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Functional Complementation of a Membrane Transport Deficiency in *Saccharomyces cerevisiae* by Recombinant ND4 Fusion Protein

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ND4 is a mitochondrially encoded component of NADH dehydrogenase (Complex I) of the respiratory chain. A cDNA encoding a fusion protein comprised of the N-terminus of *GAL10* of *Saccharomyces cerevisiae* and an N-terminally truncated form of human ND4 was isolated by its ability to functionally complement the thymidine transport deficiency of *S. cerevisiae* grown under conditions of thymidylate starvation. Epitope-tagged *GAL10*-ND4 was shown by immunological methods to be present in the plasma membranes of yeast expressing the *GAL10*-ND4 encoding cDNA. The ability of the *GAL10*-ND4 fusion protein to induce uptake of thymidine raises the possibility that native ND4, which is predicted to have 12 transmembrane domains, may function as a transporter or channel in the mitochondrial inner membrane.

Nucleosides play important roles in many physiological processes and several nucleoside analogs are used in anti-cancer and anti-viral therapies (1). Because nucleosides are hydrophilic molecules, their passage across biological membranes requires specialized transporter proteins, which, because they are present in low abundance in membranes, have been difficult to study (1). Two new families of nucleoside transporter proteins of plasma membranes have recently been recognized by molecular cloning and functional expression in *Xenopus* oocytes of cDNAs isolated from rat and human cDNA libraries (2,3). Representatives of these transporter families, which have been termed the "equilibrative" and "concentrative" nucleoside transporters, have thus far been identified in rat, human and bacterial cells (3-6).

Abbreviations used: bp, base pair; *GAL10,* UDP-galactose-4′-epimerase; nt, nucleotide; SD, synthetic-defined medium; TK, thymidine kinase; TS, thymidylate synthase.

To identify and characterize nucleoside transporter proteins, we developed a functional complementation strategy based upon a thymidine transport deficiency in the plasma membranes of *S. cerevisiae.* This strategy was used to isolate a cDNA encoding a previously unknown mouse transporter protein (MTP) of intracellular membranes (7). We report here the isolation of a human kidney cDNA that encodes an N-terminally truncated form of a human mitochondrial protein (ND4) fused to the N-terminus of the *S. cerevisiae GAL10* protein (UDP-galactose-4′-epimerase) by virtue of the ability of the fusion protein to complement the thymidine transport deficiency of *S. cerevisiae.* ND4 is a subunit of unknown function of Complex I of the mammalian respiratory chain (8,9) that has been implicated in Leber's hereditary optic neuroretinopathy (LHON), a mitochondrial genetic disease (10).

EXPERIMENTAL PROCEDURES

Library screening in yeast. Saccharomyces cerevisiae strain KY114 (genotype: MATa, *ura3-52, his3-200, trp1-63, ade2-101, lys2-801*) with a functional *Herpes simplex* thymidine kinase (TK) (7) was transformed with a human kidney cDNA expression library in the vector pYEUra3 (Clontech Laboratories), a low copy yeast/*E. coli* shuttle vector that contains the *URA3* auxotrophic selectable marker and the divergent *GAL1-GAL10* promoter (cDNA was inserted into a unique EcoRI site downstream of the *GAL10* promoter), and plated onto synthetic-defined medium (SD) that contained 2% glucose and lacked uracil and tryptophan (11). Transformants were cultured on plates with transport-selection medium that consisted of 2% galactose-SD with inhibitors of dTMP synthesis (35 μg/ml methotrexate, 5 mg/ml sulfanilamide) and 200 μM thymidine; the resulting colonies were processed exactly as previously described (7). A *URA3*-containing plasmid, termed pHKC, was rescued from a positive colony, re-transformed into TK-expressing yeast and shown to confer thymidine-dependent growth on yeast cultured on plates with transport-selection medium.

Analysis of cDNA and RNA. The cDNA insert (and its flanking regions) within pHKC was sequenced with the Sequenase V2.0 DNA Sequencing kit (United States Biochemical). DNA sequences were compared to the databases of the National Center for Biological Information and alignments were performed using the GCG software pro-

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gram (Genetics Computer Inc.). Isolation of total RNA and Northern blot analysis were performed as descibed (12) using the ^{32}P-labeled ND4 cDNA insert of pHKC as probe.

Identification of fusion protein. A double-stranded 36-nucleotide oligomer encoding the *c-myc* (EEQKLISEEDLL) epitope (13) was inserted into a unique *BstBI* site within the partial ND4 coding region of pHKC to yield pHKC-*myc*. Yeast membranes were isolated by a glass-bead disruption method (12) and immunoblotting was performed using the 9E10 (anti-*myc*) antibody as previously described (7). Intact yeast cells were prepared by the method of Harlow and Lane (14) for immunofluorescence staining using the 9E10 antibody and rhodamine B-conjugated goat anti-mouse IgG.

RESULTS

Isolation of ND4 cDNA. A detailed description of the complementation cloning strategy in *S. cerevisiae*, a cell type that normally lacks TK and a plasma membrane thymidine transporter, is provided elsewhere (7). Briefly, pharmacologic inhibition of thymidylate synthase (TS) inhibits growth because of depletion of dTMP. The co-expression in *S. cerevisiae* of *H. simplex* TK and a cDNA encoding a functional nucleoside transporter targeted to the plasma membrane reduces or eliminates the growth inhibition in the presence, but not the absence, of 200 μM extracellular thymidine. The growth inhibition can also be overcome (in TK-expressing yeast) at high concentrations ($\geqslant$500 μM) of extracellular thymidine, which enters cells by passive diffusion.

To screen for proteins that mediate membrane transport of thymidine, TK-expressing yeast were transformed with a human kidney cDNA expression library and plated onto the transport-selection medium (Figure 1). A library plasmid (designated pHKC), which contained a 1096-bp cDNA insert, was isolated by its ability to confer cell growth under conditions of TS inhibition in the presence, but not the absence, of thymidine. A plasmid lacking this cDNA insert (pYEUra3) failed to overcome the growth arrest in the presence of 200 μM thymidine. Glucose represses transcription from the *GAL10* promoter of the library plasmid, and yeast transformed with pHKC grew in transport-selection medium that contained galactose but failed to grow in transport-selection medium that contained glucose. Thus, the thymidine-dependent growth observed under the selection conditions required transcription of the cDNA insert of pHKC.

The 1096-bp cDNA insert of pHKC corresponded to nucleotides (nt) 11053-12137 of the human mitochondrial genome (15), which encodes amino acid residues 99-459 of ND4 (8), a component of Complex I of the respiratory chain. The partial ND4 cDNA differed from the ND4 DNA sequence of Anderson *et al.* (15) at three residues; a silent T-to-C substitution at nt 11335, a G-to-C substitution at nt 11447 that resulted in replacement of Val-230 with Leu, and a C-to-G substitution at nt 11450 that resulted in replacement of Leu-231

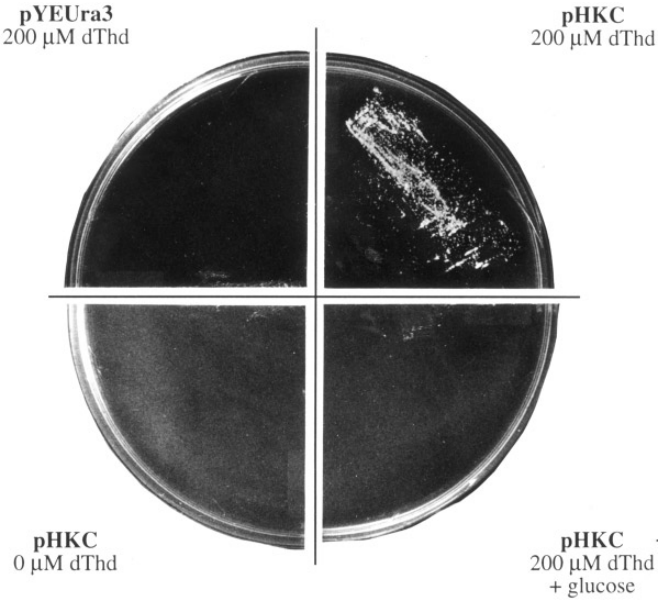

FIG. 1. Complementation of a thymidine transport deficiency in yeast. TK-expressing yeasts transformed with pYEUra3 or pHKC were plated onto transport-selection medium (2% galactose-SD, 35 μg/ml methotrexate, 5 mg/ml sulfanilamide) that contained or lacked 200 μM thymidine and/or 2% glucose.

with Val. Although yeast normally utilize the first AUG from the 5′ end of mRNA as the start codon for translation (16), the ND4 cDNA of pHKC did not contain an AUG codon within its 5′ terminus (Figure 2A). Analysis of the region encompassing the *GAL10* promoter-ND4 cDNA junction site revealed that the ND4 cDNA had been inserted (i) downstream of the *GAL10* transcriptional and translational initiation sites, and (ii) in-frame with a portion (corresponding to the N terminus) of the *GAL10* coding sequence of pYEUra3 (17). Thus, the sequence of pHKC predicted the production of a *GAL10*-ND4 fusion protein comprised of residues 1-48 of *GAL10* and residues 99-459 of human ND4.

Production of GAL10-ND4 mRNA. S. cerevisiae lack a native ND4 gene (18), and transformed yeast were examined for the production of ND4-encoding RNA transcript by high-stringency Northern blot analysis (data not shown). ND4-encoding RNA transcripts were observed in pHKC-transformed yeast grown in the presence of galactose, but not glucose, suggesting that galactose-induced transcription of the *GAL10*-ND4 cDNA was required for complementation. Cells transformed with pYEUra3 and grown in either galactose or glucose failed to exhibit any RNA transcript that hybridized to a ND4-specific probe.

Production of GAL10-ND4 fusion protein. UGA codons normally function as stop signals during protein synthesis, except in mitochondria, where they code for tryptophan (15). Since multiple UGA codons are distributed throughout the predicted ND4 RNA sequence,

A

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-182   ... ATCCAAAAAAAAGTAAGAATTTTTGAAAATTCAATATAA atg aca gct cag tta caa agt gaa   -120
                                                  Met Thr Ala Gln Leu Gln Ser Glu     8

-119   agt act tct aaa att gtt ttg gtt aca ggt ggt gct gga tac att ggt tca cac act    -63
 9     Ser Thr Ser Lys Ile Val Leu Val Thr Gly Gly Ala Gly Tyr Ile Gly Ser His Thr    27

-62    gtg gta gag cta att gag aat gga tat gac tgt gtt gtt gct gat aac ctg tcg aat     -6
28     Ala Val Glu Leu Ile Glu Asn Gly Tyr Asp Cys Val Val Ala Asp Asn Leu Ser Asn    46

-5     tcc ggA CTA ATC TCC CTA CAA ATC TCC TTA ATT ATA ACA TTC ACA GGC ACA GAA CTA     52
47     Ser Gly Leu Ile Ser Leu Gln Ile Ser Leu Ile Ile Thr Phe Thr Ala Thr Glu Leu    65

53     ATC ATA TTT TAT ATC TTC TTC GAA ACC ACA CTT ATC CCC ACC TTG GCT ATC ATC ACC    109
66     Ile Ile Phe Tyr Ile Phe Phe Glu Thr Thr Leu Ile Pro Thr Leu Ala Ile Ile Thr    84

110    CGA TGA GGC AAC CAG CCA GAA CGC CTG AAC GCA GGC ACA TAC TTC CTA TTC TAC ACC    166
85     Arg  X  Gly Asn Gln Pro Glu Arg Leu Asn Ala Gly Thr Tyr Phe Leu Phe Tyr Thr    103

167    CTA GTA GGC TCC CTT CCC CTA CTC ATC GCA CTA ATT TAC ACT CAC AAC ACC CTA GGC    223
104    Leu Val Gly Ser Leu Pro Leu Leu Ile Ala Leu Ile Tyr Thr His Asn Thr Leu Gly    122

224    TCA CTA AAC ATT CTA CTA CTC ACT CTC ACT GCC CAA GAA CTA TCA AAC TGC TGA ...    277
123    Ser Leu Asn Ile Leu Leu Leu Thr Leu Thr Ala Gln Glu Leu Ser Asn Ser  X        139
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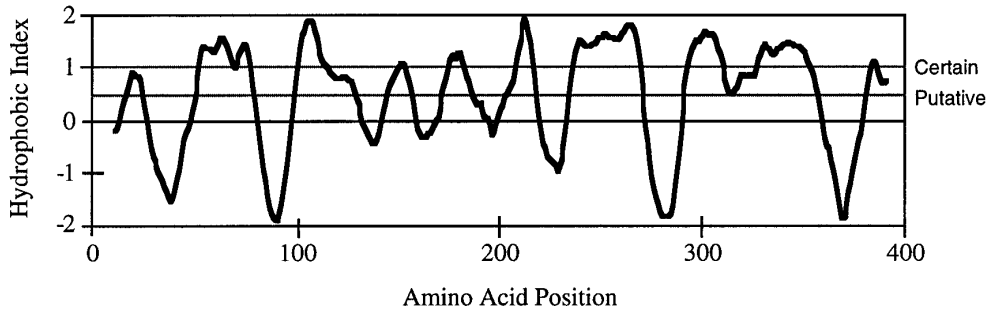
B



C

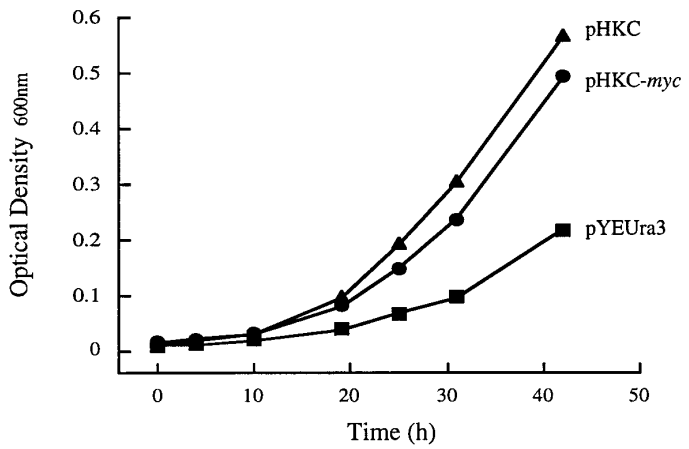


FIG. 2. The *GAL10*-ND4 coding region of pHKC and complemention ability of pHKC-*myc*. (A) Shown are the DNA and predicted amino acid sequences of pHKC indicating the site of transcription initiation of the *GAL10* promoter (underlined), the *GAL10* coding region (lower case), and ND4 coding region to its second TGA codon (upper case). The BstBI site used for *myc* epitope insertion within the ND4 region is indicated in bold. (B) Eisenberg hydrophobic moment analysis of the predicted full length *Gal*10-ND4 fusion using the TopoPred II program (31). (C) TK-expressing yeasts transformed with pYEUra3, pHKC, or pHKC-*myc* were innoculated at equal density in transport-selection medium containing 300 μM thymidine and the OD_{600} of each culture was measured thereafter.

translation of GAL10-ND4 transcripts would be expected to be highly inefficient and to result in polypeptides of variable lengths. To detect *GAL10*-ND4 fusion polypeptides in pHKC-expressing yeast, a sequence encoding an immunoreactive epitope (*c-myc* peptide) was engineered into the *GAL10*-ND4 sequence at a position upstream of the first predicted UGA codon, thereby producing pHKC-*myc*. Complementation was observed with yeast transformed with pHKC-*myc* on plates (not shown) and in liquid cultures containing transport-selection medium (Figure 2B), although with less efficiency than was observed with pHKC.

The presence of *myc*-tagged *GAL10*-ND4 fusion polypeptides in yeast was examined with a *myc* epitope-specific antibody in immunoblots and intact cell immunofluorescent staining. Repeated analysis of membrane fractions of pHKC-*myc* transformed yeast demonstrated the presence of very faint, yet reproducible, immunoreactive bands of 10-11 and 16-17 kDa; both bands were absent in membranes of pHKC-transformed yeast (Figure3A). The electrophoretic mobilities of the immunoreactive bands were those predicted for *myc*-tagged *GAL10*-ND4 polypeptides that would be translationally terminated at either the first (10.5 kDa) or second (16.3 kDa) UGA codon. These results demonstrated translational read-through of the first UGA codon, however the weak immunoblot reactivity limited our ability to detect larger *GAL10*-ND4 fusion polypeptides that might also have been produced.

In contrast to the weak immunoblot reactivity exhibited by *myc*-tagged *GAL10*-ND4 fusion polypeptides, the pHKC-*myc* transformed yeast showed a strong immunofluorescence staining that was absent in the pHKC-transformed yeast (Figure3B). A "general" immunostaining pattern over the entire cell, consistent with the presence of the *GAL10*-ND4 fusion protein in the plasma membrane, and possible sites of intracellular localization were observed. Similar discrepancies between immunoblot and immunofluorescence reactivities of heterologous membrane proteins produced at low levels in yeast have previously been attributed to rapid degradation of heterologous protein during processing of yeast in preparation for electrophoresis and immunoblotting (19).

DISCUSSION

Our results demonstrated that one or more of the *GAL10*-ND4 fusion proteins induced permeability towards thymidine. The fusion protein(s) may have upregulated the activity of a pre-existing low-activity permease in yeast, however previous authors concluded that *S. cerevisiae* lack the capacity for thymidine transport (20), and we have not detected mediated cellular uptake of [^{3}H]-thymidine in *S. cerevisiae* (7). The alternative explanation is that the *GAL10*-ND4 fusion protein(s) functioned as a membrane permease or channel. To do so, the fusion protein(s) must have had structural features that allowed localization to the plasma membrane and formation of a functional permeation site(s).

The two *myc*-tagged *GAL10*-ND4 fusion polypeptides that were produced in sufficient quantities to be immunologically detectable in yeast membranes encompassed the first 48 amino acids of the *S. cerevisiae GAL10* (UDP-galactose-4′-epimerase) protein (21) fused to fragments of the human ND4 protein (amino acid residues 99-135 or 99-190). UDP-galactose-4′-epimerase, a soluble protein whose structure is highly conserved between procaryotes and eucaryotes, converts UDP-galactose to UDP-glucose by an epimerase reaction involving a transient reduction of NAD^+. The *GAL10* region of the fusion protein contained residues (Asp^{42}, Asn^{46}, Ser^{47}) that are responsible, by virtue of hydrogen bonding with the C2′ and C3′ hydroxyl groups of the ribosyl moiety of adenosine, for the anchoring of NAD^+ (22). Although this region may have contributed to the binding of thymidine (via its 3′ ribosyl hydroxyl group), it seemed unlikely (because of its hydrophilicity) to have contributed to the membrane localization and transport characteristics of the fusion protein(s).

ND4 is localized to the inner mitochondrial membrane as a subunit of mammalian mitochondrial Complex I of the respiratory chain (8,9) and, while its precise function remains uncertain, has been postulated to act as a structural "scaffold" for the assembly of Complex I (23, 24). The two immunologically detectable *GAL10*-ND4 fusion polypeptides (10.5 kDa, 16.3 kDa) contained the first two (residues 99-135) or three (residues 99-190) of the predicted hydrophobic transmembrane domains (25, 26) of human ND4. Larger fusion polypeptides, resulting from read-through of multiple UGA codons of the ND4 sequence, may have also been produced. These hydrophobic domains of ND4 likely provided a basis for localization of the fusion protein(s) in the plasma membrane. While the creation of a thymidine permeation site by the ND4 region(s) may have been coincidental, we speculate that it was a consequence of the normal function of the ND4 protein within the mitochondrial inner membrane.

Indirect evidence suggests a role for the ND4 protein in transport of a precursor or metabolite of NAD^+ across the inner mitochondrial membrane, which is itself considered to be impermeable to NAD^+ (27). A single point mutation at nt 11778 in human mitochondrial DNA, resulting in the replacement in ND4 of Arg at residue 340 with His ($ND4^{H340}$), gives rise to Leber's hereditary optic neuroretinopathy (LHON), a mitochondrial genetic disease that results in blindness of affected individuals (10). Mitochondria that contain $ND4^{H340}$ are unaffected in electron transport and NADH binding (an indication that Complex I is structurally intact) yet exhibit severely decreased rates of

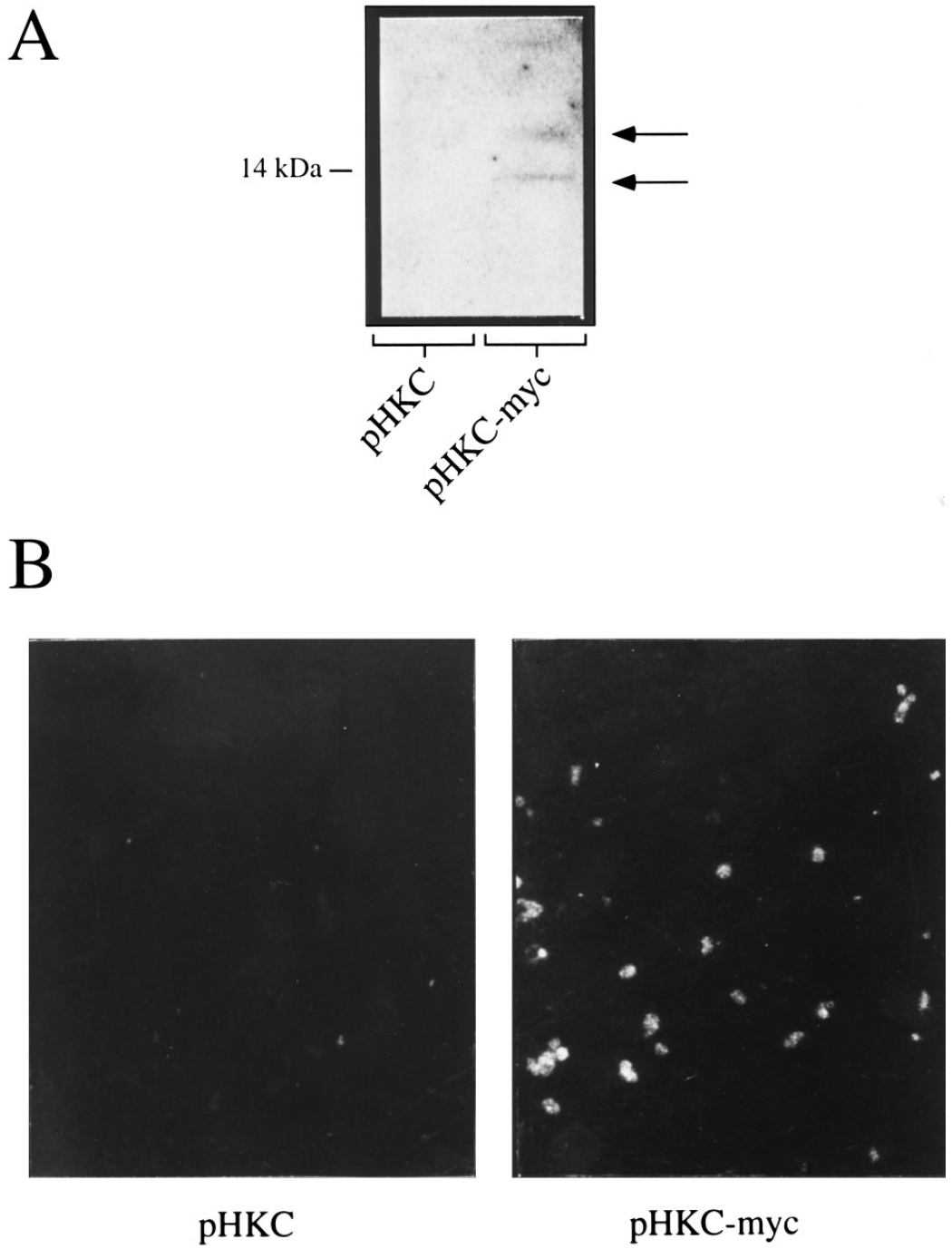


FIG. 3. Immunological studies of *myc*-tagged *GAL10*-ND4 fusion proteins. (A) Membranes prepared from of TK-expressing yeast transformed with pHKC or pHKC-*myc* were subjected to immunoblot analysis using a *myc* epitope-specific antibody. Arrows indicate specific immunoreactive bands. (B) Intact yeasts were subjected to immunostaining with *myc* epitope-specific and secondary rhodamine B-conjugated antibodies. Shown are representative fluorescence fields (50× magnification) that contain approximately equal numbers of cells.

oxidation of NAD^+-linked substrates and oxygen consumption (24, 28, 29). A similar phenotype has been observed in intact mitochondria of normal human cells that were starved of NAD^+ (30), wherein the restoration of severely decreased rates of NAD^+-linked substrate oxidation and oxygen consumption was associated with influx of a NAD^+ metabolite into the mitochondrial matrix. Thus, it is tempting to speculate that mitochondria with $ND4^{H340}$ may contain inadequate NAD^+, because of a defect in mediated influx of a NAD^+ metabolite. Within this context, it is of interest to note that the structure of the nicotinamide-ribose portion of

NAD^+ generally resembles that of thymidine. Furthermore, the hydropathic analysis of known eucaryotic ND4 homologs predicts 12 hydrophobic transmembrane domains (25, 26), a structural feature that is consistent with the prediction that ND4 is a member of the 12-transmembrane family of transporter proteins described by Henderson (31). These observations, combined with the results described in this study, suggest that ND4 may be involved in the transport of molecules across the mitochondrial inner membrane.

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