly reducing function (4), whereas the cyto c_1 precursor contains a typical matrix-targeting signal of the type that is known to function only when exposed at the N terminus. Thus, an N-terminal NLS-cyto c_1 fusion protein could probably be imported into mitochondria only after the NLS was proteolytically removed. In addition, a rapid and efficient oligonucleotide transformation method has been developed that allows the alteration of N-terminal region of cyto *c*, for example, by inserting various NLSs. This has allowed us to show that the level of holo-NLS-cyto *c* in yeast mitochondria is inversely proportional to the relative strength of the NLS fused to cyto *c*, providing an estimate of NLS strength in yeast (Z.G., F.S., and D.S.G., unpublished results).

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