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ANTI-MITOCHONDRIAL AUTOANTIBODIES OF PRIMARY BILIARY CIRRHOSIS AS A NOVEL PROBE IN THE STUDY OF THE BIOSYNTHETIC REGULATION OF THE YEAST 2-OXO ACID DEHYDROGENASE COMPLEXES

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SUMMARY. Autoantibodies present in the disease primary biliary cirrhosis react by immunoblotting with four major yeast mitochondrial antigens of 58 kDa, 55 kDa, 52 kDa and 45 kDa, tentatively identified as the lipoate acetyl transferases (E2) of the pyruvate dehydrogenase, component X of E2 pyruvate dehydrogenase, E2 of 2-oxo glutarate dehydrogenase and E2 of branched-chain 2-oxo acid dehydrogenase complexes respectively. The synthesis of these antigens is sensitive to catabolite repression. The reactive antigens are present in *mit*$^-$ mutants of yeast which have specific defects in the mitochondrial apocytochrome *b*, cytochrome oxidase subunit II and H^+-ATPase subunits 8 and 9, and in mtDNA-less *rho*^{0} petite mutants, but a significant increase in the sensitivity to catabolite repression was observed in these mutants in particular in the mtDNA-less strains.

It is recently reported that anti-mitochondrial autoantibodies (AMA) directed against trypsin-sensitive (M2) antigens of the inner mitochondrial membrane, found characteristically in sera of patients with primary biliary cirrhosis (PBC)[1,2,3,4], react specifically to the lipoate acetyl transferase (E2) components of the pyruvate dehydrogenase (PDC), the 2-oxo glutarate dehydrogenase (OGDC) and the branched-chain 2-oxo acid dehydrogenase complexes (BCOADC) [5,6,7]. These PBC autoantibodies crossreact with mitochondria from a wide phylogenetic range including yeast [8,9,10]. The autoantibodies of PBC, therefore, could be used as a novel immunoprobe in the study of the 2-oxo acid dehydrogenase complexes.

We have exploited the above findings and employed the PBC antibodies in the present study to investigate the levels of the 2-oxo acid dehydrogenases, in various respiratory deficient *mit*$^-$ and mtDNA-less (*rho*^{0}) mutants of *Saccharomyces cerevisiae*. The assembly of functional mitochondria requires the coordinated expression of a large number of mitochondrial as well as nuclear genes [see Ref 11,12 for recent reviews]. Studies in the yeast *Saccharomyces cerevisiae*

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indicate that regulatory interactions exist between the various mitochondrial and nuclear genes involved in this assembly process; a mutation in a mitochondrial gene coding for a subunit of a respiratory enzyme complex, for example, frequently has secondary effects on the assembly of other components of the respiratory chain. It is of interest, therefore, to investigate whether such pleiotropic effects are also extended to other enzyme complexes involved in oxidative metabolism, in particular on the synthesis of the 2-oxo acid dehydrogenases which are central to the flow of the fermentative intermediates into the Krebbs cycle, and thus an important point of regulation in many organism [13,14].

MATERIALS AND METHODS

PBC Sera. Sera containing AMA obtained from patients diagnosed as PBC according to established clinical and histological criteria and with positive AMA as determined by immunofluorescence [8].

Strains of Yeast and Culture Conditions. The strains of the yeast *Saccharomyces cerevisiae* used include a wild-type haploid strain J69-1B (α *ade1 his5* [*rho*$^+$]) [15], the *mit*$^-$ strains 37-16-6 (α *ade1 his* [*cob-box*]) [16], 38.6.1 (α *ade1 his* [*oli1 mit*$^-$]) [17], M26-10 (α *ade1 his* [*aap1 mit*$^-$]) [18], 1203 (α *ade1 his* [*oxi1 mit*$^-$]) [19] and three *rho*^{0} respiratory deficient mutant J69-1Bρ^0 (α ade1 his5 [*rho*^{0}]), D253-2Aρ^0 (α ade1 his1 trp1 [*rho*^{0}]) and 70Mρ^0 (α ade1 lys1 trp1 [*rho*^{0}]) [20]. The wild type cells were grown in 2 L fluted erlenmeyer flasks in 600 ml of medium containing 10 g/l glucose (or 54 g/l for catabolite repression studies), yeast extract (10 g/l), a salts mixture [21] and the auxotropic requirements (100 μg/ml adenine, 50 μg/ml histidine, 50 μg/ml tryptophane, 50 μg/ml lysine). The *mit*$^-$ and the *rho*^{0} mutant strains were grown in either batch cultures as above but with 2 g/l glucose or, in glucose-limited chemostat cultures at 28 ^{0}C at a dilution rate of 0.1 h^{-1} as described by Marzuki and Linnane [22]. Cells grown in the batch cultures were harvested after around 5 hours in the stationary phase while cells grown in the glucose-limited chemostat cultures were harvested after at least 8 generations in the steady state.

Isolation of Mitochondria. Intact yeast mitochondria were isolated from spheroplasts prepared by zymolyase digestion of the cell wall as described previously [23] in the presence of protease inhibitors phenylmethylfluoroanelsulphonate (2 mM), ϵ-aminocaproic acid (5 mM) and p-aminobenzamidine HCl (5 mM). For the isolation of human mitochondria, term placentae were obtained from a local hospital within two hours of delivery. The placental mitochondria were isolated as described by Hare et al. [24] except that all buffers contained the protease inhibitors as above.

Polyacrylamide Gel Electrophoresis and Western Immunoblotting. Polyacrylamide gel electrophoresis was carried out on 0.75 mm thick slab gels (Biorad Miniprotean II) in the presence of 0.1% SDS essentially as described by Laemli [25] and Studier [26]. After separation by electrophoresis, mitochondrial proteins were transferred to nitrocellulose filter (Schleicher & Schuell, Dassel, W. Germany) [27]. Immunoblotting was carried out essentially as described previously [28].

RESULTS AND DISCUSSION

Four *mit*$^-$ mutants, shown in previous studies to exhibit different degrees and characteristics of pleiotropic effects, were examined. The mutations in these strains have previously been defined

Table I. *Mit*$^-$ and *rho*^{0} mtDNA-less mutants of *Saccharomyces cerevisiae* employed in the present study

Strain	Genotype	Primary Defect	Pleiotropic Effects	Ref
37-16-6	ade1 his5 [*cob-box*]	apocytochrome *b*	decreased synthesis of cytochrome oxidase subunit I	16
38-6-1	ade1 his5 [*oli1 mit*$^-$]	H^+-ATPase subunit 9; early termination → truncated product of 7 aa residues	defective assembly of cytochrome oxidase, defect in the synthesis of H^+-ATPase subunit 6	17
26-10	ade1 his5 [*aap1 mit*$^-$]	H^+-ATPase subunit 8; early termination → truncated product of 18 aa residues	defective assembly of cytochrome oxidase	18
1203	ade1 his5 [*oxi1*]	cytochrome oxidase subunit II	decreased coQ-cyt *c* reductase	19
J69-1Bρ^0	ade1 his5 [*rho*^{0}]			20
D253-2Aρ^0	ade1 his5 trp1 [*rho*^{0}]	Defective synthesis of all mitochondrial translation products		
70Mρ^0	ade lys trp1 [*rho*^{0}]			

and the biochemical defects associated with the genetic lesions characterized, as summarized in Table I. Strain 1203 has a mutation in the *oxi1* gene coding for subunit II of the cytochrome oxidase complex [19], which lead to a cytochrome oxidase deficiency without any secondary effect on the synthesis and assembly of the other respiratory enzyme complexes. Strain 37-16-6 carries a mutation in the first exon of the cytochrome *b* gene [16] which in the yeast *Saccharomyces cerevisiae* is split into 6 or 3 exons, depending on the yeast strain, by intervening sequences (introns), some of which suggested to code for RNA splicing enzymes designated maturase. The maturase is essential for the processing of pre-mRNA for the cytochrome *b* apoprotein and for the cytochrome oxidase subunit I. The *cob-box* mutation has been shown to increase significantly the sensitivity of the cytochrome oxidase subunit I to catabolite repression. The *oli1 mit*$^-$ strain 38.6.1 [17] and *aap1* strain M26-10 [18] carry mutations in the structural genes of the H^+-ATPase subunits 9 and 8 respectively. In both cases the mutations lead to an early termination during the synthesis of the H^+-ATPase subunits, resulting in truncated products of only 7 and 18 amino acid residues for the subunit 9 and for the subunit 8 mutants respec-

tively. In both cases, the mutations which lead to the formation of non-functional H^+-ATPase, secondarily affect the assembly of the cytochrome oxidase complex, and to a lesser extent the respiratory complex III (ubiquinol-cytochrome *c* oxidoreductase). In addition to the *mit*$^-$ strains, three mtDNA-less petite mutants (strains J69-1Bρ^0, D253-2Aρ^0 and 70Mρ^0), were also analyzed. The complete loss of mtDNA in these strains leads to the inability of the strains to synthesize all mitochondrial translation products which in yeast include: three subunits of the cytochrome oxidase, the apocytochrome *b*, a protein of the small subunit of the mitochondrial ribosomes (the *var*1 protein) and three subunits of the H^+-ATPase [see Ref 29 for review].

Several PBC sera were initially tested, and one serum, used for the entire study reported here, was selected as it reacted with the maximum number of antigens when tested against human placental mitochondria (Fig. 1). The major 70 kDa ("a") and 52 kDa ("c") antigens observed have previously been shown to be the E2 (lipoate acetyl transferase) of PDC and the E2 of BCOADC. A component of the PDC-E2 with a mobility of between 50-52 kDa (designated component X) has also been shown to react with PBC antibodies; this antigen (designated antigen "b")

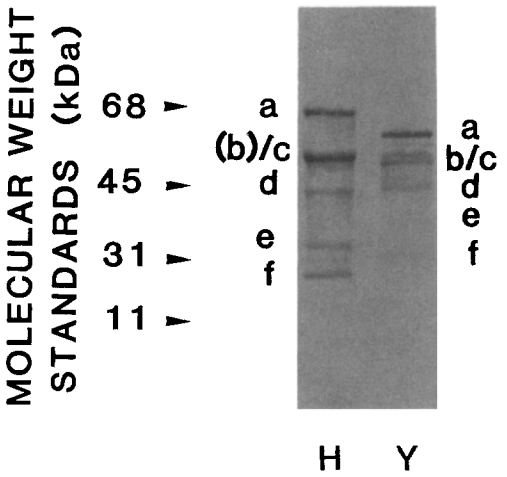

Fig. 1. Comparison of the Reactivity of Human and Yeast Mitochondrial Antigens with PBC Antibodies. Mitochondria were isolated from human placenta [24] and from the yeast *Saccharomyces cerevisiae* [23]. Mitochondrial proteins were then separated by electrophoresis in a polyacrylamide gel in the presence of SDS [25,26], electrophoretically transferred onto nitrocellulose filters [27], and immunoblotted with an AMA reactive serum. Horse-radish peroxidase labeled sheep anti-human Ig was used as a second antibody to visualize the reactive polypeptides, with 4-chloro-1-naphtol as a substrate [28]. Standard proteins used were bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and cytochrome *c* (11 kDa).

presumably co-migrates with the 52 kDa E2 of BCOADC ("c") in our gel system. The PBC antibodies have also been shown to react with the E2 of OGDC, but the mobility of this antigen varied widely between different laboratories, ranges from 43-50 kDa [5,6], and most likely to be the 45 kDa reactive protein ("d") in Fig. 1. A 33 kDa ("e") and a 28 kDa ("f") mitochondrial reactive proteins were also detected but the identities of these antigens have not been elucidated.

Four major reactive proteins were observed when the PBC serum was used in a Western immunoblotting experiment against the wild-type strain J69-1B. The largest antigen, was significantly smaller (about 58 kDa) than that observed for human, but could be shown by employing antibodies to pyruvate dehydrogenase to be the E2 of the yeast enzyme, and thus the equivalent of the human 70 kDa antigen (H. Sudoyo, S. Marzuki and G. Brown, unpublished data). The other three yeast major antigens had the electrophoretic mobilities of 55 kDa, 52 kDa and 45 kDa proteins. By comparison with the electrophoretic mobilities of the human autoantigens [5,7], these reactive proteins were tentatively identified as component X of E2 PDC ("b"), E2 of BCOADC ("c") and E2 of OGDC ("d") respectively. The PBC serum also reactive with a 38 kDa ("e") and a 31 kDa ("f") mitochondrial proteins.

Since the respiratory deficient *mit*$^-$ and *rho*^{0} mutants of yeast have to be grown in the presence of a fermentative energy source such as glucose, it is important to establish whether the synthesis of the lipoate acetyl transferases in the wild-type strain is sensitive to catabolite repression. For this purpose the yeast cells were grown in the presence of 2% glucose as an energy source to allow the release of the cells from catabolite repression to be followed during growth. In this growth medium, the wild type strain exhibited a biphasic growth curve, the first phase of which represents the fermentative growth (division time 1.8 h) on glucose. Around four hours of a lag period was then observed, during which the cells were released from catabolite repression in preparation for an oxidative growth, which occurred following the lag period (division time 6 h; Fig. 2 top). Mitochondria isolated from cells harvested during the fermentative growth showed weak reactivity with the PBC antibodies although the three major reactive proteins (58, 55 and 52 kDa) could be clearly detected (Fig. 2a); the 45 kDa antigen was not visible in this Western-immunoblot due to the low amounts of mitochondrial proteins used to allow the quantitative assessment of the larger antigens. An increase in the mitochondrial levels of the three antigens

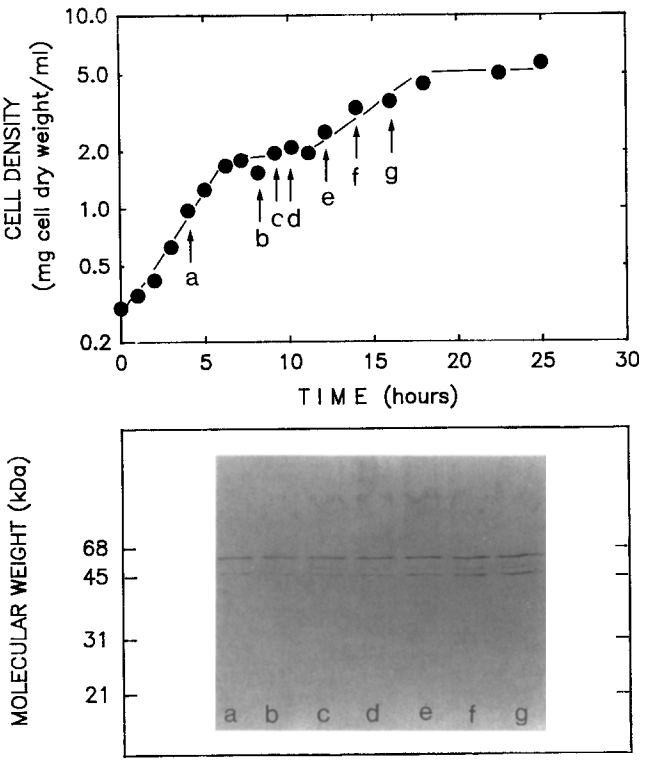

Fig. 2. Release from Catabolite Repression of the Yeast Reactive Antigens. Cells of the wild type strains of J69-1B were grown to the stationary phase in a batch culture, in the presence of 2% glucose. Samples were taken at intervals to determine the cell density (top) and mitochondria were isolated from certain samples as indicated (a to f) and immunoblotted with PBC serum as in Fig. 1 (bottom). Standard proteins used were bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and trypsin inhibitor (21 kDa).

was observed during the derepression period (Fig. 2b, c, d). A slight increase in these levels was further observed during the oxidative growth (Fig. 2e, f, g).

To examine the levels of the M2 antigens in mitochondria of the *mit*$^-$ and *rho*^{0} mutants, the mutant strains were grown in the first instance in batch cultures with 2% glucose as an energy source, and harvested four hours after entering the stationary phase of growth. This corresponded with the end of the non-growing derepression period between the fermentative and oxidative growth shown above for the wild-type strain, and as expected, mitochondria of the wild-type strain contained normal levels of the major 58, 55 and 52 kDa M2 antigens (Fig. 3a). The mitochondrial levels of these antigens, however, were significantly lower in the mutants, in particular in the mtDNA-less *rho*^{0} strains. Thus, the three antigens could be detected in trace amounts only in one of the *rho*^{0} mutants examined, and were almost undetectable in mitochondria

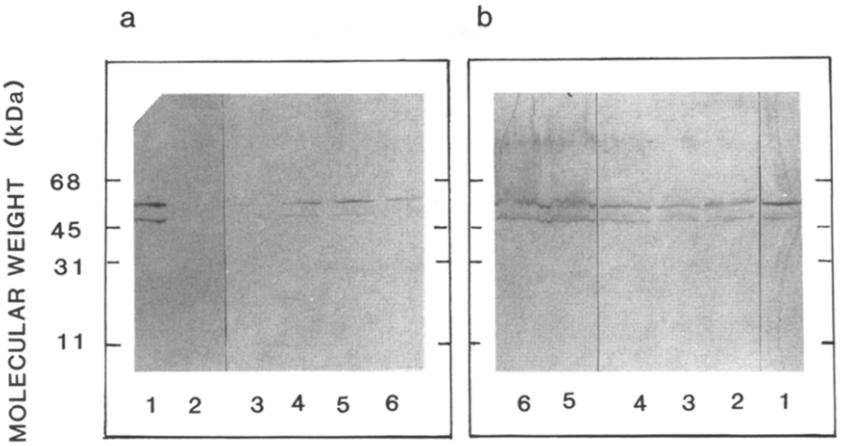

Fig. 3. Yeast Reactive Antigens in *mit*$^-$ and mtDNA-less *rho*^{0} Mutants. Yeast cells were grown in (a) batch cultures in the presence of 2% glucose as the energy source and (b) in glucose limited cultures to minimize catabolite repression [22]. Mitochondria were isolated and PBC reactive antigens were examined by Western immunoblotting. Shown are the western immunoblots of the *mit*$^-$ strains with mutations in the structural genes of the apocytochrome *b* (3), cytochrome oxidase subunit II (4) and H^+-ATPase subunit 8 (5), and subunit 9 (6), as well as the mtDNA-less *rho*^{0} strain J69-1Bρ^0 (2) and the wild type strain J69-1B (1). The other two *rho*^{0} strains examined (strain D253-2Aρ^0 and 70Mρ^0) also showed a significant increased in their sensitivity to catabolite repression.

isolated from the other two *rho*^{0} strains. When the mutant strains were grown in glucose-limited chemostat cultures under conditions which minimize catabolite repression, however, all three reactive proteins were found to be present at levels comparable to that of the wild-type strain (Fig. 3b). Thus, it appears that the reduction of the levels of the M2 antigens apparent in the mutant strains when grown in batch cultures was due to an increase in the sensitivity of the 2-oxo acid dehydrogenases, or at least their lipoate acetyl transferase components, to glucose or tabolite repression. It is of interest to note that the increase in the sensitivity to catabolite repression is significantly more severe in the mtDNA-less *rho*^{0} strains indicating the existence of a nucleo-mitochondrial regulatory interaction. Furthermore, of the four *mit*$^-$ strains examined the *cob-box* strain 37-16-6 exhibited a significantly higher sensitivity to catabolite repression. Strain 37-16-6 carries a mutation in the first exon of the cytochrome *b* gene, and in common with other mutants with genetic lesion in this region, has been shown to be severely affected in its ability to synthesize subunit I of the cytochrome oxidase, due to an increase in its sensitivity to catabolite repression. The present study, which illustrates the utility of the autoantibodies of PBC in the study of 2-oxo acid dehydrogenases, suggests that this effect is not specific to the cytochrome oxidase, but mediated by a more generalize mechanism.

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