An ABC transporter in the mitochondrial inner membrane is required for normal growth of yeast

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In an attempt to identify a mitochondrial ATP binding cassette (ABC) transporter, we have used the polymerase chain reaction to amplify 10 DNA fragments homologous to members of the ABC family from the yeast *Saccharomyces cerevisiae*. We disrupted five of the corresponding genes and found that one of the resulting null mutants barely grew on rich medium and failed to grow on minimal medium. This gene, termed *ATM1*, encodes a putative 'half-transporter' of 694 amino acids. *Atm1p* is synthesized with an N-terminal mitochondrial matrix-targeting signal and is located in the mitochondrial inner membrane, with its C-terminal ATPase domain exposed to the matrix. Cells lacking a functional *ATM1* gene have an unstable mitochondrial genome and have white mitochondria that completely lack cytochromes. *Atm1p* is the first mitochondrial member of the ABC family to be identified and the only eukaryotic ABC transporter that has been shown to be necessary for normal cellular growth.

Key words: cytochrome/epitope tagging/immunofluorescence/petite mutation/*Saccharomyces cerevisiae*

Introduction

Communication between mitochondria and the nucleus is of central importance to mitochondrial biogenesis and cell growth. Some of the nuclear signals affecting mitochondria are transmitted through nuclear-encoded proteins which are imported into mitochondria, where they control the expression of mitochondrial genes (Attardi and Schatz, 1988; Glick and Schatz, 1991; Wienhues and Neupert, 1992). However, almost nothing is known about the pathways by which mitochondria transmit signals back to the nucleus. One such signal might be heme, which is synthesized from protoporphyrin inside the mitochondria and exported to the nucleus, where it participates in the activation of genes encoding mitochondrial proteins (Guarente and Mason, 1983). There is also evidence that the status of the mitochondrial genome affects the transcription of some nuclear genes (Forsburg and Guarente, 1989; Liao and Butow, 1993), but the mechanism and physiological significance of this 'retrograde regulation' remain unknown.

A putative system which could transmit signals such as heme from the mitochondria to the nucleus might include components that belong to the ABC superfamily of mem-

brane transport proteins. ABC proteins contain a conserved stretch of 200 amino acids, the 'ATP binding cassette', which is usually linked to or associated with six to eight membrane-spanning domains. ABC transporters catalyze the ATP-driven transport of a wide range of substrates across membranes (Higgins, 1992). The steadily growing ABC superfamily seems to be ubiquitous; ABC transporters have been found in organisms ranging from bacteria, bearing the largest number of identified ABC transporters, to humans, where over-expression or dysfunction of ABC transporters may cause resistance to cancer chemotherapy or lead to various diseases, including cystic fibrosis, Zellweger syndrome, adrenoleukodystrophy and respiratory bacterial infections (Endicott and Ling, 1989; Riordan *et al.*, 1989; Gärtner *et al.*, 1992; Mosser *et al.*, 1993; de la Salle *et al.*, 1994). The first ABC transporter found in yeast, Ste6p, mediates the export of the mating pheromone α -factor across the plasma membrane (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989). Since then, several other yeast ABC transporters have been identified, several of them involved in drug resistance (Purnelle *et al.*, 1991; Kuchler *et al.*, 1992; Ortiz *et al.*, 1992; Servos *et al.*, 1993; Balzi *et al.*, 1994; Bissinger and Kuchler, 1994; Dean *et al.*, 1994; Dujon *et al.*, 1994; Johnston *et al.*, 1994). A few of the eukaryotic ABC transporters are located in organelles, e.g. the TAP1/TAP2 MHC-encoded peptide transporter of the mammalian endoplasmic reticulum (Monaco, 1992), the 70 kDa mammalian peroxisomal protein (Kamijo *et al.*, 1990) and a yeast vacuolar protein involved in heavy metal resistance (Ortiz *et al.*, 1992).

As mitochondria have apparently evolved from bacteria (Cavalier-Smith, 1987), we thought it likely that they would also contain ABC transporters. Mitochondrial ABC transporters might transmit signals to the nucleus, for example by exporting mitochondrially encoded peptides (Loveland *et al.*, 1990). They might also be involved in the passage across the mitochondrial inner membrane of metabolites for which no transporter has been identified. It is not yet known how heme is exported from the mitochondrial matrix, but two reports suggest that its export across the bacterial plasma membrane is mediated by ABC transporters (Ramseier *et al.*, 1991; Beckman *et al.*, 1992).

We have identified a mitochondrial ABC transporter by exploiting the sequence homology among members of this protein family. We used degenerate oligonucleotide primers to amplify fragments of novel ABC genes from yeast genomic DNA. We then used the amplified fragments to disrupt some of the corresponding chromosomal genes. One of these genes turned out to be required for normal growth. After cloning and sequencing this novel ABC gene, we showed that its protein product is located in the mitochondrial inner membrane.

Primer 1	
Ste6p(N)	SGSGKST LSNLLRLRFYDGYNGSISING.....HNIQTIDQKLLIEN.ITVVEQRCT..LFN.DTLRKNILGSG
Ste6p(C)	SGTGKST LVLLLTLYNCEVKGKIDG.....TDVNDWNLTSRKE.ISVVEQKPL..LFN.GTIRDNLTYGL
Mdr1(N)	SGCGKST TVQLMQRLYDPTGEMVSDG.....QDIRTINVRFLEI.IGVVSQEPV..LFA.TTIAENIRYGR
HlyB	SGSGKST TKLIQRFYIPENGQVLIDG.....HDLALADPNWLRQ.VGVVLQDNV..LLN.RSIIDNISLAN
HisP	SGSGKST FLRCINFLEKPSGAIIVNGQINLVLDKDGQKLVADKNQLRLLRTR.LTMVFQHFN..LWSHMTVLNVMEAP
MalK	SGCGKST LLRMIAGLETITSGDLFI.G.....EKRMDTPPAERG..VGMVFSQYA..LYPHLSVAVNMSFGL
PstB	SGCGKST LLRTFNKMFELYPEQRAE.G.....EILLDGDNLTNSQDIALLRK.VGMVFQKPT..PFP.MSIYDNIAGFV
OppF	SGCGKST FARAIIGLVKATDGKVAWL.G.....KDLLGMKADEWREVRSD.IQMIFQDPLASLNPRMTIGEIIAEPL
OppD	SGSGKST TAFALMGLLATN.GRIGGSA.....TFNGREILNLPERELNLTAEQISMIFQDPMSTSLNPMYRVEQQLMEVL

Primer 2	
Ste6p(N)	TDSVRNADCTNENRHLIKDACQMALLDRLFDLPDGLTETLIGTGGVTLSSGGQQQRVAIARAFIRDTPILFL DEAVSALD
Ste6p(C)	QDEILEI.....BM...YDALKYVGIHDFVISSPQGLDTRI.D.TTLLSGGQAQRLCIARALLRKSILIL DECTSALD
Mdr1(N)	ENVTMD.....EI.....EKAVKEANAYDFIMKLPKHFDTLVGERGAQLSGGQKQRIARALVNNPKILL DEATSALD
HlyB	PGMSVE.....KVIYAAKLAGAHDFISELREGYNTIVGEQAGLSGGQQRVAIARALVNNPKILL DEATSALD
HisP	IQV.....LGLSKHDARERALKYLAKVGIDERAQGYVPHLSGGQQRVSIARALAMEPDVLLF DEPTSALD
MalK	KPAGA.....KK...EVINQVRNQ.V..A.EVLQLAHLDRKPKALSGGQQRVAIARALVNNPKILL DEATSALD
PstB	RLF.....BKL.SRADMDERVQWALTAAALWNETKDLHQSGYSLSGGQQRVSIARALAMEPDVLLF DEPTSALD
OppF	RTYH.....PK...LSRQDVRDRVKAMMLKVLGLPLNIRYPHEFSGGQQRVAIARALVNNPKILL DEATSALD
OppD	MLHKGM.....SK...AEAFESVRML.DAVKMPKARKRMKMPHEFSGGMRQVRMIAMALLCRPKLLIA DEPTTALD

Fig. 1. Design of oligonucleotide primers for PCR. Sequence comparison of the ATP binding cassette of several ABC transporters and regions chosen for primer design (shaded areas). The sequences were from the following sources: Ste6p (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989), Mdr1 (Chen *et al.*, 1986), HlyB (Felmlee *et al.*, 1985), HisP (Higgins *et al.*, 1982), MalK (Gilson *et al.*, 1982), PstB (Surin *et al.*, 1985), OppF and OppD (Hiles *et al.*, 1987). Conserved amino acids are printed in bold. ATP binding boxes A and B (Walker *et al.*, 1982) are underlined. The region just upstream of primer 2 displays high homology between ABC transporters and allowed for easy analysis of PCR subclones by sequencing at this end. The oligonucleotide sequences are given in Materials and methods.

Results

ABC transporters have three particularly conserved regions: ATP binding box A (Walker *et al.*, 1982); a region preceding ATP binding box B which corresponds to loop 3 in a structural model proposed for the ABC domain of ABC proteins (Hyde *et al.*, 1990); eight residues beginning with the aspartic acid of ATP binding box B (Figure 1). Two of these regions were used to design degenerate oligonucleotides, which were then used as primers for a polymerase chain reaction (PCR) with genomic DNA. A similar approach to identify new ABC transporters in yeast and humans has been used by others (Kuchler *et al.*, 1992; Dean *et al.*, 1994; Luciani *et al.*, 1994).

Ten distinct DNA fragments homologous to the ABC family were isolated (Figure 2). Only one of these fragments (9) was part of a gene present in the EMBL database; it corresponded to *ADP1*, a gene of unknown function identified in the course of sequencing yeast chromosome III (Purnelle *et al.*, 1991). Fragments 3 and 4 correspond to genes which were subsequently described by others (Kuchler *et al.*, 1992; Dean *et al.*, 1994), named *MDL1* and *SSH1/MDL2* respectively. Surprisingly, our PCR screen failed to yield a fragment of *STE6*, even though the primers matched the gene perfectly. We therefore did not obtain fragments from all the ABC genes potentially amplifiable with our primers.

As a first step towards identifying the functions and intracellular locations of the proteins corresponding to the amplified gene fragments, we used five of the 10 fragments (4–7 and 10) to disrupt the chromosomal genes, with the expectation that the insertion of a disrupting marker within the ATP binding cassette would abolish transporter function. An *ADP1* disruptant was obtained from A.Goffeau (Louvain). The remaining four fragments were either too small to be used for gene disruptions or

1		50	
#6	.....IMS	ALYRLNELTA	GKILIDNVDI
#8	.....	.....	.....
#1	.....	.....	.....
#3	.....NCV	FVAQYDVNS	GSIEFGDEDI
#5	.....	.....	.....
Mdr1	SGCGKSTTVQ	LMQRLYDPTG	GMVSVDDQDI
#4	.....IAV	LLRLTYNPTT	GTITIDWQDI
Ste6p	SGSGKSTLSN	LLRLFYDQYN	GSISINOHNI
#7	.....	.....	.....
#9	.....	.....	.....
#2	.....	.....	.....
#10	.....	.....	.....
51		100	
#6	LFRGTIRKML	DPFNERTDDE	LWDALVRGGA
#8	.....	.....	.....
#1	.....	.....	.....
#3	LFWGTILDNI	.....LYCIP	PEIRABOD...
#5	LFWDTIWEHV	.....KFORI	DAYDDE...
Mdr1	LFATTIAENI	.....RYORE	WVTWDE...
#4	LMSGTIRDNI	.....TYGLT	YFPTKER...
Ste6p	LFWDTLRKNI	.....LLGST	DSVRNADCTN
#7	HVIMAYLPQQ	DVLSFRLTCT	ETLKFADLKL
#9	.....	.....	.....
#2	.....	.....	.....
#10	.....	.....	.....
101		150	
#6	GKMHKPHLDQ	AVEEESGNFS	LOERQLLALT
#8	.....	.....	.....
#1	.....	.....	.....
#3	ANFPD.GLQT	MVGARGAQLS	GQEQQRALIA
#5	KKLPG.GFDT	IVGERGLMIS	GQEQQRALIA
Mdr1	MKLPH.KFDT	LVGERGAQLS	GQEQQRALIA
#4	TKFPK.TYDT	VIGLTDTLSS	GQEQQRALIA
Ste6p	LDLDP.GLET	LIGTGGVTLSS	GQEQQRALIA
#7	LGLKDCADTL	VGDNSHRGLS	GQEQQRALIA
#9	LRIIDIKDRI	IGNEFDRRIS	GQEQQRALIA
#2	LCMQNTAEAL	VGKT.GRGLN	VQRKLSIG
#10	LKLP..SDIL	VA.....DLN	PTQRKLSIG

Fig. 2. Amino acid sequence comparison of 10 PCR fragments with homology to the ABC superfamily, along with the corresponding N-terminal regions of human Mdr1 and yeast Ste6p. Cloned PCR fragments were sequenced from both ends using forward and reverse sequencing primers corresponding to appropriate regions of the vector pUC18 (Yanisch-Perron *et al.*, 1985). The deduced protein sequences were aligned by the GCG Pileup algorithm. Subclone 8 is truncated due to an internal *Bam*HI site in the original PCR fragment. Subclone 9 corresponds to yeast gene *ADP1* and was not further sequenced. An incomplete sequence was obtained for subclone 1 and only the C-terminal part is given.

Spores

Fig. 3. Haploid spores from a diploid heterozygous *ATM1* disruptant. The diploid yeast strain MH339 with one copy of *ATM1* disrupted by an insertion of the *URA3* gene (*atm1::URA3/ATM1*) was sporulated and eight tetrads were dissected on a YPD plate which was then incubated for 4 days at 30°C. All tetrads except one contained two wild-type and two slow-growing spores. The slow-growing spores were *URA*⁺ and thus carried the disrupted *ATM1* gene.

failed to yield unambiguous disruptions, as determined by Southern blotting. Four of the five successful disruptions were performed in a haploid yeast strain. The frequency of transformants strongly suggested that the ability to obtain haploid disruptants was not due to extragenic suppressors, however, we cannot formally rule out such a phenomenon. None of the haploid disruptants showed a noticeable growth defect on synthetic media containing either glucose or a non-fermentable carbon source such as ethanol/glycerol or L-lactate. However, the gene corresponding to subclone 5 could not be disrupted in a haploid strain. When a diploid strain bearing one disrupted copy of the gene was dissected on YPD medium (Figure 3), spores carrying the disruption displayed a dramatic growth defect and failed to grow at all on minimal medium. The cells did grow (albeit very slowly) on rich medium containing ethanol/glycerol as the carbon source, suggesting that the growth defect was not caused by a lack of oxidative phosphorylation.

To clone the gene, PCR fragment 5 was used to screen a yeast genomic library by hybridization and a clone was isolated which restored normal growth to the corresponding haploid disruptant. The sequence of the gene encompassed that of the PCR fragment and encoded a 694 amino acid protein with high homology to members of the ABC family (Figure 4). For reasons described below, the gene was named *ATM1*. A comparison of the translated amino acid sequence of *ATM1* with the database revealed highest homologies with eukaryotic ABC transporters such as Mdr1 (Chen *et al.*, 1986) and yeast Hmt1p (Ortiz *et al.*, 1992). Atm1p and the N-terminal half of human Mdr1 are 50% identical within the ATP binding cassette and about 27% identical overall. Atm1p contains an N-proximal region with several putative membrane-spanning stretches and a C-proximal hydrophilic region containing the ATP binding cassette. The protein is thus a 'half-transporter', which probably functions as a homo- or heterodimer, in line with the usual four-domain structure of ABC transporters (Hyde *et al.*, 1990).

[illegible]

Fig. 4. Nucleotide sequence of *ATM1* and deduced amino acid sequence of Atm1p. The first nucleotide of the open reading frame is numbered +1.

In order to localize Atm1p within the cell, we tagged its C-terminus with a c-myc epitope and expressed the tagged Atm1p in yeast cells under the control of its own promoter from both a single-copy and a multi-copy vector. The tagged gene in either vector complemented the slow-growth phenotype of the disruptant, indicating that the tagged protein was functional *in vivo*. Transformants with the construct in the single-copy centromeric vector yielded no detectable signal by immunofluorescence, presumably because *ATM1* is expressed only at a very low level from its native promoter, but cells expressing tagged Atm1p from the multi-copy vector revealed an immunofluorescence pattern which co-localized with mitochondrial DNA, as visualized by the DNA stain DAPI (Figure 5A and B). Double immunofluorescence with antiserum against the mitochondrial outer membrane protein porin revealed a very similar staining pattern in those cells expressing detectable levels of tagged Atm1p (Figure 5C). Although over-expression of a protein can lead to mislocalization, we consider it extremely unlikely that an over-expressed protein would be mislocalized specifically to mitochondria.

To confirm the mitochondrial localization of Atm1p, we purified mitochondria from cells expressing tagged Atm1p. The purified mitochondria were enriched for Atm1p, as they were for Isp45, a component of the mitochondrial inner membrane protein import machinery (Maarse *et al.*, 1992; Scherer *et al.*, 1992; Horst *et al.*, 1993). In contrast, they lacked Sec61p, a protein of the endoplasmic reticulum (Stirling *et al.*, 1992), and Gas1p, a plasma membrane protein (Nuoffer *et al.*, 1991) (Figure 6A). *ATM1*, which stands for ABC transporter of mito-

Fig. 5. Immunofluorescence of yeast cells expressing Atm1p-c-myc from a multi-copy vector reveals co-localization of the c-myc epitope with mitochondrial DNA and mitochondrial porin. (A) Staining of nuclear and mitochondrial DNA with 4,6-diamidino-2-phenylindole (DAPI). Mitochondrial DNA appears as dots or bands at the cell periphery. (B) Monoclonal antibody Myc1-9E10 (Evan *et al.*, 1985), recognizing a c-myc epitope, followed by a rhodamine-conjugated secondary antibody. Note that not all cells expressed high levels of Atm1p-c-myc. (C) Antiserum recognizing the mitochondrial outer membrane protein porin, followed by a fluorescein-conjugated secondary antibody. Magnification $\sim 2000\times$.

chondria, thus encodes the first known mitochondrial member of the ABC transporter family.

As the mitochondrial outer membrane pores allow free diffusion of molecules of up to about 6 kDa (Benz, 1985), we expected Atm1p to be situated in the inner membrane. This expectation was confirmed by the demonstration that the C-terminal c-myc tag of Atm1p is inaccessible to protease in mitochondria as well as in mitoplasts (mitochondria whose outer membrane has been disrupted) (Figure 6B). This result also indicates that the C-terminal half of the protein, containing the ATP binding cassette, is located in the mitochondrial matrix. The band recognized by the c-myc antibody was not reduced in size in mitoplasts treated with protease, suggesting that the presumed inter-

Fig. 6. Subcellular localization of Atm1p. (A) Atm1p is enriched in mitochondria purified on a Nycodenz density gradient. Mitochondria were prepared from yeast expressing Atm1p-c-myc from a multi-copy vector. SDS-PAGE was performed on equal amounts of protein from four stages in the mitochondrial preparation: whole yeast, the low-speed supernatant, crude mitochondria and gradient-purified mitochondria. Immunoblotting was performed with the following antibodies: monoclonal antibody Myc1-9E10, recognizing c-myc, followed by a rabbit anti-mouse secondary antibody; antiserum against Isp45/Mpi1p, a mitochondrial inner membrane protein; antiserum against Sec61p, a protein of the endoplasmic reticulum; antiserum against Gas1p, a plasma membrane protein. All blots were developed with ^{125}I -coupled protein A and radioautography. Atm1p-c-myc and Isp45 were both enriched in the purified mitochondria, while Sec61p and Gas1p were virtually undetectable or completely absent. (B) The c-myc epitope of Atm1p-c-myc is protease-protected in mitoplasts. Crude mitochondria from the yeast transformant mentioned above were subjected to one of four conditions: no treatment; addition of proteinase K; proteinase K in the presence of 1% of the detergent octyl glucoside to disrupt both mitochondrial membranes; proteinase K under conditions of osmotic shock to selectively disrupt the outer membrane. Samples were precipitated with 5% trichloroacetic acid and subjected to SDS-PAGE, followed by immunoblotting with the following antibodies: c-myc monoclonal antibody Myc1-9E10, followed by a rabbit anti-mouse secondary antibody; antiserum against the mitochondrial outer membrane protein porin; antiserum against the intermembrane space protein cytochrome b₂; antiserum against the matrix protein α -ketoglutarate dehydrogenase (KDH). The c-myc epitope remained intact in mitoplasts in the presence of protease, strongly suggesting that it is situated in the matrix. Atm1p-c-myc was digested by protease in the presence of detergent. The efficiency of mitoplasting was $>90\%$, as seen by the disappearance of cytochrome b₂, and the intactness of the inner membrane in mitoplasts was demonstrated by the inaccessibility to protease of KDH, which is inherently highly protease-sensitive. Porin, which is protease-resistant even in the presence of detergent, was present in equal amounts under all conditions.

Fig. 7. The first 53 amino acids of Atm1p fused to the mature region of yeast cytochrome oxidase subunit IV (COXIV) restore respiratory growth to the COXIV-deficient strain BYL2. BYL2 was transformed with plasmid pYCX11 (Allison and Schatz, 1986), encoding presequence-free ('mature') COXIV, or with plasmid pMC4 (E. Hurt, Heidelberg, Germany), equivalent to pYCX11 but encoding the authentic COXIV precursor, or with pYCX11, with the N-terminal 53 residues of Atm1p fused upstream to mature COXIV. Transformants were grown at 30°C in liquid cultures in ethanol/glycerol and growth was monitored by optical density at 600 nm. Cells expressing the presequence-free COXIV failed to grow and were not included in the results, while *ATM1-COXIV* transformants (open circles, average of four cultures) grew as fast as transformants containing the authentic COXIV gene (closed squares, average of two cultures).

membrane space loops of Atm1p are not accessible to proteolysis.

Atm1p contains a potential mitochondrial targeting signal (Schatz, 1987) at its N-terminus. However, it was uncertain whether Atm1p is targeted to mitochondria via this N-terminal signal or via internal sequences, as has been observed with other mitochondrial inner membrane proteins, such as the ADP/ATP carrier (Pfanner *et al.*, 1987; Smagula and Douglas, 1988) and the phosphate carrier (Zara *et al.*, 1992). To assess the mitochondrial targeting function of the N-terminal region, we fused the first 53 amino acids of Atm1p, which precede the first putative transmembrane domain, to the mature portion of cytochrome oxidase subunit IV (COXIV), a mitochondrial protein which requires an N-terminal targeting sequence for import into the matrix and assembly into the inner membrane. When a respiratory-deficient *COXIV*-deletion strain was transformed with the *ATM1-COXIV* fusion gene, the cells became respiratory-competent and grew as fast on ethanol/glycerol as cells expressing a COXIV protein with its authentic presequence (Figure 7), while cells transformed with a gene encoding mature COXIV failed to grow. Atm1p thus contains a functional N-terminal mitochondrial targeting signal. Restoration of the wild-type growth rate on ethanol/glycerol can be achieved even with an inefficient N-terminal presequence (Lemire *et al.*, 1989) and we do not exclude the possibility that internal sequences within the protein also contribute to the targeting of Atm1p to mitochondria. However, we have found that the first 53 amino acids of Atm1p also transport a cytoplasmic protein (Ura3p with a c-myc epitope at its C-terminus) to mitochondria when fused to its N-terminus (data not shown), further confirming that

Fig. 8. Mitochondria from the *atm1::URA3* strain are white. Mitochondria were prepared from the *atm1::URA3* strain, the parental wild-type strain and the parental wild-type strain made ρ^- by acriflavine treatment. All strains were grown in rich galactose medium to late logarithmic phase. Pellets containing crude mitochondria from the three strains are shown: on the left, wild-type mitochondria; in the middle, *atm1::URA3* mitochondria; on the right, wild-type ρ^- mitochondria. Wild-type mitochondria are pale brown; ρ^- mitochondria, lacking cytochromes b and aa₃, are pink; *atm1::URA3* mitochondria are white, indicating a lack of all major cytochromes.

the Atm1p presequence is sufficient for mitochondrial targeting.

The *atm1::URA3* cells quickly lost the ability to grow on non-fermentable carbon sources because they spontaneously became ρ^- , meaning that their mitochondria had lost some of their DNA: the cells were unable to complement the respiratory deficiency of a ρ^0 strain (lacking mitochondrial DNA) upon mating, while DAPI staining revealed the presence of mitochondrial DNA. Subsequent analysis of newly dissected *atm1::URA3/ATM1* diploids showed that this instability of the mitochondrial genome is a reproducible phenomenon and that very few *atm1* cells are still able to grow on ethanol/glycerol plates several days after the dissection.

As a step towards determining the function of Atm1p, we prepared mitochondria from the haploid ρ^- *atm1::URA3* cells, from parental wild-type cells and from wild-type cells which had been rendered ρ^- by treatment with acriflavine. The three different strains showed a marked difference in color and this color difference was accentuated in the mitochondrial pellets (Figure 8). Wild-type mitochondria were pale brown, as they contain green cytochrome aa₃, red cytochrome b and pink cytochromes c and c₁. ρ^- mitochondria were pink, as they lack cytochromes aa₃ and b, but have normal or even elevated levels of cytochromes c and c₁ (Pon and Schatz, 1991). Mitochondria from the ρ^- *ATM1* disruptant were white, suggesting that they lacked all major cytochromes. Cytochrome spectra of the mitochondrial preparations confirmed the absence of cytochromes, including cytochromes c and c₁, in the disruptant and immunoblotting showed a greatly reduced level of the heme-free apo-cytochrome c (data not shown).

The absence of cytochromes in the disruptant suggested that Atm1p might be a heme transporter. However, the addition of neither hemin nor Tween 80 and ergosterol, which satisfy the fatty acid and sterol requirements of heme-deficient mutants (Bard *et al.*, 1974; Gollub *et al.*, 1977), reversed the slow growth phenotype. This result could still be explained if hemin failed to penetrate these cells or if the phenotype were caused by a toxic accumulation of heme within the mitochondrial matrix.

However, when we combined the *ATM1* disruption with a *HEM1* disruption (which removes the first enzyme of the heme biosynthetic pathway), the resulting doubly disrupted haploid cells still grew very slowly in the presence of exogenous hemin, which relieved the heme deficiency of singly disrupted *hem1* cells (data not shown). This result suggests that the primary defect in the *ATM1* disruptant is not a failure to export heme across the mitochondrial inner membrane.

Discussion

The conservation of the ABC motif throughout evolution and its appearance in such a large number of proteins probably reflects a particularly successful means of coupling biological processes to ATP hydrolysis. The vast majority of ABC proteins are involved in the transport of substrates across membranes, although a few appear to have other functions (Husain *et al.*, 1986; Qin *et al.*, 1990; Sandbaken *et al.*, 1990). The chemical species transported by various members of the ABC family are extremely diverse, including ions, sugars, amino acids, small hydrophobic molecules, peptides and even large proteins. Nonetheless, individual transporters usually catalyze unidirectional transport of a narrowly defined substrate.

The majority of bacterial ABC transporters mediate either the uptake of nutrients from the surroundings, often via an additional periplasmic binding protein (Ames *et al.*, 1990), or the secretion of pathogenic toxins. In contrast, most eukaryotic ABC transporters identified until now appear to be involved in export with respect to the ATP binding cassette, often as a means of reducing the intracellular concentration of potentially harmful substances such as heavy metals (Ortiz *et al.*, 1992), chemical mutagens (Servos *et al.*, 1993) and toxic hydrophobic compounds.

A few eukaryotic ABC transporters catalyze tissue-specific reactions, such as the transport by TAP1/TAP2 of peptides into the endoplasmic reticulum in antigen-presenting cells, chloride ion secretion by the CFTR protein in lung tissue and the development of eye pigment in *Drosophila* (O'Hare *et al.*, 1984). A more universal function may be performed by the mammalian peroxisomal protein PMP70, which has been proposed to be involved in protein import into peroxisomes (Kamijo *et al.*, 1990).

The identification of *ATM1* extends the range of the ABC transporter family to include the mitochondrion and, to our knowledge, represents the first known ABC transporter which is required for the normal growth of a eukaryotic cell, although the yeast elongation factor EF-3, which contains the ABC motif, has been shown to be essential (Qin *et al.*, 1990; Sandbaken *et al.*, 1990). The function of Atm1p remains unclear, despite our observation of a specific absence of cytochromes *c* and *c*₁ in an *ATM1* disruptant. The protein does not appear to mediate the export of heme from mitochondria. Its function also cannot be restricted to the biogenesis of mitochondrial cytochromes, as a loss of these cytochromes would not by itself yield the dramatic growth inhibition observed on rich glucose medium.

There are, of course, many potential substrates for the Atm1p transporter. The high homology with the mammalian multidrug resistance proteins suggests such

possibilities as an export pump for toxic substances which accumulate in the mitochondrial matrix, a phospholipid exporter or flippase (Higgins and Gottesman, 1992), as has been proposed for murine *mdr2* (Smit *et al.*, 1993), or an ion channel, by analogy to the human Mdr1 protein, which has been shown to have separate chloride channel and drug transport activities (Gill *et al.*, 1992). The tendency of *atm1::URA3* disruptants to become ρ^- suggests that the loss of Atm1p adversely affects the mitochondria themselves and that the growth defect might be due to the accumulation of a substance within the mitochondria, rather than its depletion from the rest of the cell. Although the inability of *atm1* cells to grow on minimal SD medium might suggest that the defect is due to the absence of a necessary factor which is present in rich YPD medium, the growth phenotype is already severe on YPD, suggesting that the primary defect cannot be compensated for by the addition of exogenous factors and that the inability to grow on minimal SD medium reflects the additional stress placed on already compromised cells.

The possibility remains that Atm1p is, in fact, necessary for signaling from the mitochondria to the nucleus, perhaps by exporting mitochondrially derived peptides (Loveland *et al.*, 1990). If the expression of nuclear genes encoding mitochondrial proteins were severely perturbed, due to an absence of positive signals emanating from the mitochondria, a growth defect of the degree observed here might result. Genes which are essential for mitochondrial biogenesis are essential for life (Baker and Schatz, 1991) and thus even a partial reduction in the expression of nuclear genes for essential mitochondrial proteins could have a severe effect on growth.

We have observed that the *atm1::URA3* disruptant spontaneously generates a suppressor activity within days after dissection of the heterozygous diploid, reducing somewhat the severity of the growth phenotype. The cell can thus partly adapt to the absence of Atm1p. In fact, we have observed a variation in the strength of the null mutant phenotype in different disruptions, as mentioned in the Materials and methods section, consistent with the existence of one or more suppressor genes. One such suppressor of the *atm1::URA3* disruptant appears to segregate as a separate gene in tetrad dissections. We are trying to identify this suppressor in the hope of learning more about the function of Atm1p.

The nucleotide sequence of *ATM1* has been entered in the EMBL Nucleotide Sequence Database under accession number X82612.

Materials and methods

Yeast strains

The following *Saccharomyces cerevisiae* strains were used in this study: BSL1-2C (*MAT α* , *lys2*, *his4-519*, Δ *ura3*, *leu2-3,112*) and BYL2 (*MAT α* , *his4519*, Δ *ura3*, *ade2*, *coxIV::LEU2*), from B.Lemire; YKB5 (*MAT α* , Δ *ura3*, *his4-519/his4-519*, *leu2-3,112/leu2-3,112*, *lys2/LYS2*, *ade2/ADE2*), from K.Baker; and MH339 (*MAT α* , *ura3*), from M.Hall.

Oligonucleotide primer design

The following oligonucleotide primers corresponding to the shaded regions of Figure 1 were synthesized. Forward primers: 5'-GGC GG-ATCC TCI GGI T/GC/GI GGI AAA/G AGC/T AC-3' and 5'-GGC GGATCC TCI GGI T/GC/GI GGI AAA/G TCI AC. Downstream primers: 5'-CGG GAATTC TC IAG/A IGC A/GCT IAC IGC/G T/CTC A/GTC, 5'-CGG GAATTC TC IAG/A IGC A/GCT IGT IGC/G T/CTC

A/GTC, 5'-CGG GAATTC TC IAG/A IGC IGA IAC IGC/G T/CTC A/GTC and 5'-CGG GAATTC TC IAG/A IGC IGA IGT IGC/G T/CTC A/GTC. *Bam*HI and *Eco*RI restriction sites were included in the upstream and downstream primers respectively for ease of cloning, along with a few extra nucleotides to improve cutting efficiency.

PCR

PCR was performed on genomic DNA from the yeast strain BSL1-2C with all the degenerate oligonucleotide primers pooled and used at concentrations such that each unique primer sequence was present at 5 pmol/reaction. A program was used of 30 cycles of 1 min at 94°C, 2 min at 45°C and 3 min at 72°C, followed by 7 min at 72°C. The PCR reaction products were run on agarose gels and individual bands or regions were cut out and the DNA purified and cloned into the pUC18 vector using the *Bam*HI and *Eco*RI sites. Individual clones were analyzed by restriction digestion and limited sequencing for uniqueness and homology to the ABC family. Clones of interest were sequenced further.

Gene disruptions

Gene disruptions using the cloned PCR fragments were performed via two different schemes. In one scheme, the cloned PCR fragment was subcloned via the *Bam*HI and *Eco*RI restriction sites into the yeast integrating vector YIp5 (Struhl *et al.*, 1979), which carries the *URA3* gene. This construct was then cut within the PCR fragment at a unique site and used to transform the *ura3* yeast strains YKB5 or BSL1-2C. In the other scheme, the PCR fragment was cut at a unique internal restriction site while in the pUC18 vector. A yeast marker gene (*URA3* or *LEU2*) was cloned into this site and the PCR fragment disrupted by the marker was cut out of pUC18 with *Bam*HI and *Eco*RI and used to transform yeast strain YKB5, BSL1-2C or MH339. All disruptions were confirmed by Southern blotting, using the cloned PCR fragments as hybridizing probes.

The *atm1::URA3* disruption in strain MH339 described in this study involved the insertion of the *URA3* gene at a unique *Nhe*I site within the region encoding the ATP binding cassette (after the codon for amino acid 554), a disruption likely to abolish protein activity.

In addition to the *atm1::URA3* disruption in strain MH339 described in the text and in Figure 3, two other disruptions of *ATM1* at the same *Nhe*I site were performed in strain YKB5, using both the YIp5 scheme and a linear fragment interrupted by the *LEU2* marker. The YIp5 disruption yielded a similarly dramatic phenotype, while the *LEU2* disruption showed only a weak-to-moderate phenotype. The *atm1::LEU2* disruptant was used for complementation by *ATM1* and expression of Atm1p-c-myc, as it had additional markers present and was more amenable to transformation with DNA. Furthermore, the *atm1::LEU2* disruptant did not spontaneously become ρ^- , as did *atm1::URA3*.

A disruption in *ATM1* in which 960 base pairs of the open reading frame were deleted (after the codon for amino acid 232) was performed after the work reported here was completed. These disruptants showed the same phenotype found for the *atm1::URA3* disruptant.

Cloning of ATM1

PCR fragment 5 was excised from the pUC18 vector with *Bam*HI and *Eco*RI and used to screen yeast genomic library R1HC33A (R. Henriquez and M. Hall) by colony hybridization using the ECL system (Amersham). Three positive clones which passed two further screens were used to transform an *atm1::LEU2* disruptant. One of the clones yielded transformants which grew normally, indicating complementation of the *ATM1* disruption. This clone contained an insert of about 11 kb which was used for initial restriction analysis. A 6.5 kb *Eco*RI fragment was subcloned and found also to complement the *atm1::LEU2* disruption. The *ATM1* gene contained within the *Eco*RI fragment was sequenced by a combination of subcloning and successive primer construction.

Tagging of Atm1p with a c-myc epitope

The 6.5 kb *Eco*RI fragment containing the *ATM1* gene was subcloned into the yeast centromeric and 2 μ vectors YCplac33 and YEplac195 (Gietz, R.D. and Sugino, 1988). The two plasmids containing *ATM1* were both cleaved with *Kpn*I and *Sma*I, removing the C-terminal 500 bp of *ATM1* beginning at the unique *Kpn*I site and the downstream sequence extending to the *Sma*I site of the polylinker. Approximately 1.5 kb of *ATM1* was amplified by PCR, using a reverse primer (5'-CGCCG GAGCTC TTA CAA GTC TTC TTC AGA AAT AAG CTT TTG TTC TAG TTC TTG CTG GTC TTT-3') that contained the C-terminus and 10 adjacent codons encoding the c-myc epitope, EQKLISEEDL, and a forward primer corresponding to a region 1.5 kb upstream. The PCR product was filled-in with Klenow DNA polymerase and cleaved with

*Kpn*I, releasing a 500 bp C-terminal fragment containing the c-myc epitope. The *Kpn*I/blunt end fragment was cloned into the *Kpn*I/*Sma*I-cut vectors, regenerating the C-terminus of the gene but with the c-myc epitope now following the final sense codon.

Cloning of an ATM1 presequence upstream of mature COXIV

The first 159 nucleotides of *ATM1* were amplified by PCR, using primers containing *Eco*RI and *Xba*I sites respectively at their 5' ends. The PCR product was cloned into vector pYCX11 (Lemire *et al.*, 1989), creating an in-frame fusion of the first 53 amino acids of Atm1p upstream of mature COXIV.

Miscellaneous

Published methods were used for growth of yeast strains and dissection of tetrads (Guthrie and Fink, 1991), growth of heme-deficient yeast strains in the presence of hemin or Tween 80 and ergosterol (Bard *et al.*, 1974; Gollub *et al.*, 1977), DNA manipulation (Sambrook *et al.*, 1989), isolation of mitochondria (Daum *et al.*, 1982) and their purification on a Nycodenz gradient (Lewin *et al.*, 1990), generation of mitoplasts and protease treatment of mitoplasts and mitochondria (Glick, 1991; Glick *et al.*, 1992), protein extraction from whole yeast (Yaffe and Schatz, 1984), immunoblotting (Haid and Suissa, 1983), yeast transformation (Gietz, R. *et al.*, 1992), preparation of yeast chromosomal DNA (Riezman *et al.*, 1983), recovery of plasmids from yeast (Rose, 1987) and immunofluorescence (Hall *et al.*, 1990).

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