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Expression of the Primary Biliary Cirrhosis Antigens in Yeast: Aspects of Mitochondrial Control

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Abstract

The mitochondria of 21 yeast strains were tested for the expression of primary biliary cirrhosis (PBC) specific antigens. The amounts of the antigens in the mitochondrial preparations varied with the strains. Genetic analysis of the strain differences in antigen expression indicated nuclear control which was complex. Those strains expressing the least amounts of antigens exhibited coagulating mitochondria in organellar preparations. Additional evidence relating expression of antigens to the physiological/structural state of mitochondria was that cells grown in the presence of the mitochondrial uncoupling agent, 2,4-dinitrophenol (DNP), failed to produce any antigens, and that glucose repression of mitochondria suppressed antigen expression. Blockage of mitochondrial protein synthesis either through *petite* mutation or by culture in the presence of erythromycin decreased the content of antigens in the mitochondria but did not competely block antigen production. The presence of the PBC antigen in the mitochondria of these cells with nonfunctional mitochondrial synthesizing machinery further indicates that these antigens are cytoplasmically synthesized. Analysis of the pre- and postmitochondrial fractions of all homogenates confirmed that the antigens are not only cytoplasmically synthesized but also have an extramitochondrial location in cells, probably in the plasma membrane.

Key Words: Primary biliary cirrhosis (PBC); mitochondrial antigen; antimitochondrial antibodies (AMA); mitochondrial biogenesis; yeast genetics; *petite* mutation; glucose repression.

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Introduction

Primary biliary cirrhosis (PBC)[4] is a liver disease of unknown etiology with many features pointing toward an autoimmune disorder (Doniach, 1972). From the many autoantibodies described in sera of PBC patients, anti-mitochondrial antibodies (AMA) are routinely demonstrated by indirect immunofluorescence (IFL), (Mackay and Ritts, 1979) and are present characteristically in PBC (Doniach *et al.*, 1966; Munoz *et al.*, 1981). The reactivity of AMA associated with PBC has been shown to be directed primarily against a trypsin-sensitive polypeptide of the inner mitochondrial membrane (Berg *et al.*, 1980, 1982), termed M2. However, no demonstrable mitochondrial function has been found to be associated with this antigen (Baum and Palmer, 1985). A large portion of the major PBC antigen from rat has recently been cloned (Gershwin *et al.*, 1987), but its peptide sequence is unrelated to that of any known mitochondrial constituent.

Attempts have been made by ourselves and other workers to characterize the PBC-specific antigen(s), but very little work has been done to identify and/or characterize the genes coding for them. There are claims, however, that the HLA complex of genes (Arriaga *et al.*, 1980; Yasuhiro *et al.*, 1982) is associated with these antigens.

We have previously reported briefly that these antigens are present in yeast mitochondria and have presented preliminary evidence that they are coded by genes located in the nucleus (Ghadiminejad and Baum, 1985; Uzoegwu *et al.*, 1984). Results further indicated that the expression of these antigens was reversibly inhibited when the mitochondria of the cells were glucose repressed. These initial studies were suggestive of a mitochondrial role in the expression of these antigens. There is well documented evidence from our studies on yeast cells that mitochondria have a function in the modulation of the activity of nuclear genes which specify cell surface components (Wilkie and Evans, 1982; Wilkie *et al.*, 1983) and that aberration or inhibition of function or assembly of the organelle can disturb this function. It is of interest in this connection that we have presented evidence that the PBC antigens are also located at the cell membrane of certain cell types, including yeast (Ghadiminejad and Baum, 1987a).

The present work gives an account of the PBC-specific yeast mitochondrial antigens and their affinities with their mammalian counterparts. It also sets out to characterize in more detail the genes coding for the PBC

[4]Abbreviations: DNP, 2,4-dinitrophenol; EDTA, ethylenediaminetetraacetic acid; mt DNA, mitochondrial deoxyribonucleic acid; MW, molecular weight; PBC, primary biliary cirrhosis; SDH, succinate dehydrogenase (11.3.99.1); SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; YED, 1% yeast extract, 2% glucose culture medium; YEG, 1% yeast extract, 4% glycerol culture medium.

antigens and to investigate the possible role played by mitochondria in their expression.

Materials and Methods

Yeast Strains

Twenty-one haploid strains of *Saccharomyces cerevisiae*, of this laboratory, were used. The characteristics of these strains have been previously described (Egilson *et al.*, 1979). The species is a facultative anaerobe.

Culture Conditions

Yeast cultures were grown in liquid media containing 1% yeast extract with either 2% glucose (YED) or 4% glycerol (YEG), as carbon and energy source. Cells were grown in shake culture at 30°C. In the case of glucose repression, the concentration of glucose was maintained at the original levels with further additions of concentrated glucose solution at 12-hour intervals.

Mitochondrial Inhibition

Blockage of mitochondrial function was achieved in yeast cells (strain D6) by growth in YED medium (1% yeast extract, 2% glucose) in the presence of 2,4-dinitrophenol (DNP), an uncoupler of mitochondrial oxidative phosphorylation. Mitochondrial specificity of DNP was manifest in the ability of cells to grow on fermentable substrates, i.e., glucose, but their inability to grow on nonfermentable substrates such as glycerol in the presence of the inhibitor. (This phenomenon was used to select a yeast strain for specific mitochondrial inhibition by DNP, i.e., strain D6.) Cells were grown to stationary phase (growth cycle) in YED containing 100 μg/ml DNP for 1, 2, and 3 growth cycles. The cells were subfractionated as below.

Erythromycin

Erythromycin selectively blocks mitochondrial protein synthesis. Yeast cells of strain D6 were grown to stationary phase in YED media containing 100 μg/ml erythromycin (Sigma, Chemical Ltd).

Petite Yeast Cells

Yeast cells (strain D6) were grown in YED media containing 20 μg/ml of acriflavine, an intercalating agent, which dramatically increases the formation of *petite* mutation (Egilsson *et al.*, 1979). This high rate makes

their identification straightforward and unmistakable. *Petite* cells plated on standard agar medium containing glucose as energy source are able to grow and give rise to colonies but are unable to grow on medium containing glycerol, a substrate of the mitochondrial energy-producing pathways. The *petite* colonies were further subcultured for homogeneity and were then grown to stationary phase in YED media at 30°C.

Spectra

Spectra of the yeast cells with mitochondrial inhibition and of those strains of particular interest were obtained using a Unicam SP 1805 Program Controller spectrophotometer. Cells were scanned at a concentration of 10^8 cells/ml in cuvettes of 1 cm path length.

Genetic Analysis

Standard methods were used in crossing strains and tetrad analysis (Mortimer and Hawthorne, 1969). Zygotes were micromanipulated after 2 hours mating onto YED agar and incubation to give diploid clones. A de Fonbrune micromanipulator was used for zygote isolation and also for the microdissection of ascospores in tetrad analysis.

Subfractionation of Yeast Cells

Cells were centrifuged, washed, and homogenized in a Mickel shaker, using balletini beads 0.2–0.3 mm in size. Differential centrifugation of cell homogenates was carried out using standard methods (Massari *et al.*, 1972). The "mitochondrial," "premitochondrial," and "postmitochondrial" fractions were stored in small aliquots at -20°C.

Protein Estimation

The protein concentrations of samples were determined using the previously described method of Bradford (1976).

Marker Enzyme

Succinate dehydrogenase was used as a marker for the mitochondrial inner membrane. The enzyme was assayed by the established method of Pennington (1961), modified according to the method of Jenkins and Peters (1978). In the case of *petite* yeast cells and where cells were grown in the presence of mitochondrial inhibitors, the cells were subfractionated according to the protocol established for other yeast strains.

Immunological Tests

Serum. A large quantity of PBC serum from one patient was obtained when plasma exchange was performed. The patient was clinically classed to be in stage 4 of the disease at the time of plasma exchange. The reactivity of this PBC serum with mitochondrial antigens was extensively characterized and was found to be typical with respect to the number and pattern of bands detected on the immunoblot.

Immunoblotting. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to standard procedures (Laemmli, 1970; Laemmli and Faver, 1973). The gel was electroblotted onto nitrocellulose (pore size 0.45 μm, Sartorious Membranes Ltd.) as described by Towbin *et al.* (1979). The reaction of the patient's serum antibodies (diluted 1 : 100) with antigenic species on the nitrocellulose blot was detected indirectly by the binding of radiolabelled antisera; rabbit anti-human antibodies (anti total immunoglobulins, DAKO-immunoglobulins, Denmark) were iodinated by the chloramine T method (Hunter and Greenwood, 1962; Greenwood *et al.*, 1963). The assay was standardized by the addition (where possible) of 100 μg of protein per well for all fractions assayed and by using equal exposure time for autoradiography in all cases. The intensities of the antigenic species were visually assessed as compared with the standard track. (Direct counting of the labelled blots, or densitometric scans of the plates, confirmed the validity of this mode of recording the approximate relative antigenicity of the bands). Where it was not possible for 100 μg of protein to be applied, the amount applied was noted and was adjusted for in the presentation of results. The reaction of beef heart mitochondria against PBC serum has been extensively characterized in our laboratory, and these mitochondria were therefore used as control tracks for determining the approximate MW of the yeast antigenic species.

Control normal serum was routinely tested against replicate immunoblots to confirm that the antigens detected were indeed PBC-specific. Controls were also carried out with the PBC serum following adsorption with beef heart mitochondria (Ghadiminejad and Baum, 1987b), to confirm that all reactivity against yeast antigen was abolished and that these antigens may thus be properly designated as "cross-reacting" with AMA.

Results

Antigen Expression in Different Yeast Strains

The antigenicity of the fractions obtained from the differential centrifugation of yeast cell homogenates of 21 yeast strains was estimated in terms

of the intensity of the antigen species observed on the immunoblot compared to that of the standard. The relative intensities of the antigenic species for the mitochondrial fractions were then numerically assessed and are given in Table I, where the antigenic band of R_f value 0.3 is the major PBC-specific antigen. These results and all subsequent findings were repeatedly obtained, confirming that the varying antigen production was a stable characteristic of each strain. From Table I, it can be seen that the mitochondria from two of the yeast strains failed to express any antigenicity. Close observation of the samples treated prior to gel electrophoresis (final concentration 0.05 M Tris-HCl, pH 6.8, 2 mM EDTA, 2% SDS, 0.5 mM dithiothreitol, 0.001% Bromophenol Blue) showed that the mitochondria from these strains exhibit coagulation in the denaturing medium. This would obviously affect both the amount of protein in each aliquot to the gels and the amount of protein in such an aliquot which was fully denatured. All samples were checked for this feature, and the problem of application was partly overcome by vortexing the samples immediately before application on the gel. Counterstaining of such gels with Coomassie Blue revealed that there was no significant difference

Table I. Expression of PBC Antigens in Mitochondria from Various Yeast Strains[a]

	R_f values of antigens and relative amounts				
Strains	0.09	0.15–0.25	0.3	0.37	0.45–0.55
D6	3	1	15	10	2
B21	–	–	2	–	–
188 wt	–	–	5	2	–
A30	–	3	3	–	–
D22	–	–	–	–	–
D18	–	–	–	–	–
22.701[b]	–	1	–	–	–
B/B	–	–	1	–	1
6–81[c]	–	5	2	–	–
D11	–	–	8	–	2
B41	2	–	8	4	2
D4	–	2	3	1	–
B27	1	–	10	8	4
2180A	–	4	5	–	–
2180B	1	–	2	–	1
41/161	–	–	2	–	–
B/A	–	–	2	–	–
A285	–	–	12	10	2
517b	–	1	8	6	1
D26	–	2	4	–	–
D75	–	3	2	–	1

[a]The main antigenic species are at R_f values 0.3 and 0.37. The relative amounts of the antigens are numerically valued, where 15 is given to the antigenic band with the highest intensity.
[b]Mitochondrial drug-resistant mutant of D22.
[c]Mitochondrial drug-resistant mutant of D6.

(with respect to the amount of protein applied) in the amount of protein entering the gels. Nevertheless, the coagulating ability of the mitochondria from different yeast strains did seem to correspond inversely to the amount of PBC antigens expressed in those mitochondria, i.e., the lower the antigen expression the greater seemed to be the tendency for mitochondrial coagulation. However, the "premitochondrial" fractions of these yeast strains, although containing some mitochondrial contamination (as assessed by succinate dehydrogenase), expressed a relatively high amount of the PBC antigens, not accountable for by such contamination. These results are consistent with there being both a mitochondrial and another cellular site for the disposition of these antigens. That cellular site has been localized to the plasma membrane (Ghadiminejad and Baum, 1987a).

Interestingly, the low expression of antigen in certain strains, for example 22.701, D18, and D22, correlated with an apparently lower cytochrome content than that of strain D6 (Fig. 1).

Effects of Mitochondrial Inhibitors and the Petite Mutation on Antigen Production

Erythromycin. The antibacterial antibiotic erythromycin selectively blocks protein synthesis on mitochondrial ribosomes thus precluding the translation of mitochondrial genes carried in the organellar chromosome (mt DNA). These specify components of the inner membrane assembly particularly of cytochromes aa_3 and *b* of the respiratory chain and hydrophobic constituents of the ATPase complex. However, the synthesis of mitochondrial proteins coded by nuclear genes (and these comprise over 90% of mitochondrial proteins) is apparently unaffected, as exemplified in the production of cytochrome *c* (Fig. 1). Likewise, the production of PBC antigens continues (albeit to a lesser extent than in control) in cells growing in the presence of erythromycin (Fig. 2, track 3), indicating cytoplasmic synthesis of the PBC antigens. The relatively low antigen content in the mitochondria of these cells as compared to control tracks (Fig. 2, tracks 1 and 8) is discussed later.

Petite Mutation. The *petite* mutation is due to an extensive deletion in mitochondrial DNA which occurs with a high frequency in yeast cells. The mechanisms of aberration are unknown but *petite*-type mitochondria are unable to carry out protein synthesis. Thus, *petite* mutations are somewhat analogous to cells growing in the presence of erythromycin, i.e., they produce cytochrome *c* but not cytochromes aa_3 and *b* (Fig. 1). As in erythromycin-grown cells (and as previously reported; Ghadiminejad and Baum, 1985; Uzoegwu *et al.*, 1984), PBC antigens were expressed (Fig. 2, track 2).

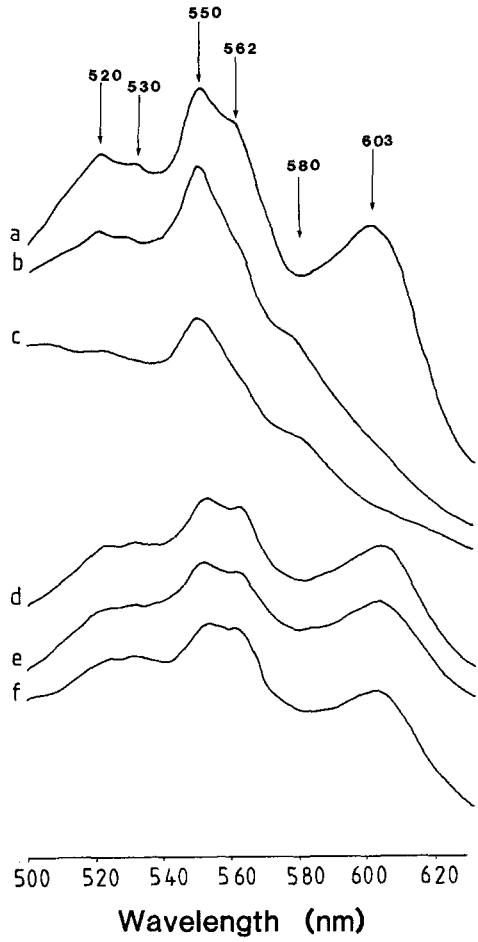

Fig. 1. Absorption spectra of various yeast cultures (10^8 cells/ml). (a) Strain D6 in glycerol medium (1% yeast extract, 4% glycerol) and (b) in glucose medium (1% yeast extract, 2% glucose) with 1 mg/ml erythromycin; (c) *petite* cells obtained from strain D6, in glucose medium; (d–f) strains 22.701, D18, and D22, respectively, in glycerol media. Peaks at 603, 562, and 550 nm are α peaks of cytochromes $a + a_3$, b and c respectively, while the β peaks of cytochromes b and c occur at 530 and 520 nm, respectively. The peak at 580 nm in the spectra of cells with erythromycin and *petite* cells (b and c, respectively) is thought to arise from the cytoplasmically synthesized precursors of the cytochrome $a + a_3$ complex.

DNP. The "mitochondria" and "pre-" and "postmitochondrial" fractions of yeast cells (strain D6, see method for selection) grown in the presence of DNP were examined for the expression of the PBC antigens. The subfractions of cells from the first growth cycle showed considerably decreased antigen expression, as compared to subfractions of yeast cells grown on

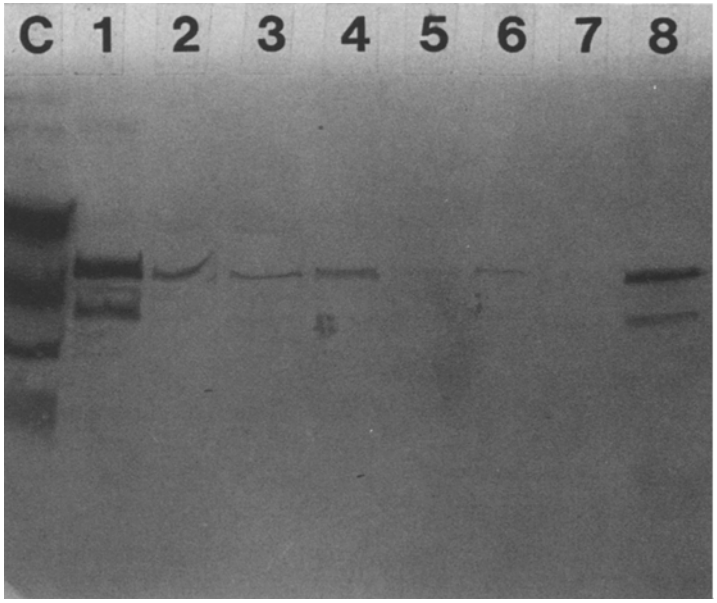

Fig. 2. Reaction of PBC serum with the mitochondrial fractions of yeast strain D6. (1) Cells grown in YEG medium; (2) *petite* cells of strain D6; (3) cells grown in the presence of 1 mg/ml erythromycin; (4) cells grown in DNP, first growth cycle; (5) cells grown in DNP, second growth cycle; (6) cells repressed with 2% glucose; (7) cells repressed with 5% glucose (mitochondrial repression was achieved by maintaining the glucose concentrations at 2 and 5%, respectively, with further additions of concentrated glucose solution at 12-hour intervals); (8) cells grown normally in YED. Beef heart mitochondria were used as the standard track (C).

YED alone (Fig. 2, track 4 and Fig. 3). The subfractions of yeast cells from the second and third growth cycle failed to express any antigenecity (Fig. 2, track 5 for mitochondria; results for other fractions not shown). These findings are consistent with the fact that 2,4-dinitrophenol abolishes membrane potential-dependent transfer of molecules across the mitochondrial membranes. In this case, the impairment of mitochondrial assembly was such that loss of antigens paralleled the loss of cytochome *c* (Fig. 4). It is noteworthy that under these conditions antigen did not accumulate in any other cellular compartment, and this will be discussed below.

Glucose Repression. Mitochondrial proteins fall into two categories: those that are repressible and those that are constitutive. The most potent conditions for repression are anaerobiosis and the continuous presence of relatively high concentrations of glucose. Under these conditions, mitochondrial protein synthesis is considerably reduced (Fig. 4), and is halted if glucose concentrations are very high.

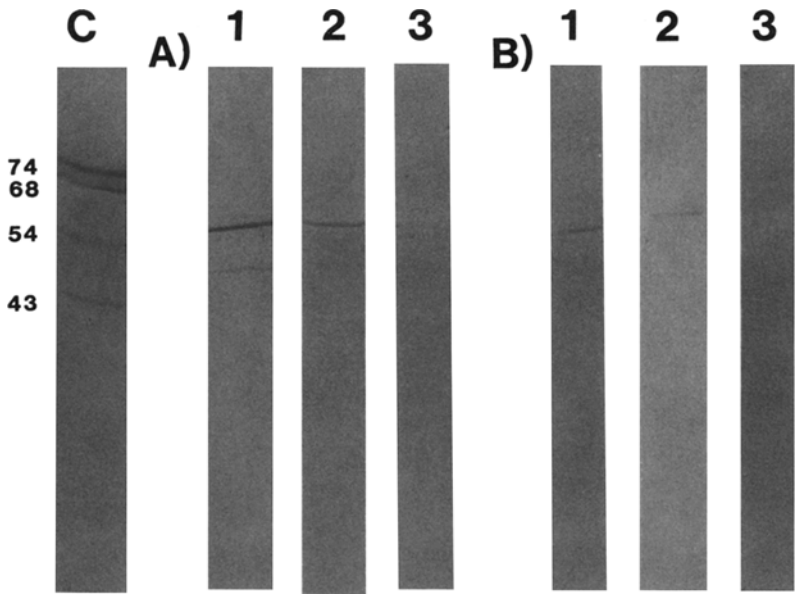

Fig. 3. Expression of PBC antigens in subfractions of cells. (A) Grown in YED medium; (B) grown in YED medium containing 100 μg/ml of DNP (first growth cycle). (C) Beef heart mitochondria were used as the standard track. In each case track one is the "mitochondrial" fraction and tracks 2 and 3 the "pre-" and "postmitochondrial" fractions respectively. The numbers alongside track C correspond to the approximate MW's (kD) of the antigenic bands of beef heart mitochondria.

The mitochondria of yeast cells (strain D6) were glucose repressed to varying degrees. The mitochondria of these cells were then examined for antigen expression. The extent of antigen expression (in comparison to nonrepressed mitochondria) decreased with increasing glucose repression of the mitochondria (Fig. 2, tracks 6 and 7).

Genetic Analysis

The diploids obtained by crossing the yeast strain 22.701 (mating type, a, a minimum producer of PBC antigen) with the strains A285, B27, and B/B (mating types, α, the first two being good producers, the third exhibiting very low antigen expression) showed low amounts of antigen (Fig. 5). The presence of the PBC antigens in the "premitochondrial" fraction of the parental strain 22.701 (Fig. 6, track 1) indicates that the antigens are produced and again are consistent with the presence of a second antigenic

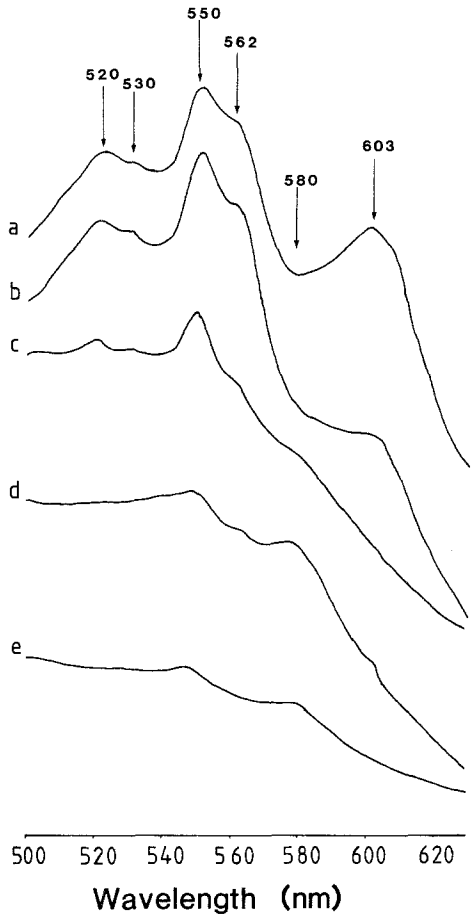

Fig. 4. Absorption spectra of strain D6 (10^8 cells/ml) in (a) YEG medium, (b) YED medium, (c) glucose repressed medium (1% yeast extract, 10% glucose), and (d, e) YED medium containing 100 μg/ml DNP, from the first and second growth cycle, respectively.

site. The high-molecular-weight (MW 80 kD) antigenic species detected in the "premitochondrial" fractions of all the samples might suggest that these are precursors of the PBC antigens, prior to uptake and proteolytic processing.

The diploid from the cross 22.701 × B27 was chosen for further investigation. This diploid was sporulated, and ascospore cultures of two tetrads were analyzed for the presence of antigenic species. In the first tetrad (Fig. 7, 1A–1D), mitochondrial preparations from 1A, 1C, and 1D showed the presence of the main antigen (MW 63 kD) in amounts similar to parental strain B27 in the case of 1C and 1D but to a greater extent in 1A. On the other

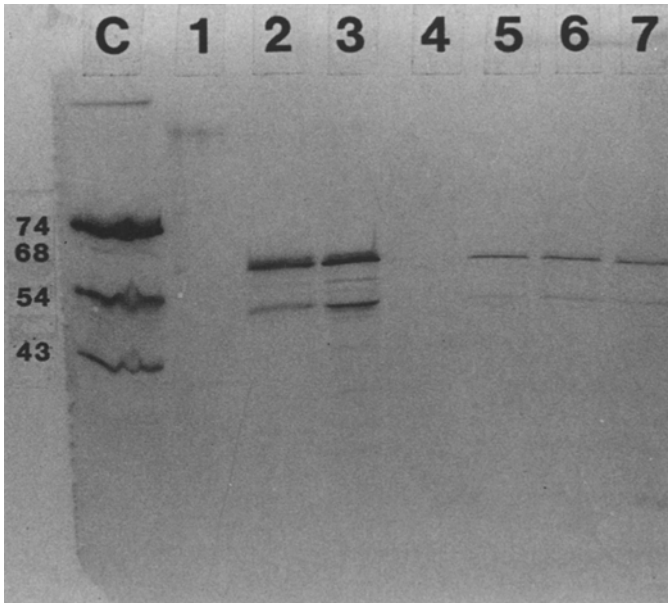

Fig. 5. Expression of PBC antigens in the mitochondria of haploid yeast: (1) 22.701, (2) B27, (3) A285, and (4) B/B; and of dipoloids: (5) 22.701 × B27, (6) 22.701 × A285, and (7) 22.701 × B/B. (C) Beef heart mitochondria used as the standard track. Numbers alongside track C correspond to the approximate MW's (kD) of the antigenic bands of beef heart mitochondria.

hand, the mitochondria from 1B had very little if any of the main antigen. In tetrad 2 (2A–2D), all four meiotic products showed the presence of the main antigen in mitochondrial fractions, but 2A, 2C, and 2D produced greater amounts than B27 while 2B was similar to the parental strain in this respect. These results indicate that both parental strains possessed the structural gene for the main antigen and that its expression was controlled by a regulatory gene or genes. Recombination of regulatory genes could explain the extra high and low levels of antigen in the recombinant types.

With respect to the amounts produced of the second antigenic species (MW 48 kD), there seems to be a good correlation between these and amounts of the main antigen. These results suggest that both species come under the same regulatory system (indeed, they might both represent "post-translational" derivatives of the same precursor gene product).

Tests were made of the "pre-" and "postmitochondrial" fractions from these tetrad cultures for the presence of antigens. There was a good general

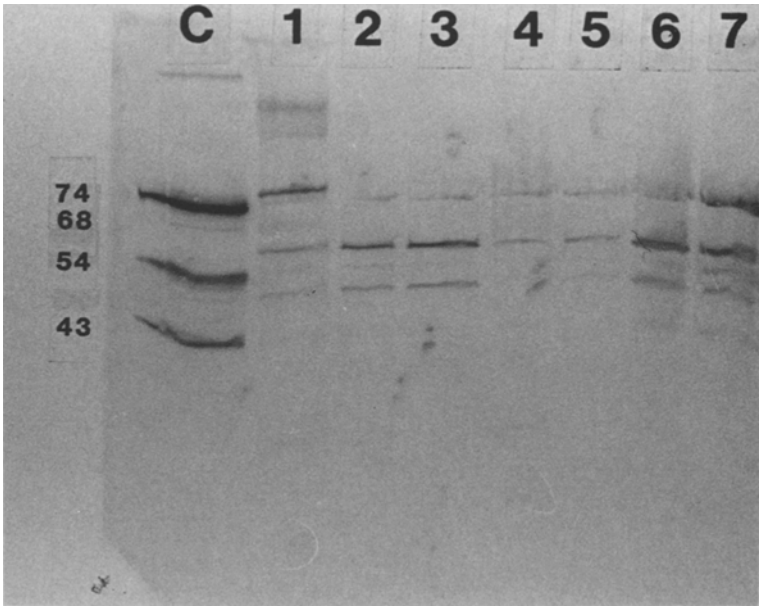

Fig. 6. Expression of the PBC antigens in the premitochondrial fraction of strains (1) 22.701, (2) B27, (3) A285, and (4) B/B; and of the diploids (5) 22.701 × B27, (6) 22.701 × A285, and (7) 22.701 × B/B. (C) Beef heart mitochondria were used as the standard track. Numbers alongside track C correspond to the approximate MW's (kD) of the antigenic bands of beef heart mitochondria.

correlation between antigen expression in these fractions and that in the corresponding mitochondrial preparations (results not shown).

Discussion

The large variation in the expression of PBC antigens detected in the mitochondria of 21 yeast strains tested (Table I) in conjunction with the appearance of these antigens in the "premitochondrial" fractions, particularly of strains 22.701, D22, and D18 (results for D22 and D18 are not shown), suggests that the nuclear genes coding for these antigens may be under a regulatory system, which determines not only the extent of expression of the antigen, but also, directly or indirectly, their subcellular distribution.

The yeast strains with low mitochondrial antigen expression exhibited coagulating mitochondria (after sample treatment). Indeed, in a general

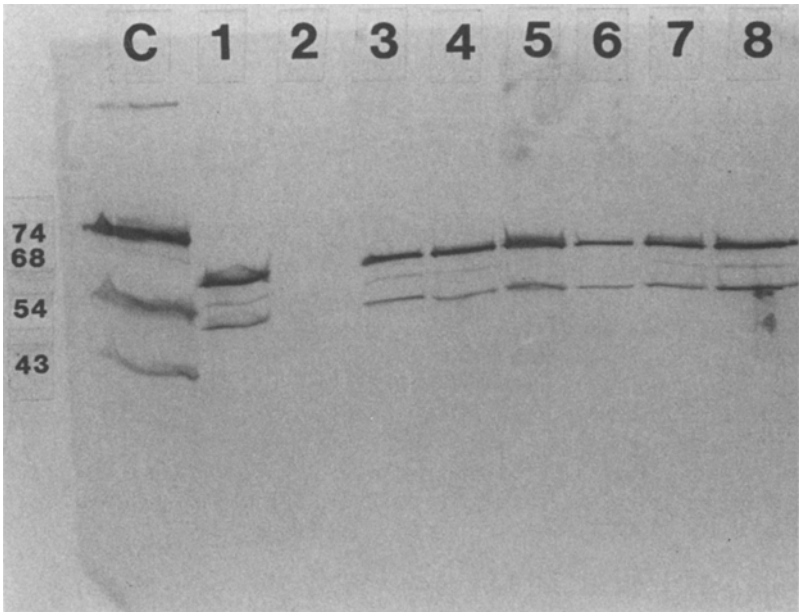

Fig. 7. The mitochondrial expression of the PBC antigens in the spores from the sporulation of the diploid 22.701 × B27. Lanes 1–4 represent the spores 1A to 1D (from the first sporulation) and lanes 5 to 8 represent the spores 2A to 2D (from the second sporulation). (C) Beef heart mitochondria were used as the standard track. Numbers alongside track C correspond to the approximate MW's (kD) of the antigenic bands of beef heart mitochondria.

qualitative sense, the coagulating ability of the mitochondria appeared to correspond inversely with the degree of antigen expression. These findings might have been taken to suggest that the absence of the PBC antigens in the mitochondria of these strains is the cause of their tendency to coagulate. An alternative explanation is that the coagulating mitochondria may lack the ability (possibly due to the physiological/structural state of the mitochondrial membrane) to take up these antigens. This latter suggestion of impaired assembly is supported by the detection of comparatively low concentrations of cytochrome *c* in the mitochondria in these strains (Fig. 1), even though the conditions of growth were made favorable for high mitochondrial activity. It is of particular interest, however, that these strains, although seemingly with no mitochondrial PBC antigens, do show antigen expression in the "premitochondrial" fraction. Indeed the antigen expressed in these fractions has now been localized at least in part to the plasma membrane (Ghadiminejad and Baum, 1987a). However, their expression indicates that the genes coding for these antigens are active under these growth conditions.

Detection of the PBC antigens in the mitochondria of yeast cells grown in the presence of erythromycin, a specific inhibitor of the mitochondrial protein-synthesizing machinery, further suggests that the genes coding for these polypeptides are nuclear. This was also confirmed by the detection of PBC antigens in the mitochondria of the *petite* yeast mutants (Fig. 2). The considerably decreased antigen content detected in the mitochondria of *petite* mutants and cells grown in the presence of erythromycin (Fig. 2, tracks 2 and 3) suggests that the abnormal structure and function of the mitochondrial membrane, caused by the lack of constituents such as cytochromes $a + a_3$ and b (Fig. 1), decreases the mitochondrial ability to take up the PBC antigen and possibly other cytoplasmically synthesized, mitochondrially destined precursors. However, the detection of the antigens in the premitochondrial fractions in amounts not greatly reduced from those in controls (results not shown) indicates that the responsible genes are switched on, and further confirms the presence of a second site for these antigens (Ghadiminejad and Baum, 1987a). This in turn supports the earlier interpretation that the structural state of the mitochondria determines the extent of antigen uptake.

Glucose repression of the mitochondria repressed the expression of PBC antigens to an extent that correlated with the extent of mitochondrial repression as monitored spectroscopically. Under glucose repression the decrease in the mitochondrial expression of the PBC antigens was associated with a corresponding decrease in the "premitochondrial" fractions (results not shown). Hence there was a net decrease in antigen production rather than a redistribution between cellular sites. Although these findings might be taken to suggest that it is the respiratory activity of the mitochondria that is responsible for the regulation of antigen expression, the earlier studies with the 21 yeast strains grown in YEG media eliminates mitochondrial energy production as a specific effector, since all the strains grow well under conditions requiring aerobic metabolism.

2,4-Dinitrophenol (DNP) is a specific uncoupler of the mitochondrial membrane in yeast which consequently inhibits the potential-dependent uptake of cytoplasmic precursors of mitochondrial proteins (Schatz, 1979). However, incorporation of (or response to) this uncoupler is slow. This is demonstrated by the detection of residual peaks at 603 and 562 nm in addition to the new peak at 580 nm corresponding to the cytoplasmically synthesized precursors of cytochrome $a + a_3$ in the cells from the first growth cycle. Only the latter is seen (together with smaller amounts of cytochrome c as indicated by the residual peak at 530 nm) in the cells from the second growth cycle (Fig. 4). This may explain the small amounts of PBC antigens detected in the corresponding mitochondrial fractions of these cells (Fig. 2, tracks 4 and 5). The absence of accumulated PBC antigens in the

other subcellular fractions in these cases suggests that the gene(s) coding for the PBC antigens are switched off when the mitochondrial membrane is rendered inactive. This, in conjunction with the glucose repression studies, suggest that the antigen production is inhibited by some kind of feedback mechanism. This may not be exerted by the gene product itself since in the case of some of the "nonproducing" yeast strains studied PBC antigens are detected in the "premitochondrial" fractions. Genetic analysis of the diploids, parental haploids and the spores, strongly suggests that all strains, regardless of the extent of the antigen expression, possess the structural genes for the main antigen and that its expression is controlled by a regulatory gene or genes.

In conclusion, it seems that the genes coding for the PBC antigens (eventually located both in mitochondrial and other cellular membrane sites) are inhibited by a feedback mechanism. The ability to switch these genes off (together with other genes concerned with mitochondrial assembly) needs to be fully investigated. However, the situation is certainly complex, and control by regulatory genes and by the physiological/structural state of the mitochondria may both individually or cooperatively play an important role in the final regulation of the PBC antigen production.

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