Accumulation of Viruslike Particles in a Yeast Mutant Lacking a Mitochondrial Pore Protein

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Received 29 September 1988/Accepted 14 December 1988

The lack of mitochondrial porin is not lethal in *Saccharomyces cerevisiae*, but it impairs some respiratory functions and, therefore, growth on nonfermentable carbon sources such as glycerol. However, after a lag phase porinless mutant cells adapt to growth on glycerol, accumulating large amounts of an 86-kilodalton (kDa) protein (M. Dihanich, K. Suda, and G. Schatz, EMBO J. 6:723–728, 1987) and of a 5-kilobase RNA. Immunogold labeling localized the 86 kDa-protein exclusively to the cytosol fraction, although most of it cosedimented with the microsome fraction in earlier cell fractionations. This discrepancy was resolved when the 86-kDa protein was identified as the major coat protein in viruslike particles (VLPs) which is encoded by a double-stranded RNA (L-A RNA). Elimination of VLPs in the original porinless strain by introduction of the *mak10* or the *mak3* mutation increased the respiratory defect and prolonged its lag phase on nonfermentable carbon sources. The fact that the simultaneous loss of VLPs and respiratory functions are the introduction of *mak10* or *mak3* occurred even in some porin-containing wild-type strains suggests that there is a link between VLP and mitochondrial functions.

The most abundant and best characterized protein of the outer mitochondrial membrane is porin, which is the only general diffusion pore known to exist in mitochondrial outer membranes. Because of its lack of specificity (only weak anion selectivity) and relatively large molecular weight cut-off (about 6,000 daltons [Da]), it is assumed to be the major, if not the only, gate for hydrophilic metabolites across the outer membrane (1).

Porinless mutant HR125-2A grows on glucose medium as well as the wild type at 30°C and more slowly than the wild type at 37°C. It fails to grow for at least 3 days if it is plated onto medium containing nonfermentable carbon sources such as glycerol. This defect can be explained by a dramatic reduction in the levels of all mitochondrial cytochromes. Growth on glycerol starts only after several days and is accompanied by partial restoration of the levels of

TABLE 1. Yeast strains used in this study

Strain	Genotype	ds RNA	Source
HR125-2A	<i>MATα gal2 his3 his4 leu2 trp1 ura3 por::LEU2</i>	L-A	M. Dihanich
HR125-2B	<i>MATα gal2 his3 his4 leu2 trp1 ura3</i>	L-A	M. Dihanich
HR125-2D	<i>MATα gal2 his3 his4 leu2 trp1 ura3</i>	L-A	M. Dihanich
1937	<i>MATα arg9 leu2</i>	L-O	R. Wickner
1937α	<i>MATα arg9 leu2</i> (HO-transformed strain 1937)	L-O	This study
POR ⁹⁵	<i>MATα arg9 leu2 por::LEU2</i>	L-O	This study
2107	<i>MATα ade2 can1 his3 leu2 mak10</i>	L-O	R. Wickner
297	<i>MATα his1 mak3</i>	L-O	R. Wickner
1101	<i>MATα his4 kar1</i>	M, L-A, L-B, C	R. Wickner
5x47	<i>MATα/MATα</i> killer-sensitive tester	ND ^a	R. Wickner

^a ND, Not determined.

The biogenesis of porin is well characterized; in *Saccharomyces cerevisiae* it is encoded by a single nuclear gene with an open reading frame of 849 base pairs (13). Disruption of this gene by deletion-insertion mutagenesis does not affect the viability of yeast cells (5), but it does lead to a temporary loss of respiratory functions, the severity of which is strain

mitochondrial cytochromes and the accumulation of large amounts of an 86-kDa protein. Since this protein accumulates in an extramitochondrial cellular compartment, it cannot simply be an alternative outer membrane pore. Its high level of overproduction, in fact, suggests that it plays a role similar to that of stress proteins such as heat shock and glucose-regulated proteins (2, 4, 16). However, although the 86-kDa protein and the well-known heat shock protein hsp90 are similar in size, they are not identical (5).

In this study we identified the 86-kDa protein as the major coat protein of viruslike particles (VLPs) and propose that there is a novel connection between the functions of VLPs and mitochondria.

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FIG. 1. Cytosolic localization of the 86-kDa protein demonstrated by immunogold labeling of yeast cells grown on glucose or glycerol. (a) Glucose-grown mutant; (b) glycerol-adapted mutant; (c) glucose-grown wild type; (d) glycerol-grown wild type. N, Nucleus; V, vacuole; arrowheads, mitochondria.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used in this study are listed in Table 1. Glucose-, glycerol-, and lactate-containing media were the same as those described by Dihanich et al. (5). Killer test plates (YPD plates buffered to pH 4.8 with sodium citrate containing 0.003% methylene blue) were made as described by Woods and Bevan (26).

Immunogold labeling of yeast cells. Yeast cells were fixed and embedded in Lowicryl for immunogold labeling as described by van Tuinen and Riezman (19).

Negative staining. Samples (5 μ l) were spread onto glow-discharged collodion-carbon-coated copper grids, washed with buffer A (50 mM Tris [pH 7.5], 10 mM MgSO_4 , 150 mM NaCl, 390 mM KCl), blotted dry, and incubated with 1% uranyl acetate for 1 min. The liquid was withdrawn and the grids were viewed in an electron microscope (109; Zeiss) at 80 kV.

Preparation and sequencing of CNBr-cleaved peptides. Glycerol-adapted *por*⁰ mutant cells were grown to the late-log phase in either rich medium containing 3% glycerol or

FIG. 2. Strategy used for making oligonucleotide probes.

semisynthetic medium containing 2% lactate, harvested by centrifugation, and subfractionated (5). The pellet obtained from centrifugation at $100,000 \times g$ that was rich in the 86-kDa protein was dissolved in 75% formic acid and loaded onto a high-pressure liquid chromatographic (HPLC) column (RP-4). Elution was performed in a gradient of 20 to 70% propanol in 20% formic acid over 20 min. The major 86-kDa peak was well separated from the bulk of the microsomal proteins and eluted at about 55% propanol. Propanol was removed from the eluate in a stream of N_2 , and the protein was digested to completion with CNBr (about 1 mg/100 μ g of protein) in 70% formic acid at room temperature. The cleavage peptides were separated on a column (RP-18) in 0.1% trifluoroacetic acid in a gradient of 15 to 45% acetonitrile over 60 min. Only the five smallest peptides (<3 kDa) were well resolved; three of those were subjected to gas-phase sequencing. Sequence determination was performed by Edman degradation with a gas-phase sequencer (470A; Applied Biosystems) (12) with an on-line analyzer (120A PTH; Applied Biosystems) and a data-handling system (900A; Applied Biosystems).

Screening with oligonucleotide probes. A degenerate oligonucleotide probe was synthesized from a DNA sequence deduced from a 13-amino-acid stretch of peptide 3. Application of the yeast codon usage rules reduced the number of possible DNA sequences from about 2,000 to 8:

The resulting mixture of 39mers was purified by HPLC, end-labeled with $[\gamma-^{32}P]ATP$ and polynucleotide kinase, and subsequently hybridized to DNA (colony hybridization was done as described by Grunstein and Hogness [11]) or RNA (Northern hybridization; see below). Hybridization conditions were the same in both cases. Prehybridization was done in $10\times$ Denhardt medium and $6\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 N sodium citrate) at $65^\circ C$ for 30 min (DNA) or 5 h (RNA); hybridization was done in $6\times$ SSC and 1×10^6 cpm of probe per filter (specific activity, 1.1×10^8 cpm/ μ g) starting at $66^\circ C$; it was then slowly cooled to the actual hybridization temperature (45 to $55^\circ C$) overnight. Washes after hybridization were done in $6\times$ SSC for three

times for 10 min each time at room temperature and then two to three times for 10 min each time at the end temperature of hybridization.

Preparation and Northern analysis of total yeast RNA. RNA was prepared from log-phase cells that were grown in rich medium containing either 2% glucose or 3% glycerol (14). RNA equivalent to equal amounts of cells (about 20 μ g of total RNA per lane) was separated by electrophoresis on formaldehyde-agarose gels and transferred to membranes (Gene Screen Plus; Dupont, NEN Research Products, Boston, Mass.) as recommended by the manufacturer.

Preparation of VLPs. VLPs were prepared by a modification of the method described by Welsh et al. (20). The pellet obtained by centrifugation at $100,000 \times g$ and cell fractionation of glycerol-grown cells (see above) was suspended in buffer G (50 mM Tris hydrochloride [pH 7.5], 10 mM $MgSO_4$, 0.15 M NaCl, 0.39 M KCl, 1 mM dithiothreitol, 1 mM EDTA, and 20% glycerol) and centrifuged through a 20 to 50% sucrose gradient. Fractions of 1 ml were collected, and portions were assayed for the presence of the 86-kDa protein on dot blots. Portions (0.5 ml and 50 μ l) from each fraction were used for RNA extraction and negative staining, respectively.

V8 protease digestion. Digestion of the 86-kDa protein and VLPs with staphylococcal V8 protease was performed by the method of Cleveland et al. (3).

Other techniques. Protein was extracted from yeast by using NaOH for cell lysis (27). Protein determination, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblots, and production of anti-86-kDa antibodies were performed as described previously (5).

RESULTS

Identification of the 86-kDa protein. Two major strategies were pursued to identify the 86-kDa protein, the first one

FIG. 3. Overexpression of a 5-kb poly(A)⁺ RNA in adapted *por*⁰ mutant cells. Ethidium bromide-stained gels (lanes 1 to 4) and Northern blots (lanes 5 and 6) are shown. Lanes 1 and 2, Total RNA from glycerol-grown mutant and wild-type cells, respectively; lanes 3 and 4, size standards (rRNAs from *E. coli* and calf liver, respectively); lanes 5 and 6, Northern hybridization with oligonucleotide probe oligo1 against total RNA from wild-type (w.t.) and mutant cells, respectively.

FIG. 4. VLPs (visualized by negative staining) were found in sucrose gradient fractions containing ds RNA. VLPs were purified from glycerol-grown wild-type and mutant cells by sucrose density gradient centrifugation (see text). RNA extracted from portions of each fraction of the gradient was size fractionated on nondenaturing agarose gels (A and B). Lanes 1, Size standard (*Hind*III-cut λ DNA); lanes 2 to 16, fractions 2 to 16, respectively, from sucrose gradients;

being its localization by immunocytochemistry. Previous localization by subcellular fractionation (differential centrifugation [5]) gave ambiguous results. Most of the protein cofractionated with the microsomes in a pellet obtained by centrifugation at $100,000 \times g$, but a large part was also found in the soluble fraction. Since the nuclear pellet was contaminated with unbroken cells, it was also unclear whether the 86-kDa protein was present in the nucleus. An unambiguous result was obtained by immunogold labeling of mutant and wild-type cells grown under different conditions. The 86-kDa protein was exclusively cytosolic under all conditions (Fig. 1). Even when it was overexpressed as much as that shown in Fig. 1b (in *por*⁰ cells grown on glycerol for more than 5 days), no gold label was found to be associated with any of the organelles. The discrepancy between this result and that obtained by differential centrifugation suggests that the protein must form aggregates.

The second strategy for identification of the 86-kDa protein is shown in Fig. 2. Large amounts of the 86-kDa protein were purified by HPLC in order to obtain enough material for protein sequencing. Since the NH₂ terminus of the intact protein was found to be blocked, CNBr was used to generate a number of small peptides which could be sequenced. From three peptide sequences that were obtained, one was selected which gave the least ambiguous DNA sequence (see above). The resulting oligonucleotide probe was a mixture of eight oligonucleotide sequences (referred to as oligo1). On Northern blots it recognized two RNA species: a 5-kilobase (kb) RNA which was overproduced about 50-fold in glycerol-adapted *por*⁰ cells and a 10-kb RNA (Fig. 3). Because overproduction of the 5-kb RNA correlated with overproduction of the 86-kDa protein, we were confident that the 5-kb RNA encoded the 86-kDa protein, even though it seemed to be too large. *Escherichia coli* colonies carrying a yeast genomic library in the vector YEp13 were screened with oligo1, but no clones hybridizing to the overproduced RNA were found. Closer investigation revealed that the 5-kb RNA lacked a poly(A) tail, as judged from its inability to bind to oligo(dT)-cellulose, and that it was not an rRNA-precursor, since it failed to hybridize to various rRNA clones.

Another poly(A)⁻ RNA in *S. cerevisiae* of approximately the same size (4.5 to 4.7 kb on nondenaturing gels [17]) is the large (L) double-stranded (ds) RNA found in VLPs known as killer virus. With a size of 78 to 88 kDa, the major coat protein of these VLPs would also match the 86-kDa protein.

To test whether the 86-kDa protein was associated with VLPs, *por*⁰ mutant and wild-type cells were grown on glycerol-containing medium to induce overexpression of the 86-kDa protein and then subfractionated following a protocol for VLP purification. Each fraction of the sucrose gradient was tested for the presence of the 86-kDa protein by dot blots (data not shown) and for the presence of overproduced 5-kb RNA on nondenaturing agarose gels (Fig. 4A and B). The 86-kDa protein peaked in fractions 7 to 11 with the mutant and in fractions 8 to 10 with the wild type, which is in good agreement with the peak fractions of the overproduced 5-kb RNA (note that the 5-kb RNA ran as a 4.3-kb RNA on nondenaturing gels). Portions of fractions 1, 5, 8, 9, 10, and 15 (from *por*⁰ cells) were also negatively stained and examined under an electron microscope. Virions were seen

equivalent amounts were loaded onto each lane. (A) *por*⁰ mutant; (B) wild type; (C) negative stain showing VLPs in sucrose gradient fraction 5 (a) and in the absence of VLPs in fraction 1 (b).

FIG. 5. L-A VLPs and the 86-kDa (86 k) protein gave identical cleavage patterns with staphylococcal V8 protease. Lanes 1 to 7, Coomassie blue-stained gel; lanes 8 to 18, immunoblot incubated with anti-86-kDa protein antibody; lanes 2 to 4 and 9 to 12, digestion of L-A VLPs with 1.0, 0.1, 0.01, 0.001, and 0.0 µg of staphylococcal V8 protease, as indicated; lanes 5 to 7 and 14 to 18, digestion of L-A VLPs with 0.0, 0.001, 0.01, 0.1, and 1.0 µg of staphylococcal V8 protease, as indicated; lanes 1 and 13, molecular weight (mw) standards (phosphorylase *b*, 92,000; bovine serum albumin, 68,000; chicken ovalbumin, 45,000; carbonic anhydrase, 31,000; chymotrypsinogen, 24,000; horse heart cytochrome *c*, 12,000; the 24,000-molecular-weight standard is missing in lane 13).

in all fractions except fraction 1; most of them were found in fraction 5 to 11, and only a few were found in fraction 15. Fractions 1 and 5 are shown in Fig. 4C.

Direct evidence for the identity of the 86-kDa protein was obtained by comparing its proteolytic one-dimensional fingerprint with that of the major protein of purified VLPs. The same fragments appeared in both cases, whether the gel was stained with Coomassie blue (Fig. 5) or blotted onto nitrocellulose and incubated either with anti-VLP antibody or anti-86-kDa antibody (Fig. 5; anti-86-kDa protein antibody). Thus, the 86-kDa protein and the major coat protein of the yeast killer virus are identical.

This result was confirmed by recent sequence data obtained from T. Icho and R. Wickner. All three peptide sequences determined by us were matched by highly homologous regions in the sequence of L-A cDNA (Fig. 6).

VLPs and respiration. Since the 86-kDa protein was encoded by a viral RNA genome rather than by chromosomal

DNA, we could test directly whether growth of porinless cells on glycerol was influenced by this protein. Many yeast strains can be cured on most of their ds RNAs by growth at high temperature (39°C [21]). Since our original porinless mutant (strain HR125-2A) was partly temperature sensitive for growth (it still grew poorly at 37°C but not at all at 39°C), this approach did not work. The few cells that survived at 39°C overproduced the 86-kDa protein instead of losing it (data not shown).

As an alternative approach, we eliminated VLPs from the original *por*⁰ mutant by crossing in the *mak10* mutation, which causes the loss of VLPs from yeast cells (8, 23, 24). Loss of VLPs from the *por*⁰ mutant greatly prolonged the time for adaptation to glycerol. Growth on this carbon source only started after 5 to 10 days (Fig. 7 a and b and Fig. 8, lanes 1 to 4). In addition, the cells grew more poorly even on glucose. To our surprise, elimination of VLPs by the *mak10* mutation impaired mitochondrial respiration even

101	GNAVASHAYNITSWRWYDNHVALLMNMLRAYHLQVLTEQQQYSAGDIPMY	150
	PEPTIDE 3	1 LKAYHLQVLTEQQQY R AG 18
301	MKPVPNCAEGYAWLMHDAVNIPKFGSIRGRYPFLLSGDAALIQATALED	350
1	KPVPN R AEGYAXL I	14 PEPTIDE 1
351	WSAIMAKPELVFTYAMQVSVALNTGLYLRRVKKTGFGTTIDDSYEDGAFL	400
1	AKPELVFTYA I	11 PEPTIDE 2

FIG. 6. Comparison of the L-A VLP DNA and 86-kDa peptide sequences. Bold letters indicate mismatches; underlined mismatches might have been caused by sequencing errors.

TABLE 2. Segregation of a glycerol-negative phenotype in crosses with *mak10* or *mak3* strains

Cross	% Glycerol positive	% Glycerol negative	% <i>mak10</i> that were glycerol negative	No. of tetrads analyzed
Theory (wild type $\times$ <i>mak</i>)	75	25	50	
2D $\times$ 2107	80	20	47	15
2D $\times$ 297	80	20	46	15
Theory (<i>por</i> ⁰ $\times$ <i>mak</i>)	38	62		
2A $\times$ 2107	40	60	ND ^a	11
2A $\times$ 297	38.5	61.5	ND	7

^a ND, Not determined.

with porin-containing cells (Fig. 7 c and d and Fig. 8, lanes 5 to 9). To better measure the respiratory defect, we also monitored growth in liquid medium with glycerol as the sole carbon source (Fig. 9). All strains eventually started to grow; but growth of *mak10* mutants 12C, 25B, and 25D was significantly delayed. However, the porin-containing *mak10*

mutant 12D as well as parent strain 2107 did not exhibit the same respiration defect. Further analysis (growth on glycerol and immunoblots to detect the 86-kDa protein and porin) of tetrads obtained from the cross HR125-2D (wild type) $\times$ 2107 (*mak10*) showed that 20% of the offspring were both *mak10* and negative for growth on glycerol (Table 2). This indicates that the effect of the *mak10* mutation on respiration is only seen in the presence of a second unknown nuclear gene *x*, which stems from the genome of HR125 strains. A cross between 25B and 25D would therefore give a diploid that was homozygous for both *mak10* and gene *x* and that should only lead to respiratory-deficient offspring. This was indeed the case (data not shown). Thus, elimination of both porin and VLPs does not have the same effect in all yeast strains. In addition, when the VLP-less strain 1937 was rendered *por*⁰ by gene conversion, its growth on glycerol at 30°C was not altered, indicating that the need for porin and VLPs can be bypassed in some genetic backgrounds.

Since the respiratory defect associated with some *mak10* mutants could be caused by a specific function of the *MAK10* gene rather than a function of VLPs, the same experiments

FIG. 7. Growth on glycerol is impaired after *mak10*-induced loss of VLPs. (a) Immunoblot showing segregation of *mak10* and *por*⁰ in a cross between HR125-2A (*por*⁰) and 2107 (*mak10*). Lanes 1 to 4, Strains 12A, 12B, 12C, and 12D four siblings of a tetrad, respectively; strains 12C and 12D lost most of their 86-kDa proteins because of the *mak10* mutation (small amounts were still present but could not be cured, were not inducible, and probably stemmed from an unrelated protein); lane 5, HR125-2A (*por*⁰); lane 6, HR125-2B (wild type [w.t.]). In the blot showing the 86-kDa protein, there was 20 μ g of total yeast protein per lane; in the blot showing the 29-kDa porin, there was 30 μ g of total yeast protein per lane. Protein was extracted from glycerol-grown cells. (b) Slower adaptation to glycerol of the *mak10 por*⁰ sibling 12C (growth after 4 days). (c) Immunoblot showing segregation of *mak10* in a cross between HR125-2D (wild type) and 2107 (*mak10*) (25A, 25B, 25C, and 25D, which are four siblings of tetrad given in lanes 1 to 4, respectively). (d) Growth of siblings 25A, 25B, 25C, and 25D on glycerol after 3 days.

FIG. 8. *mak10*-induced loss of the 86-kDa protein was accompanied by loss of the L-A RNA. A Northern blot with total RNA (20 µg per lane) after hybridization with oligo1 for detection of the L-A RNA (A) and the same blot after methylene blue staining showing rRNAs as a control (B) are shown. Lanes 1 to 4, Siblings 12A, 12B, 12C, and 12D, respectively (cross HR125-2A *por*⁰ × 2107 *mak10*); lanes 5 to 8, siblings 25A, 25B, 25C, and 25D (cross HR125-2D wild type × 2107 *mak10*), respectively; lane 9, parent 2107 *mak10*.

were performed with *mak3* as well. *mak3* is another mutation that causes strains to have no ability to maintain L-A VLPs (24). Also, when the *mak3* mutation was introduced into the background of HR125 strains, it caused the strains to have an inability to grow on glycerol (Table 2). This supports the hypothesis that one function of VLPs might be to contribute to full respiratory competence.

One of the functions encoded by the yeast viral RNA is the production of killer toxin, a dimer consisting of 9- and 9.5-kDa polypeptides (18). The toxin is produced only by strains carrying M ds RNA in addition to L ds RNA and enables them to inhibit growth of sensitive nonkiller strains. If the original *por*⁰ mutant were derived from a killer strain (a

strain carrying both L and M RNAs), stress from glycerol might lead to the induction of not only L but also M RNA synthesis and overproduction of killer toxin. To test this, mutant and wild-type strains that were pregrown on glucose or glycerol were streaked onto a lawn of a killer-sensitive tester strain (5×47). The killer-positive control (a strain, 1101, containing both L and M RNAs) produced a larger halo, whereas the negative control (5×47) as well as the wild-type strain HR125-2B and the isogenic *por*⁰ cells did not form halos (data not shown). The porinless mutant is thus not a K₁ killer strain.

DISCUSSION

VLPs containing L ds RNA of about 4.7 kb are found in most strains of *S. cerevisiae*, but only a minority of them have the killer phenotype; this phenotype is caused by a second toxin-encoding ds RNA (M ds RNA; 1.5 to 1.9 kb) which allows the cells bearing it to kill sensitive nonkiller strains and to be immune to their own toxin (18, 23). At least three different classes of L RNAs are known (L-A, L-B, and L-C) which encode different forms of the viral coat protein. L-B and L-C are homologous but are unrelated to L-A, which is the most abundant form. Apart from the fact that L RNA is necessary for the maintenance of M RNA, no phenotype was found for the host cells that carried it. The presence of at least one species of L RNA in so many different yeast strains is surprising, since infection of cells with VLPs is only possible after removal of the cell wall or during mating (6).

The 86-kDa protein that accumulated in *por*⁰ cells is a virus protein. In this study we showed that the 86-kDa protein that accumulated in porinless yeast cells is the major coat protein of the killer virus. Since the peptides obtained by staphylococcal V8 protease digestion of the 86-kDa protein and of a coat protein derived from cells carrying L-A RNA were identical, our 86-kDa protein was apparently encoded by L-A RNA. This was confirmed by a comparison of the amino acid sequences of three CNBr-cleaved peptides with the DNA sequence of cDNA clones made from L-A RNA (T. Icho and R. Wickner, personal communication). The average homology between the two sequences was at least 86%; the mismatches might reflect the existence of different variants of the L-A RNA (23).

Is there a new function for VLPs? Our finding that respiratory-deficient *por*⁰ mutant cells dramatically overproduce a viral coat protein was unexpected and raised the question of whether there is a link between VLPs and respiration. Even in wild-type strains, L RNA synthesis is stimulated up to threefold on medium containing ethanol, which is a nonfermentable carbon source (15). However, porinless mutant cells adapted to nonfermentative growth accumulated the protein at 50-fold higher levels than wild-type cells. In these mutant cells the 86-kDa protein represented about 10 to 20% of the total cell protein. Overproduction of the VLPs could indicate a stress response of the virus itself (maybe an evolutionary remnant from the times when the virus was still infectious), in which case no benefit for the host would be expected. Indeed, some yeast strains lacking both mitochondrial porin and L ds RNA grew on glycerol, indicating that in their genetic background neither VLPs nor porin is essential for respiration and might be replaced by host factors with similar functions. However, in other yeast strains (e.g., HR125-2A), deletion of porin led to a more pronounced phenotype: no growth on glycerol for at least 3 days. Loss of L RNA from porinless cells not only prolonged the lag for

FIG. 9. Growth curves of cells in rich medium containing glycerol. (A) Growth curves of strains 12A (*por*⁰), 12B (wild type), 12C (*por*⁰ *mak10*), 12D (*mak10*), 2B (HR125-2B wild type), 2A (HR125-2A *por*⁰). (B) Growth curves of strains 25A (wild type), 25B (*mak10*), 25C (wild type), 25D (*mak10*), and 2107 (*mak10*).

growth on glycerol but also slowed down growth on glucose. Even in some wild-type strains (e.g., HR125-2D) *mak10*-induced loss of VLPs correlated with respiratory deficiency; in these VLP-less cells the mitochondrial genome was retained, but growth on glycerol started only after a lag, despite the presence of porin. The same effect was also produced by *mak3*, another mutation that led to the loss of VLPs. In some genetic backgrounds the presence of VLPs thus confers an advantage to the host and stimulates adaptation to nonfermentable carbon sources, indicating a connection between VLPs and respiratory functions.

Since our immune localization studies did not reveal any association between VLPs and mitochondria, even when VLPs were overproduced, we must think of other ways to explain how the presence of VLPs can influence the mitochondrial respiratory system. Since killer toxin acts by making the plasma membrane leaky (18), a killer toxin precursor that is overproduced in *por⁰* cells could perhaps create additional mitochondrial pores. This seems unlikely, since the original porinless mutant was derived from a nonkiller strain. The 86-kDa protein itself does not have any pore-forming activity (M. Dihanich and R. Benz, unpublished data).

We consider it more likely that VLPs interact with mitochondria indirectly, e.g., by affecting the expression of nuclear genes for mitochondrial proteins or by competition between regulatory sequences of VLP and mitochondrial genes for a binding protein. The *MAK10* product could be such a protein (22).

If one assumes that the respiratory defect caused by the loss of porin is caused by the impaired traffic to a regulatory molecule (e.g., heme or Ca) across the outer mitochondrial membrane, leading to slower induction of the biosynthesis of respiratory proteins on nonfermentable carbon sources, one can imagine the interaction of a molecule like this with a VLP- or nuclear-encoded (*MAK10*?) regulatory protein. Higher amounts of VLPs would stimulate more efficient formation of such a regulatory complex. Loss of VLPs would increase the need for the regulator molecule, and therefore the time lag for induction of mitochondrial proteins.

An interaction like this need not be caused by the 86-kDa protein itself. With a length of almost 5 kb, the L-A RNA can potentially encode proteins with a total size of 167 kDa; only half of this coding capacity is used by the coat protein. Recent results by Esteban et al. (7) and Fujimura and Wickner (10) indicate that L-A encodes a second, larger polypeptide whose sequence partially overlaps that of the 86-kDa protein. Biochemical and genetic data (7, 9, 10, 15, 17, 25) suggest that there is an association between an RNA-dependent RNA polymerase and a single-stranded RNA-binding activity with VLPs and the existence of at least three genetic variants of L-A ds RNA. The full sequence of the L-A RNA will be available soon (T. Icho and R. Wickner, personal communication); it should help to determine those regions of the RNA that mediate the unexpected effects describe here.

ACKNOWLEDGMENTS

We are grateful to G. Schatz, in whose laboratory most of this work was carried out. We also thank R. Wickner, T. Icho, and T. Fujimura for sending us anti-VLP antibodies, purified VLPs, and a series of useful yeast strains and for communicating results before publication; H. Riezman, in whose laboratory the immuno-EM studies were done; P. Wagner, and M. Hall for helpful discussions;

and K. Suda, W. Oppliger, and D. Aquila for excellent technical assistance.

This study was supported by grants 3.335-0.86 and 3.139-1.85 from the Swiss National Science foundation and Public Health Service grant GM 37803 from the National Institutes of Health.

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