The Human Mitochondrial ADP/ATP Carriers: Kinetic Properties and Biogenesis of Wild-Type and Mutant Proteins in the Yeast *S. cerevisiae*†

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ABSTRACT: The mitochondrial adenine nucleotide carrier, or Ancp, plays a key role in the maintenance of the energetic fluxes in eukaryotic cells. Human disorders have been found associated to unusual human *ANC* gene (*HANC*) expression but also to direct inactivation of the protein, either by autoantibody binding or by mutation. However, the individual biochemical properties of the three HAncp isoforms have not yet been deciphered. To do so, the three *HANC* ORF were expressed in yeast under the control of the regulatory sequences of *ScANC2*. Each of the three *HANC* was able to restore growth on a nonfermentable carbon source of a yeast mutant strain lacking its three endogenous *ANC*. Their ADP/ATP exchange properties could then be measured for the first time in isolated mitochondria. *HANC3* was the most efficient to restore yeast growth, and HAnc3p presented the highest V_M (80 nmol ADP min^{-1} mg protein^{-1}) and K_M^{ADP}(8.4 μM). HAnc1p and HAnc2p presented similar kinetic constants ($V_M \approx 30-40$ nmol ADP min^{-1} mg protein^{-1} and $K_M^{ADP} \approx 2.5-3.7$ μM), whose values were consistent with *HANC1*'s and *HANC2*'s lower capacity to restore yeast growth. However, the *HANC* genes restored growth at a lower level than *ScANC2*, indicating that HAncp amount may be limiting in vivo. To optimize the HAncp production, we investigated their biogenesis into mitochondria by mutagenesis of two charged amino acids in the N-terminus of HAnc1p. Severe effects were observed with the *D3A* and *D3K* mutations that precluded yeast growth. On the contrary, the *K10A* mutation increased yeast growth complementation and nucleotide exchange rate as compared to the wild type. These results point to the importance of the N-terminal region of HAnc1p for its biogenesis and transport activity in yeast mitochondria.

The adenine nucleotide carrier (Ancp[1]) plays a key role in the energy metabolism of eukaryotic cells. It is an integral protein of the mitochondrial inner membrane (MIM) that exchanges cytoplasmic ADP^{3-} for mitochondrial ATP^{4-} under conditions of oxidative phosphorylation. This transport is electrogenic and driven by the membrane potential. Ancp are nuclear encoded proteins of 30−35 kDa, and the functional unit is a homodimer. These proteins are very similar throughout the eukaryotic kingdom, and various isoforms (one to three) have been identified depending on the organism (for review, see ref *1*). Up to now, three isoforms of the adenine nucleotide carrier have been found in human, beef, *A. thaliana* and *S. cerevisiae* (*2*−*5*).

Regulation of mammalian gene transcription depends on the tissue and the state of cell differentiation. For example, in human, the amount of *HANC1*, the heart and muscle specific isoform, is modulated by the cell energetic requirements (*6*, *7*). *HANC2* is ubiquitously expressed to a level depending on the oxidative metabolism (*8*, *9*), whereas *HANC3* is weakly expressed in normal tissues but strongly induced in highly proliferative cells (*10*).

Unusual *HANC* transcription levels are associated with certain pathologies such as cancers or myopathies (*11*−*13*), and direct alterations of HAncp activity are induced by antibody binding in autoimmune pathologies. Recently, point mutations of *HANC1* have been identified in several families of patients and in one sporadic patient presenting an autosomal dominant progressive external ophthalmoplegia (adPEO) (*14*, *15*). AdPEO is a rare human disease that shows a Mendelian inheritance pattern. They are three different autosomal loci for this disorder; one of them comprises the *HANC1* gene. The authors have shown indirectly that one

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[1] Abbreviations: adPEO, autosomal dominant progressive external ophthalmoplegia; *ANC*, mitochondrial adenine nucleotide carrier coding gene; Ancp, mitochondrial adenine nucleotide carrier protein; ATR, atractyloside; B, beef; EtBr, ethidium bromide; H, human; MCF, mitochondrial carrier family; MIM, mitochondrial inner membrane; Mo, Mouse; PCR, polymerase chain reaction; PK, proteinase K; Sc, *Saccharomyces cerevisiae*; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMS, transmembrane segment; YPD, rich yeast extract, peptone, dextrose medium.

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of the mutations, A114P, could impair HAnc1p activity, but the effects on HAnc1p function of the two other ones, V289M and L98P, were not evaluated (*14*, *15*).

The amount of tissue that can be obtained by human biopsies is usually too low to perform all the experiments necessary to characterize the kinetic properties of mutant HAncp associated with pathologies. Moreover, human cells cannot be used to evaluate functional consequences of *HANC1* mutations, mainly because *ANC1* is not expressed in cultured cells, even in myoblasts (*8*, *14*). Thus, it is of crucial importance to set up a system for nonlimiting HAncp production by heterologous expression. Bacterial cells such as *E. coli* were used with no success in an attempt to produce HAnc1p (*16*). However, yeast has proved to be convenient in various cases of human protein production although this is generally very difficult for membrane proteins. For example, it was necessary to create chimaeric proteins between beef Anc1p (BAnc1p) or HAnc1p and ScAnc2p (*17*, *18*) to obtain significant levels of mammalian carriers in yeast mitochondria.

Expression of the adenine nucleotide carrier genes (*ScANC*) in *S. cerevisiae* (Sc) is regulated by carbon source and by oxygen. *ScANC1* is weakly expressed, whatever the nature of the carbon source is (*19*), whereas *ScANC3* is specifically induced under anaerobic conditions (*5*, *20*), thus allowing entry of glycolytic ATP into mitochondria. *ScANC2* expression is high or repressed in cells growing on a nonfermentable or on a fermentable carbon source, respectively. However, it can sustain yeast growth whatever the growth conditions in the absence of both *ANC1* and *ANC3* (*21*). They are not essential since a yeast mutant, the three *ScANC* of which were inactivated, is viable, but only under aerobiosis and on a fermentable carbon source (*5*, *22*).

In this study, expression of the three *HANC* genes was achieved in a yeast *anc* triple mutant. ADP/ATP exchange kinetics of the three human carriers could be analyzed for the first time, thus pointing to the potential interest of this system to explore mutant forms of human carriers. Because HAncp content was lower in yeast mitochondria than ScAnc2p content (2−3 times less), we tried to improve HAnc1p production by mutating two charged residues in its cytosolic N-terminal region. We can infer from our studies that the presence of a negatively charged amino acid in this region is important for efficient production of HAncp in *S. cerevisiae*, but the nucleotide exchange activity can be substantially increased by removing a positively charged amino acid.

MATERIALS AND METHODS.

Construction of the Yeast anc *Triple Mutant* JL1-3Δ2. The *Saccharomyces cerevisiae* strain used in this study is *JL1-3Δ2* (*Matα leu2-3,112 his3-11,15 ade2-1 trp1-1 ura3-1 can1-100 anc1::LEU2 Δanc2::HIS3 anc3::URA3*). To construct this strain, the *ANC2* locus of *JL1-3ANC2* previously named 2N1-3 (*23*) was replaced with the *HIS3* marker obtained by polymerase chain reaction (PCR) amplification using Yip203 (*24*) as the matrix and the following primers, complementary to *HIS3* and to the noncoding regions of *ANC2* (underlined, from nucleotide −43 to −1 and from nucleotide +955 to +995): 5′-ATACTTCAGAATCATACATTAACATACATATAAGCAAATAGCCTCTTGGCCTCCTCTAG-3′ and 5′-CCAAAGAAGAAAAAAGGAAAATGTGAGAAAGAATTTAGATTTCGTTCAGAATGACACG-3′. The PCR fragment was used to replace, by homologous recombination in yeast (*25*), the *ANC2* ORF of pMD101 (*26*) partially digested first with *Hin*dIII (at position +302 of *ANC2*) and then with mung bean nuclease. It was controlled that the resulting vector did not contain *ANC2* ORF. This plasmid was then digested with *Pst*I and *Sal*I, and the fragment of interest was used to replace *ANC2* locus by nonselective transformation of *JL1-3-ANC2* (*23*). Replacement of *ANC2* by *HIS3* was controlled by Southern blot analysis of the transformants that could grow in the absence of histidine but not in the presence of lactate or glycerol.

Media and Transformations. Escherichia coli strains used for plasmid propagation were TG1 (Δ(lac-pro) supE thi hsd5 F′ [traD36 proAB$^+$ lacI^q lacZΔM15]) and XL1-Blue (recA1 endA1 gyrA96 (Nal^r) thi hsdR17 (r_K^- m_K^+) supE44 relA1 lac$^-$ F′ [Tn10 (tet^r) proAB$^+$ lacI^q lacZΔM15]). Bacterial strains were grown as described in ref *26*. Bacteria were transformed according to standard methods either with calcium chloride (*27*) or by electroporation.

Yeast cells were cultivated as described (*26*, *28*). Where indicated, ethidium bromide (EtBr) was added at 20 μg/mL in YPD (1% yeast extract, 2% peptone, 2% glucose). Yeast transformation was carried out by lithium chloride method (*29*).

Chemicals. [^{3}H]atractyloside (ATR) was synthesized as previously described (*30*). Protein concentration was determined using the bicinchoninic acid reagent kit from Pierce.

Cloning of the Human HANC cDNA. The three HANC cDNA, about 900 bp each, cloned into the bacterial pBluescript KSII (Stratagene) and kindly provided by G. Stepien, were amplified by PCR using the following primers, which allowed creation of unique restriction sites (underlined) upstream the initiation codon and downstream the stop codon for subsequent cloning: 5′-GAGAATTCATGGGTGATCACGC-3′ (*Eco*RI) and 5′-GAGGATCCTTAGACATATTTTTTG-3′ (*Bam*HI) for *HANC1*; 5′-GAGAATTCATGACGGAACAGG-3′ (*Eco*RI) and 5′-GCGGGATCCTTAGATCACCTTC-3′ (*Bam*HI) for *HANC2*; 5′-GCAATTGATGACAGATGCCG-3′ (*Mfe*I) and 5′-GCCCGGGTTATGTGTACTTC-3′ (*Xma*I) for *HANC3*. *ScANC2* promoter (1350 bp), *HANC*, and *ScANC2* terminator (1100 bp) were sequentially subcloned into the bacterial pBluescript KSII. For *HANC3* cloning, a compatible restriction site was introduced by amplifying *ScANC2* terminator using the following primers: 5′-CCCGGGAATCTAAATTCTTTCTC-3′ (*Xma*I site underlined) and 5′-CCACTAGTCTAGGTCGACGTC-3′ (*Spe*I site underlined). The resulting fragment, 3′ *ScANC2*, was 500 bp. The [*Kpn*I-*Not*I] fragment (3.3 kbp for *HANC1* and *HANC2* or 2.8 kbp for *HANC3*) containing *HANC* and the 5′- and 3′-flanking DNA sequences (Figure 1A) was introduced into the low copy number phagemid pRS314 (*TRP1/CEN6/ARSH4*) (*31*) or into the multicopy phagemid pRS424 (*TRP1*/2μ) (*32*). The sequences of the open reading frames were controlled by DNA sequencing (ABI PRISM 310, Applied Biosystems).

Site-Directed Mutagenesis. Site-directed mutagenesis of *HANC1* was performed using the Chameleon Double Stranded kit (Stratagene) with the following mutagenic primers (mutated bases are underlined): 5′-GAATTCATGGGTGCTCACGCTTGGAGCTTCC-3′(*D3A*); 5′-GAATTCATGG-

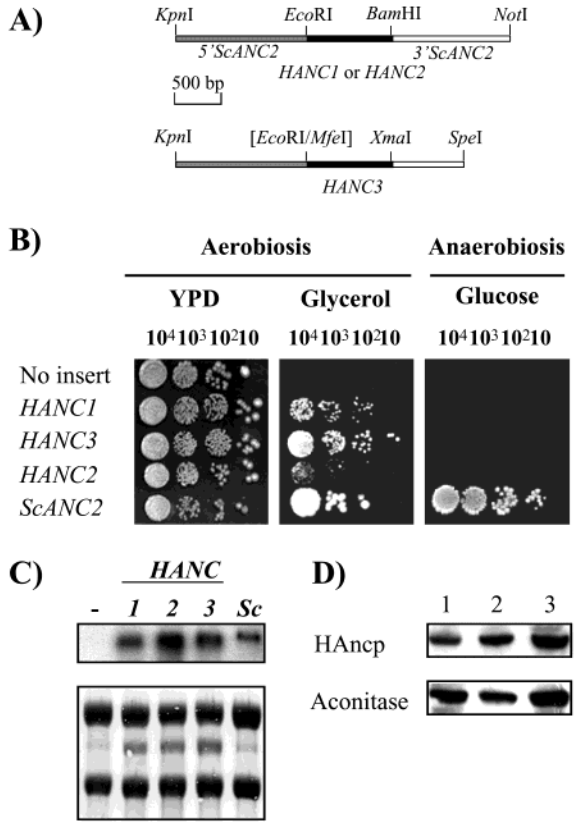

FIGURE 1: (A) Cloning of *HANC* genes under the control of *ScANC2* regulatory sequences. The *HANC* ORFs (black boxes) were PCR amplified to introduce the indicated single restriction sites and cloned between the *ScANC2* promoter (1335 bp; shadowed boxes) and terminator (white boxes; 1158 bp for *HANC1* and *HANC2*; 490 bp for *HANC3*). (B) The *HANC* genes are fully functional in *S. cerevisiae*. *JL1-3Δ2* was transformed with the *HANC* genes cloned into a multicopy plasmid. The transformants (10^4 to 10 cells) were plated on YPD or on rich glycerol medium (glycerol) or on rich glucose medium supplemented with 0.2% Tween 80 and 12 μg/mL ergosterol (glucose). Plates were incubated at 28 °C under aerobiosis or anaerobiosis during 3 days (YPD) or 10 days (glycerol and glucose). (C) The *HANC* genes are transcribed in yeast. Total RNA was isolated from cells expressing *HANC1*, *2* or *3*, or *ScANC2* and cultivated in YPLactate. 30 μg was loaded in each lane of a formaldehyde agarose gel. After tranfert, the membrane was stained with methylene blue (lower panel) and then hybridized with an antisense oligonuleotide probe located in the 3′ noncoding region of the *ANC* (upper panel). (D) Immunodetection of HAncp in isolated yeast mitochondria. Mitochondria were isolated from *JL1-3Δ2* expressing *HANC1* (1), *HANC2* (2), and *HANC3* (3) cloned into a multicopy plasmid. Mitochondrial proteins (10 μg in each lane) were subjected to SDS-PAGE (12.5% acrylamide). After transfer onto a nitrocellulose membrane, proteins were immunostained with a polyclonal antibody directed against a synthetic peptide corresponding to amino acids 40−61 of HAnc3p. As a control, the membrane was immunostained with an antibody raised against aconitase.

GTGAACACGCTTGGAGCTTCC-3′ (*D3E*); 5′-GAATTCATGGGTAAACACGCTTGGAGCTTCC-3′ (*D3K*); 5′-CTTGGATCCTAGCTGACTTCCTGGCCGG-3′ (*K10A*); 5′-CTTGGATCCTAGATGACTTCCTGGCCGG-3′ (*K10D*); 5′-CTTGGATCCTAGAAGACTTCCTGGCCGG-3′ (*K10E*).

The selection primers were 5′-GATTACGGCATTGACATCGTCCAACTG-3′ and 5′-CCACCGCGGTGGATATCCAGCTTTTGTTCC-3′.

Isolation of Mitochondria, Cytochrome Content Determinations, and [^{3}H]ATR Binding Assays. Yeast cells were grown in YPLactate or YPGalactose rich media, and mitochondria were isolated according to ref *33* with minor modifications (*26*). [^{3}H]ATR binding assays on isolated mitochondria were carried out as described in ref *23*. Cytochrome contents in mitochondria were measured as described in ref *26*.

In Vitro Synthesis of Precursor Proteins and Import Into Yeast Mitochondria. The DNA fragments carrying the ORF of *HANC1*, *HANC2*, *ScANC2* [*Eco*RI-*Bam*HI], and *HANC3* [*Mfe*I-*Sal*I] were subcloned into the plasmid pGEM4Z (kindly provided by M. Donzeau) for in vitro transcription by SP6 polymerase (TEBU, Ampliscribe). Precursor proteins were then synthesized in a rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine. Import reactions were performed by incubating radiolabeled precursor for 30 min at 25 °C in 100 μL of import buffer in the presence of isolated yeast mitochondria (*34*). Mitoplasts were prepared by diluting mitochondria 10 times in 20 mM HEPES buffer, pH 7.2. Mitochondria or mitoplasts were further isolated by centrifugation, washed, and resuspended in the sample buffer (*26*). All the samples were separated by a 16% SDS-PAGE and the labeled proteins visualized with a PhosphorImager (Molecular Dynamics). Radioactivity of the bands was quantified using the NIH Image software.

Protein Immunodecoration. Mitochondria were lysed in the sample buffer prior to electrophoresis. Human Ancp were detected using polyclonal antibodies raised against either the entire beef Ancp or a synthetic peptide corresponding to amino acids 40−61 of HAnc3p (HASKQITADKQYKGIIDCVVRI) or of HAnc1p (HASKQISAEKQYKGIIDCVVRI). The peptides were coupled to ovalbumin as described in ref *35* and used to generate specific polyclonal antibodies (Eurogentec). HAncp3 antibodies were affinity purified on a peptide-coupled SulfoLink column (Pierce). BAncp antibodies were purified through a CNBr-activated Sepharose 4 Fast-Flow column (AP Biotech) crossed-linked with proteins from a *JL1-3Δ2* cell extract according to the manufacturer recommendations. The diluted serum (1/3000) was then incubated three times with nitrocellulose membranes coated with 100 μg of blotted *JL1-3Δ2* proteins after SDS-PAGE separation. An antibody recognizing the 14 C-terminal amino acids of ScAnc2p (*26*) was used to detect the yeast carrier (1/4000 final dilution). Yeast porin and aconitase were detected with polyclonal specific antibodies (1/4000 and 1/1500 final, respectively) (*36*, and C. Vélot, personal communication). Secondary antibody (anti-rabbit) coupled to horseradish peroxidase was purchased from Bio-Rad, and ECL reagents, from Amersham.

Northern Blot Analyses. Total yeast cellular RNA was prepared and analyzed as described in ref *37*. RNA was hybridized with an antisense ^{32}P-labeled oligonucleotide probe located in *HANC1* coding sequence (^{291}5′-CTGCTTGTACTTGTCCTTGAAGGCGAAGTT-3′^{262}), or with an antisense probe located in the 3′ noncoding region (5′-CGTGTCACTCATTAAACACC-3′). The yeast actin gene was hybridized either with a [*Xho*I-*Hind*III] fragment corresponding to nucleotides 1762−2774 or with an antisense oligonucleotide probe (^{795}5′-CGACGTGAGTAACACCATCACCGGAATCC-3′^{767}). Hybridized probes were visualized with a PhosphorImager.

Measurement of ADP/ATP Transport in Isolated Mitochondria. The method for measurement of ADP/ATP

Table 1: Comparison of Growth Properties of *JL1-3Δ2* Expressing Human or Yeast *ANC*

	lag phase (hours)	doubling time (hours)		growth yield[a]
	non adapted[b]	non adapted	adapted[b]	adapted and nonadapted
HANC1	55	36	17	4
HANC2	75	40	18	4
HANC3	45	28	15	4
HANC1(K10A)	40	20	20	7
HANC1(D3A/K10D)	nd[c]	nd	20	7
ScANC2	none	3.5	3.5	13

[a] OD_{600nm} was measured during stationary phase of the cultures. [b] Transformants were cultivated in YPLactate at 28 °C after overnight preculture in synthetic complete glucose medium minus tryptophan (non adapted) or after three successive precultures in YPLactate (adapted). The standard error value for the control experiment (ScAnc2p) was no more than 10−15%. [c] nd: not determined.

transport was based on protocol described previously (*38*). Freshly isolated mitochondria (0.5 mg) were suspended and incubated at 22 °C in 1 mL of 0.6 M mannitol, 0.1 mM EGTA, 2 mM $MgCl_2$, 10 mM KP_i, 5 mM α-ketoglutarate, 0.01 mM Ap_5A, 10 mM Tris-HCl, pH 7.4, in the presence of an ATP detecting system (2.5 mM glucose, hexokinase (1.7 E.U.), glucose-6-phosphate dehydrogenase (0.85 E.U.), 0.2 mM $NADP^+$). Externally added ADP started exchange reaction with intramitochondrial ATP mediated by ADP/ATP carrier. NADPH formation ($\epsilon_{340\ nm} = 6200\ M^{-1} \times cm^{-1}$), which is proportional to ATP efflux, was monitored continuously for 3 min by spectrophotometry at 340 nm. The rate of absorbance increase was obtained from linear part of the curve and used to calculate amount of exchanged ADP (nmol min^{-1} mg $protein^{-1}$). Mitochondrial adenylate kinase was inhibited with specific inhibitor Ap_5A. To determine K_M^{ADP} values, concentrations of free ADP were calculated using the program WinMAXC v2.05 created by Chris Patton (www.stanford.edu/%7Ecpatton/maxc.html).

RESULTS

Wild-Type HANC *Genes Can Rescue* JL1-3Δ2 *Growth on a Nonfermentable Carbon Source.* The yeast *Saccharomyces cerevisiae* triple *anc* mutant, *JL1-3Δ2* (described in Material and Methods), is viable only on a fermentable carbon source. Efficient expression of the three *HANC* can directly be assessed in vivo by their ability to restore *JL1-3Δ2* growth on a nonfermentable carbon source. The three *HANC* genes were subcloned into a low (pRS314) or a high (pRS424) copy number plasmid under the control of *ScANC2* regulatory regions (see Material and Methods). As a control, *ScANC2* was also subcloned following the same strategy. *JL1-3Δ2* was transformed with the various constructs and growth of the transformants was examined on a glycerol-containing medium. As expected, *ScANC2* but not the empty vector restored *JL1-3Δ2* growth on YPGlycerol, either from a low (not shown) or a high copy number plasmid (Figure 1B). Interestingly, a better growth was obtained with the centromeric vector compared to its multicopy counterpart (data not shown), indicating that overexpression could be deleterious for yeast.

In the presence of *HANC* genes in the pRS314 plasmid, *JL1-3Δ2* growth was poor on YPGlycerol or on minimal media plates containing lactate (not shown). Growth was improved when *HANC* genes were overexpressed from the pRS424 plasmid (Figure 1B). *HANC3* was the most efficient to restore *JL1-3Δ2* growth, and *HANC2* was the less efficient. Such differences were confirmed in liquid YPLactate (Table 1). The doubling time was 28 h in the presence of *HANC3* and 40 h in the presence of *HANC2*. This was surprising considering that the three HAncp sequences are 88% to 92% identical (for review, see ref *1*). It is noticeable that growth restored by the human genes was not as good as in the presence of *ScANC2* since in this last case the doubling time was 3 h 30 min (Table 1). Furthermore, a long lag phase was observed before *JL1-3Δ2* growth started in the presence of *HANC* but not in the presence of *ScANC2* (Table 1). We controlled that during this lag phase, there was neither loss of the plasmid nor decrease in cell viability (not shown). After successive restreakings on YPGlycerol or YPLactate plates, growth of *HANC* expressing strains was improved since it was fully developed after 4 or 6 days, depending on the isoform, instead of 7 or 11 days. In liquid YPLactate, after three successive re-inoculations, the lag phase disappeared and the doubling times reached stable values of 15−18 h for all *HANC* (Table 1). The cell yields at the end of the successive cultures remained unchanged. This process was reversible since the initial doubling times and lag phase lengths were recovered after the cells were transiently cultivated in glucose medium. The *HANC1* gene was PCR amplified from total yeast DNA and sequenced to control that no mutation was involved in such a phenomenon. This "adaptation" process was not observed in the presence of the yeast *ScANC2*, as the doubling time was constant throughout successive inoculations in YPLactate.

When the transformants were grown under anaerobiosis or on YPD medium supplemented with 20 μg/mL EtBr to induce the ρ°/ρ^- state, the *HANC* genes were unable to restore *JL1-3Δ2* growth, whether they were expressed from pRS314 (not shown) or from pRS424 plasmid. In contrast, *ScANC2* rescued growth under anaerobiosis (Figure 1B) or in the presence of EtBr (not shown).

Kinetic Properties of the Three HAncps for the ADP/ATP Exchange. To correlate the different growth characteristics conferred by the three *HANC* with the catalytic properties of the human carriers, we have investigated kinetics of the ADP/ATP exchange catalyzed by mitochondria isolated from *JL1-3Δ2* strain producing the various *HANC* and cultivated in lactate medium. As a control we carried out experiments with mitochondria isolated from *JL1-3ANC2.* The protocol used was based on (*38*) and was modified to be adapted to yeast mitochondria. It allows to measure the efflux of matrix ATP as a function of time in the presence of a constant external ADP concentration.

The three HAncp exchanged externally added ADP for mitochondrial ATP. Variation of the exchange rate as a

Table 2: ADP/ATP Exchange by HAncp and ScAnc2p in Isolated Yeast Mitochondria

	V_M (nmol ADP min^{-1} mg prot^{-1})[a]	K_M^{ADP} (μM)
HAnc1p	32.6	3.7
HAnc2p	40.4	2.5
HAnc3p	80.5	8.4
K10Ap	95.5	7.2
ScAnc2p	69.6	1.6

[a] The given values are the averages of at least two independent experiments. The standard error value for the control experiment (ScAnc2p) was no more than 10−15%.

function of ADP concentration could be analyzed with the Michaelis−Menten equation. The deduced kinetic parameters are given in Table 2. HAnc3p presented the highest K_M^{ADP} (2−3 times higher), and the K_M^{ADP} values were in the micromolar range for all the carriers. The V_M values correlated with the growth rates of the transformed *JL1-3Δ2* cells (Tables 1 and 2). For example, HAnc3p exhibited the highest V_M and *HANC3* restored the fastest yeast growth in YPLactate.

The Protein Levels More than the Transcript Levels Could Account for a Reduced Yeast Growth Rescued by HANC. Although all *HANC* were active in yeast, they were less efficient than *ScANC2*. One or several steps, including transcription, translation, import, and/or correct folding of HAncp into yeast mitochondria, could be limiting for *JL1-3Δ2* growth rescue.

We first controlled that all *HANC* genes were efficiently transcribed. Total RNA was extracted from yeast transformed with the human or yeast *ANC* genes and subjected to Northern blotting using an antisense oligonucleotide probe located in the *3′ScANC2* terminator. The amounts of the various *HANC* transcripts were not lower than that of *ScANC2* (Figure 1C) and therefore could not account for a reduced yeast growth rescued by *HANC* on a nonfermentable carbon source.

The amount of HAncp present in yeast mitochondria was then estimated first by immunodecoration of mitochondria lysates with an antibody raised against a synthetic peptide corresponding to amino acids 40−61 of HAnc3p. The three HAncp isoforms could be detected (Figure 1D) though their amino acid sequences are not exactly the same within the peptide region. To get a more precise quantitation of HAncp, we then performed ATR binding experiments with isolated mitochondria.

ATR is a very specific inhibitor of the ADP/ATP transport which binds with high affinity to Ancp. Therefore, determination of the maximum number of ATR-binding sites allows to quantitate the amount of functional Ancp present in the membrane. Since ATR blocks Ancp in a conformation proposed to be involved in the adenine nucleotide transport, a high affinity for ATR will reflect a proper folding of Ancp. Results of [^{3}H]ATR binding experiments are given in Table 3. The level of HAncp was reduced 2−3 times compared to the ScAnc2p level. The K_d values of ATR are lower for HAncp (22−49 nM) than for ScAnc2p (100 nM). They are, however, in the same range as ATR K_d values of other mammalian Ancp in isolated mitochondria (*30*, *39*). Taken together, these results demonstrate that HAncp are functional in yeast mitochondria and thus properly folded in the yeast MIM environment. Therefore, these results argue in favor of using *S. cerevisiae* as the living relay to express human *HANC* and to study with reliability properties of the proteins they encode.

Mutations of HANC1 *Identified in adPEO Impede HAnc1p.* AdPEO is a mitochondrial disorder associated with multiple mtDNA deletions. Different mutations have been identified in *HANC1* in several families and in one sporadic patient. The familial mutations produce replacement of alanine 114 or leucine 98 by a proline, and the third mutation changes valine 289 for a methionine (*14*, *15*). Alanine 114, a well-conserved residue, is located in the third transmembrane segment (TMS), and the nonconserved leucine 98 and valine 289 are located in the first cytosolic loop and in the sixth TMS, respectively. A114P has been introduced in ScAnc2p at equivalent position (*14*). It was concluded that it impaired carrier function since growth of the mutated yeast was delayed on a nonfermentable carbon source. The effect of V289M and L98P have not yet been assessed in yeast. The equivalent amino acid are respectively a serine and a methionine in the yeast ScAnc2p. It is thus difficult to predict the effect of such substitutions on ScAnc2p function.

To obtain direct evidence of HAnc1p activity impairment in adPEO, we have introduced A114P and V289M mutations into *HANC1*, and we have expressed the resultant mutants in *JL1-3Δ2*. As shown in Figure 2, none of the mutants was able to restore yeast growth on a nonfermentable carbon source. For both mutants, no HAncp could be detected by ATR binding experiments or by immunodecoration (Figure 2B), indicating total or partial lack of active HAnc1p. Facing these results, we have controlled that the corresponding genes were transcribed in yeast cells cultivated in galactose-containing medium: the levels of mutant *HANC1* transcripts were comparable to that of the wild-type gene (Figure 2C). But since the yeast cells expressing the mutant *HANC1* could grow on glucose, we can infer that there was no acute instability of mtDNA in yeast, in contrast to what was found for human cells (*14*). Indeed, the combination of ρ^- status and absence of active *ANC* is lethal for yeast cells (*40*). However, our results provide direct evidence of the involvement of both mutations in adPEO etiology.

HAncps Are Less Efficiently Imported into Isolated Yeast Mitochondria than ScAnc2p. The relatively low level of yeast growth complementation by human *ANC* could be related to limited import of HAncp into yeast mitochondria. This was suggested for two mitochondrial proteins that do not have cleavable presequence: subunit VIII of the bc1 complex (*41*) and beef Ancp (*17*). We addressed this issue by studying in vitro HAncp import into isolated yeast mitochondria; ScAnc2p import was carried out as a control (Figure 3).

In the cases of HAnc2p and HAnc3p, two bands were radiolabeled after in vitro transcription and translation. The lower molecular mass bands likely result from in vitro translation starts within the coding sequences, leading to truncated HAncps that were unspecifically imported ($\Delta\Psi$-independent). HAncp incubated with energized yeast mitochondria were partially resistant to PK digestion (Figure 3, lane 3), thus partially imported into mitochondria. Furthermore, after PK treatment of energized mitoplasts, a smaller part of the precursors was protected (Figure 3, lane 4). The amounts of radioactivity remaining in lane 4 represented 10% (HAnc1p), 7% (HAnc2p), and 13% (HAnc3p) of the amounts

Table 3: Kinetic Parameters of [^{3}H]ATR Binding with Isolated Yeast Mitochondria

	ATR_{Max} (pmol/mg prot)[a]	K_d (nM)[a]	cytochrome *b* (pmol/mg prot.)	mol ATR/ mol cyt b[a]
HANC1[b]	174	34	684	0.25
HANC2[b]	230	22	597	0.39
HANC3[b]	161	49	510	0.31
K10A[b]	148	31	634	0.24
D3A[c]	0	−	550	0
D3A/K10D[b]	244	43	616	0.4
ScANC2[b]	450	100	667	0.67

[a] [^{3}H]ATR binding was measured with mitochondria isolated from *JL1-3Δ2* expressing *HANC* genes (wild type or mutant) or *ScANC2*. The given values are the averages of at least two independent experiments. The standard error value for the control experiment (ScAnc2p) was no more than 20−25%. [b] Cells were cultivated in YPLactate. [c] Cells were cultivated in synthetic complete galactose-containing medium minus tryptophan.

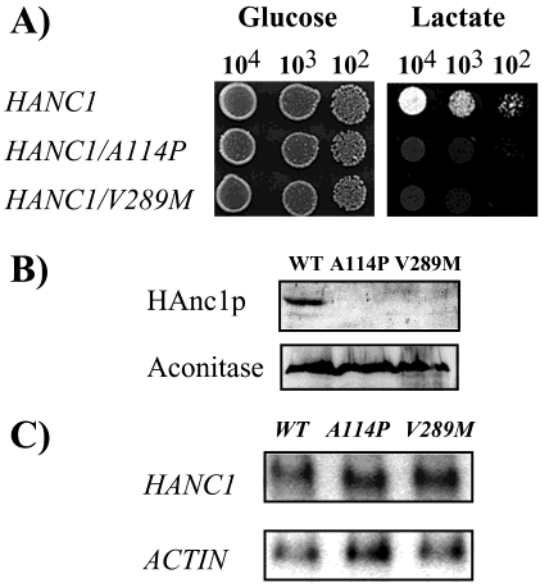

FIGURE 2: (A) The *HANC1* mutations associated with adPEO result in carrier deficiencies in yeast. *JL1-3Δ2* cells expressing *HANC1* wild-type or carrying A114P or V289M mutation were cultivated in synthetic complete glucose medium minus tryptophan. Serial dilutions of the cultures were then plated on rich medium containing glucose or lactate (from 10^4 to 10^2 cells) and grown for 13 days at 28 °C. (B) A114P and V289M mutant proteins are not detected in yeast. Mitochondria were isolated from cells cultivated in synthetic complete galactose medium minus tryptophan and expressing wild-type *HANC1* or the *A114P* and *V289M* mutants. 10 μg of proteins was loaded per lane. After transfert, the membrane was immunodecorated with an antibody raised against a synthetic peptide corresponding to amino acids 40−61 of HAnc1p peptide or against aconitase. (C) The *A114P* and *V289M* genes are transcribed in yeast. Total RNA (30 μg per lane) was isolated from cells cultivated in minimum galactose medium minus tryptophan and then loaded on a formaldehyde-containing agarose gel and transferred onto a nylon membrane. The probes used were an antisense oligonucleotide corresponding to the coding region for *HANC1* transcripts (*WT*, wild type; *A114P* and *V289M*, *Ala114Pro* and *Val289Met HANC1* mutants) and an antisense oligonucleotide probe for the actin gene.

in lane 2, but it was 47% for ScAnc2p, indicating that only 7−13% of the human precursors bound to mitochondria were imported. With de-energized mitochondria HAncp was not protected from PK treatment (Figure 3, compare lanes 7 and 6). The $\Delta\Psi$ requirement for HAncp import followed the one for ScAnc2p (*42*), but the final amount of HAncp inserted into MIM was lower than that of ScAnc2p, as can be deduced from PK treatment of energized mitochondria. These results are in agreement with the amounts of [^{3}H]ATR binding sites determined with isolated yeast mitochondria (Table 3).

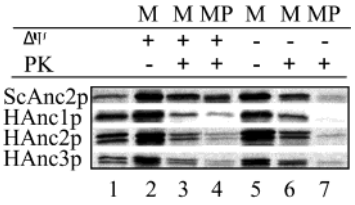

FIGURE 3: In vitro import of HAncp into isolated yeast mitochondria. [^{35}S]methionine-labeled precursors were incubated with isolated yeast mitochondria, either energized (+$\Delta\Psi$) or de-energized (−$\Delta\Psi$), for 30 min at 25 °C. Mitochondria (M, lanes 2, 3, 5, and 6) or mitoplasts (MP, lanes 4 and 7) were then treated with proteinase K (PK, 100 μg/mL; lanes 3, 4, 6, and 7) for 5 min at 4 °C. They were further isolated by centrifugation, washed, and resuspended in sample buffer before SDS-PAGE. Lane 1 corresponds to 10% of the amount of radiolabeled precursor added to each import assay.

A less efficient import of HAncp could be due to the lack of "cytosolic chaperones" in the in vitro system. These proteins increase precursor recognition by the TOM machinery, which is the mitochondria outer membrane translocase (*43*, *44*). We therefore added cytosolic yeast extracts during in vitro import experiments of HAncp precursors synthesized in wheat germ lysates known to lack these chaperones (*45*). However, no increase of precursor import into isolated yeast mitochondria was observed (data not shown).

Single Mutations in the N-Terminal Region of HAncp1 Lead to Striking Differences in JL1-3Δ2 *Growth Complementation.* Several studies have suggested that the N-terminus of Ancp plays a crucial role in the import of carriers into yeast mitochondria, even if it does not correspond to a mitochondrial cleavable presequence (*46*, *47*). The N-terminal regions of HAncp differ from the one of ScAnc2p which is about 15 amino acid longer (Figure 4). By comparing the first residues of the yeast (15−26 aa, depending on the isoform) and human (11 aa) carriers, we noticed that two charged amino acids, aspartate or glutamate 3 and lysine 10, present in the HAncps are not found in ScAncp at equivalent positions. Moreover, these amino acids are also present in all mammalian Ancps, with a prevalence of aspartate at position 3 (*1*). To evaluate their importance for HAncp biogenesis in yeast, we performed site-directed mutagenesis of *HANC1*. The mutant genes were further subcloned into the pRS424 vector under the control of *ScANC2* regulating sequences, as for the wild-type *HANC1*, and used to transform *JL1-3Δ2*.

Mutation of Asp3 into an alanine or a lysine precluded *JL1-3Δ2* growth complementation on YPLactate, unlike mutation *D3E* (Figure 5). Surprisingly, replacing lysine 10

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ScAnc1p          MSHTETQTQQ-SHFGVDFL18
ScAnc2p MSSNAQVKTPLPPAPAPKKESNFLIDFL28
ScAnc3p            MSSDAKQQETNFAINFL17
MoAnc1p               MGDQALS-FLKDFL13
MoAnc3p               MTDAAVS-FAKDFL13
RatAnc1p              MGDQALS-FLKDFL13
RatAnc3p              MTDAAVS-FAKDFL13
BAnc1p                MSDQALS-FLKDFL13
BAnc2p                MTEQAIS-FAKDFL13
BAnc3p                MTDAAVS-FAKDFL13
HAnc1p                MGDHAWS-FLKDFL13
HAnc2p                MTEQAIS-FAKDFL13
HAnc3p                MTDAAVS-FAKDFL13
```

FIGURE 4: Amino acid comparison of the N-terminus of various Ancp. Sources of the sequences (EMBL or NCBI codes) were as follows: ScAnc1p, *S. cerevisiae* (M12514); ScAnc2p, *S. cerevisiae* (X74427); ScAnc3p, *S. cerevisiae* (M34076); MoAnc1p, mouse (U27315); MoAnc3p, mouse (U27316); RatAnc1p, rat (D12770); RatAnc3p, rat (D12771); BAnc1p, beef (M24102); BAnc2p, beef (M24103); BAnc3p, beef (AB065433); HAnc1p, human (J04982); HAnc2p, human (J03592); HAnc3p, human (M57424). The two first amino acids of the first putative transmembrane segment are underlined. The number to the right of each line corresponds to the length of the amino acid sequence taken into account for comparison. For mammalian sequences, amino acids at position 3 and 10 are in bold characters.

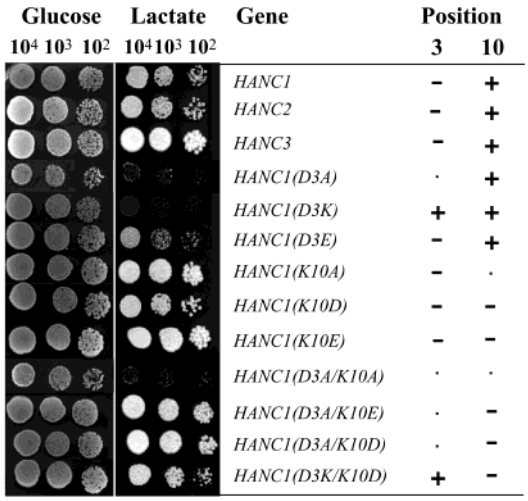

FIGURE 5: Growth phenotypes of *JL1-3Δ2* conferred by various *HANC1* mutants. Cultures of *JL1-3Δ2* cells expressing wild-type or mutant *HANC1* were diluted and plated (from 10^4 to 10^2 cells) on rich medium containing glucose or lactate. Growth was followed at 28 °C for 3 days (glucose) or 17 days (lactate). The amino acid side chain charges at positions 3, 4, or 10 of N-terminal HAncp sequences are indicated as −, +, or · for negative, positive, or no charge, respectively.

with alanine, aspartate, or glutamate increased yeast growth (Figure 5 and Table 1). However, combining mutations *D3A* and *K10A* resulted in growth impairment but the double mutant *D3K*/*K10D* was as efficient as the wild-type *HANC1* gene. Last, requirement of at least one negative charge in the N-terminus of HAnc1p for growth complementation was evidenced by better growth properties of the *D3A*/*K10D* and *D3A*/*K10E* double mutants (Figure 5 and Table 1).

Transport Activity of K10Ap Is Increased whereas D3Ap Cannot Be Detected in Yeast Mitochondria. The absence of growth in the presence of *D3A* or *D3K* could be due to the lack of the corresponding transcripts or proteins. Conversely, a faster growth restored by *K10A* could reflect increased mRNA or protein levels or higher nucleotide transport activity. As can be seen in Figure 6A, mRNA steady state

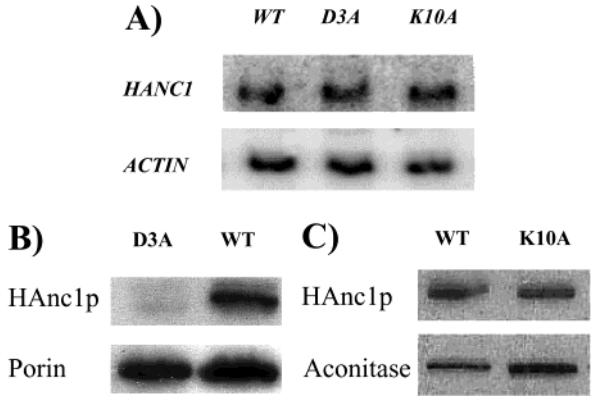

FIGURE 6: (A) Wild-type (WT) and mutant *HANC1* mRNA are equally expressed. *JL1-3Δ2* expressing *HANC1*, wild type or mutants, were cultivated in 100 mL galactose-containing rich medium at 28 °C. Cells were harvested during log phase, and total RNA was extracted. Total RNA (30 μg per lane) was then loaded on a formaldehyde-containing agarose gel and transferred onto a nylon membrane. The probes used were an antisense oligonucleotide, corresponding to the translated region of *HANC1* transcripts, and a [*Xho*I-*Hin*dIII] DNA fragment for the actin gene. (B) D3Ap is not detected in yeast mitochondria. Mitochondria were isolated from *JL1-3Δ2* cells, expressing *D3A* or *WT HANC1*, grown in synthetic complete galactose medium minus tryptophan for selection of the plasmid. Mitochondrial proteins (75 μg) were subjected to SDS-PAGE (12.5% acrylamide). Proteins were transferred onto a nitrocellulose membrane. HAncp were immunostained with an anti-BAncp antibody. As a control, the membrane was immunostained with an anti-yeast porin antibody. (C) K10Ap is detected in yeast mitochondria. 10 μg of mitochondria isolated from cells cultivated in YPLactate was solubilized in sample buffer and subjected to SDS-PAGE (12.5% acrylamide). After transfer onto a nitrocellulose membrane, carrier proteins were immunostained with an anti-BAncp antibody. As a control, aconitase was immunostained.

levels were roughly equivalent for the wild type and for two *HANC1* mutants, *D3A* and *K10A*.

D3Ap was not detected in mitochondrial lysates either by immunodecoration (Figure 6B) or by [^{3}H]ATR binding assays (not shown). In contrast, K10Ap (Figure 6C) and D3A/K10Dp (not shown) were immunodetected in mitochondria, and their amounts, determined by [^{3}H]ATR binding experiments (Table 3), were similar to that of wild-type HAnc1p. In addition, the ATR K_d value was roughly the same (Table 3).

We then investigated whether the kinetic properties of K10Ap were modified. As shown in Table 2, the V_M of ADP/ATP exchange was about 3-fold higher as compared to that of the wild-type HAnc1p, and the K_M^{ADP} for K10Ap was increased from 3.7 to 7.2 μM. These kinetic constants are close to those of HAnc3p, whose gene is the most efficient isoform restoring *JL1-3Δ2* growth. Thus removal of the positive charge in the N-terminal region increases transport activity of HAnc1p and not the amount of this carrier inserted in MIM.

DISCUSSION

The adenine nucleotide carrier belongs to the mitochondrial carrier family (MCF) whose members are involved in the transport of metabolites across the MIM and are essential for communications between mitochondria and cytosol. In humans, dysfunctions of MCF members can lead to pathologies (*48*, *49*), and characterization of the deficient carriers through elucidation of their kinetic properties and their

structure implies that the carriers can be produced in nonlimiting quantities. Mammalian cell cultures appear not to be suitable since apoptosis is induced in human cells overexpressing *MoANC1* (*50*), and furthermore, no associated disease phenotype could be detected with cultured cells of adPEO patients (*14*). Besides, several *HANC* isoforms may be expressed in the same tissue, precluding characterization of single isoform properties from human biopsies. Heterologous *HANC* expression could help overcoming this. Since HAnc1p production in *E. coli* did not succeed (*16*), yeast appeared to be a suitable organism, its mitochondria providing a near-natural environment for mammalian carriers that could facilitate their kinetic studies.

HANC genes were cloned under the regulatory sequences of *ScANC2*, which should lead to a high expression level in yeast cells growing on a nonfermentable carbon source. Under these conditions, the three *HANC* genes were functional as they rescued growth defect of the Δ*anc* yeast strain on a nonfermentable carbon source. Furthermore, it was not necessary to create chimaeric proteins unlike what was described for *BANC1* and *HANC1* expression in yeast (*17*, *18*).

The strains expressing *HANC* grew slower than the one expressing *ScANC2*. Surprisingly, the transformants exhibited an "adaptation" phenomenon which occurred after successive inoculations on a nonfermentable carbon source. As this process is reversible, it may reflect that a threshold amount of HAncp is required in mitochondria to reach a sufficient level of oxidative phosphorylation to allow yeast growth. This hypothesis is in agreement with the observation of a lag period before transformed yeast started growing.

An inactive ScAnc2p is lethal in ρ°/ρ^{-} cells or under anaerobiosis (*40*, *51*), because providing energy from glycolytic ATP to mitochondria is of crucial importance for maintenance of this organelle in the cell (*52*). Since expression of *ScANC2* promoter is repressed on glucose (*21*), the amount of HAncp may not be high enough to rescue *JL1-3Δ2* growth defect on glucose plus ethidium bromide or under anaerobiosis. However, cloning of *HANC* under the control of the *PGK* promoter, which is a strong promoter on glucose medium, could not restore yeast growth under these conditions (data not shown). This suggests that the amount of HAncp may not be the only limiting factor for yeast growth rescue.

Dissociation constants of the three HAncp for ATR binding in yeast mitochondria were close to the values reported for the bovine and rat Ancp (*30*, *39*). These results argue in favor of a proper folding of HAncp in yeast MIM and demonstrate that the yeast heterologous system is a particularly well-adapted tool for studying wild-type and mutated HAncp.

Indeed, this paper is the first report of measurement of HAncp kinetic properties. HAnc3p is distinct from HAnc1p and HAnc2p by its higher ADP/ATP exchange activity and a higher complementation ability of the corresponding gene, *HANC3*. This isoform is also markedly expressed in highly proliferative cells (*10*) and is essential during development in mammals since a knocked-out mouse for this gene is not viable (*53*). Expression of *HANC3* would be enhanced when high amount of cytosolic ATP has to be rapidly imported against matrix ADP. It would thus be interesting to get information about HAnc3p ATP affinity. Our results showed that HAnc3p is more active than HAnc1p and HAnc2p in exchanging cytosolic ADP for matrix ATP. These observations correlate with the fact that the various isoforms could play different roles in mammalian cells. Indeed, overexpression of *MoANC1* but not *MoANC3* induces apoptosis (*50*), and besides, MoAnc1p but not MoAnc3p can bind the ADP ribosylation factor-like 2 (ARL2) through its binding partner BART (*54*). Similarly, in human cells, hypothetical binding partners of HAncp could specifically interact with one of the HAncp isoforms and modulate its activity. Identification of this isoform is difficult since mammalian cells express more than one *HANC* gene and since HAncp sequences are about 90% identical. Such interactions could be directly characterized in our system which allows production of one chosen HAncp.

Ancp lacks a mitochondrial targeting sequence and the precise nature of the import signal is not yet fully understood. Like other members of the MCF, it consists of three sequence related modules (*55*) that cooperate for recruitment of the import machinery (*56*, *57*). The N-terminal region of HAncp contains three charged amino acids at positions 3, 10, and 11 (Figure 4) that could be involved in ionic interactions either for binding to components of the import machinery, Ancp stability, or dimerization within the MIM. Two of them, aspartate/glutamate 3 and lysine 10, are absent at equivalent positions in yeast Ancp. Mutations of the negatively charged aspartate 3 into alanine or lysine precluded yeast growth, and no carrier protein was detected in yeast MIM in both cases. The *D3A/K10A* double mutant was unable to grow on lactate or glycerol whereas complementation was restored when one or two negative charges were introduced back in the N-terminal region. Taken together, these results stress that the presence of a negative charge in HAncp N-terminus is required for complementation by *HANC*. Identification of intragenic second site revertants of the *D3A* mutation is likely to unveil the role of this residue either in HAncp folding or in carrier dimerization which is a requisite to its function (*58*).

Mutation of the positively charged lysine 10 into alanine, aspartate, or glutamate improved functional complementation, and we have shown that surprisingly K10Ap has an increased nucleotide exchange activity as compared to the wild-type HAnc1p, the kinetic constants being close to those of HAnc3p.

Recently, three independent mutations of HAnc1p have been identified in various families or in a sporadic case presenting adPEO (*14*). Two mutations were found in the third and the sixth TMS (A114P and V289M, respectively), and recently a third mutation was identified in the first cytosolic loop (L98P). To examine the consequences of mutations on HAnc1p activity, the authors mutated the yeast cognate gene *ScANC2* at equivalent position of Ala114 and showed that it delayed but not prevented yeast growth (*14*). We have studied the Ala128Pro mutant protein in isolated yeast mitochondria and shown that it could exchange nucleotide and bind ATR similarly to the wild-type ScAnc2p (data not shown). In contrast to these results, we have found that heterologous expression in yeast of *HANC1* in which we introduced independently each of the two mutations A114P and V289M precluded *JL1-3Δ2* growth rescue. No transporter could be detected with either ATR binding assays or immunodecoration, suggesting that HAnc1p mutants are

not present in the yeast MIM. It is reasonable to assume that this is also the case in human mutant cells.

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