Overproduction of Yeast Viruslike Particles by Strains Deficient in a Mitochondrial Nuclease

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Saccharomyces cerevisiae strains are often host to several types of cytoplasmic double-stranded RNA (dsRNA) genomes, some of which are encapsidated by the L-A dsRNA product, an 86,000-dalton coat protein. Here we present the finding that nuclear recessive mutations in the *NUC1* gene, which encodes the major nonspecific nuclease of yeast mitochondria, resulted in at least a 10-fold increase in amounts of the L-A dsRNA and its encoded coat protein. The effect of *nuc1* mutations on L-A abundance was completely suppressed in strains that also hosted the killer-toxin-encoding M dsRNA. Both *NUC1* and *nuc1* strains containing the L-A genome exhibited an increase in coat protein abundance and a concomitant increase in L-A dsRNA when the cells were grown on a nonfermentable carbon source rather than on glucose, an effect independent of the increase in coat protein due to *nuc1* mutations or to the absence of M. The increase in L-A expression in *nuc1* strains was similar to that observed in strains with mutations in the nuclear gene encoding the most abundant outer mitochondrial membrane protein, porin. *nuc1* mutations did not affect the level of porin in the mitochondrial outer membrane. Since the effect of mutations in *nuc1* was to alter the copy number of the L-A coat protein genome rather than to change the level of the M toxin genome (as do *mak* and *ski* mutations), these mutations define a new class of nuclear genes affecting yeast dsRNA abundance.

Most *Saccharomyces cerevisiae* strains are host to one or more double-stranded RNAs (dsRNAs) (2, 35; for a review, see reference 39). The L-A and M dsRNAs are associated with cytoplasmic virus-like particles and are responsible for conferring the killer phenotype (2, 30). Killer strains secrete a toxin encoded on dsRNAs of the M type (1.8 or 1.5 kilobases [kb]) that can kill sensitive yeast strains (3, 4). The coat proteins that encapsidate the dsRNAs are encoded on the separate L-A genome, which is 4.5 kb in length (1, 19, 20, 31). That host functions are necessary for maintenance and propagation of the dsRNAs is evidenced by the myriad of nuclear genes defined by mutations that eliminate toxin production (*MAK* [maintenance of killer]) (1, 36, 40) or enhance the killer phenotype (*SKI* [superkiller]) (27, 33). *mak* mutations result in the loss of either the toxin-encoding M genome alone or both the L and M genomes, whereas *ski* mutations result in an increased copy number of the toxin-encoding M genome and therefore bestow an enhanced killer phenotype.

Here, we present the finding that mutations in a nuclear gene resulted in an increased copy number of the L-A type genome and an overabundance of one product of that genome, the 86-kilodalton (kDa) coat protein. Furthermore, we show that the L-A copy number increased in mutant strains only when M, the toxin-encoding genome, was absent. These nuclear mutations are in the *NUC1* gene, which encodes the major nonspecific nuclease in yeast mitochondria (5, 34, 41). We created such a mutation in the course of our studies on *CBP1*, which is adjacent to *NUC1* and encodes a factor necessary for the stability of mitochondrial cytochrome *b* mRNA (8–13). Mutations in another nuclear gene, *POR*, which encodes the mitochondrial outer membrane protein porin, result in a phenotype similar to that of the *nuc1* mutant strains. *por* mutant strains also overproduce the 86-kDa viruslike particle coat protein (14, 15). We present evidence that *nuc1* mutations do not affect the level

of mitochondrial porin, that *nuc1* mutations are recessive with respect to the overproduction of L-A, and that L-A copy number and coat protein abundance remain sensitive to carbon source regulation in the *nuc1* background.

MATERIALS AND METHODS

Yeast strains. All of the insertion and deletion-insertion mutations shown in the figures were made in LL20 (α [*rho*⁺] *leu2-3 leu2-112 his3-11 his3-15*) (25). The Δ KG strain, mentioned only in the text, is a *cbp1* derivative of the DCL strain (13) (α [*rho*⁺] *ade1 leu2-3 leu2-112*). *CBP1* sequences are deleted in Δ KG from the *KpnI* site to the *BglII* site in the coding sequence, i.e., positions +55 to +977 relative to the AUG start codon (9). The inserted sequences include both the *LEU2* gene as a 2,200-base-pair *BglII* fragment and a 700-base-pair *KpnI-BglII* adapter fragment derived from plasmid sequences. The A12 strain (α [*rho*⁺] *trp1 ura3-52 leu2-3 nuc1-1::LEU2*) was obtained from H. P. Zassenhaus, St. Louis University, St. Louis, Mo. CB11 (32) is a *Saccharomyces carlsbergensis* strain (α [*rho*⁺] *ade1*).

In experiments in which *cbp1* strains were compared with *CBP1* wild-type strains, the [*rho*⁺] mitochondrial genomes were eliminated from all of the strains by ethidium bromide mutagenesis (7). A mitochondrial genome carrying a suppressor of *cbp1* was introduced into each strain via cytoduction from a *karl* strain harboring the [*rho*^{int4-35}] DNA (8). This replacement procedure allowed the growth of both wild-type *CBP1* strains and normally respiratory-deficient *cbp1* strains on glycerol medium.

The A12WT strain was made by replacement of the disrupted *NUC1* gene with the wild-type yeast nuclear sequence (29), which was originally isolated as a 6.7-kb fragment selected from a YEp13 library by complementation of a *cbp1* mutation (12). A total of 10⁸ A12 cells were transformed with 3 μ g of the linear 6.7-kb fragment and plated in WO medium (see below) containing 1.2 M sorbitol, 2% agar, tryptophan, uracil, and leucine (24). Colonies derived from cells surviving the procedure were transferred

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to WO plates containing tryptophan, uracil, and leucine and were replicated onto plates lacking leucine. Of 400 colonies, 2 were *leu*⁻. One of these transformants was named A12WT.

The A12-LA and A12-O strains were derived from A12 by growing the strain at an elevated temperature (37). A12 was grown in 2 ml of YPD (see below) at 39°C for 2 days. The culture was diluted, spread on YPD plates, and incubated at 30°C for 2 days. Single colonies were chosen for analysis of dsRNA. An isolate which lost M dsRNA was named A12-LA, and another isolate which lost both M and L-A dsRNAs was named A12-O. A12WT was also heat treated to yield the derivative strain A12WT-LA, which retains the L-A dsRNA and has lost the M genome.

Media. Yeast strains were grown on either YPD, YEPG, or WO. YPD is 2% peptone, 1% yeast extract, and 2% glucose. YEPG is 2% peptone, 1% yeast extract, and 3% glycerol. WO is 0.67% nitrogen base without amino acids and 2% glucose. Solid media included 2% agar, and amino acid supplements were added at 20 µg/ml (except for leucine, which was added at 30 µg/ml). Top agar used to plate spheroplasts from the transformation procedure contained 1.2 M sorbitol.

Whole-cell protein extracts and Laemmli gels. Yeast strains were grown in 2 ml of YPD or YEPG to stationary phase. After being washed with water, the cells were vortexed for 2 min in 0.1 ml of freshly diluted 20% trichloroacetic acid and 0.1 ml of glass beads (425 to 600 µm; Sigma Chemical Co.). The suspended precipitate was decanted, the protein concentration was determined by the bicinchoninic acid method (Pierce Chemical Co.), and 100 µg of protein from each sample was pelleted and suspended in 5 µl of 4× Laemmli sample buffer (0.25 M Tris chloride, [pH 6.8], 40% glycerol, 20% β-mercaptoethanol, 0.024% bromophenol blue, 8% sodium dodecyl sulfate)–3 µl of 1.0 M Tris chloride (pH 8.5)–10 µl of water. The suspensions were heated at 100°C for 3 min, and half of the volume (50 µg of protein) was loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel (22).

Differential centrifugation procedure. The yeast strains were grown to stationary phase on liquid YEPG medium, harvested by centrifugation at 2,500 × *g* for 5 min, and washed once with 1.2 M sorbitol. Spheroplasts were prepared by digestion with zymolyase (ICN Pharmaceuticals Inc.) (17) and lysed by treatment with 0.6 M sorbitol containing the following protease inhibitors: 1 mM each anti-pain, leupeptin, pepstatin, chymostatin, aprotinin, and phenylmethylsulfonyl fluoride. The lysed spheroplast mixture was centrifuged at 700 × *g* for 10 min to remove the cell debris. The supernatant was centrifuged at 25,000 × *g* for 10 min to pellet the mitochondria. The supernatant from this spin was centrifuged at 100,000 × *g* for 1 h to collect the pellet fraction containing the viruslike particles (23).

dsRNA preparation. dsRNA was prepared from whole cells by the method of Fried and Fink (18).

Outer mitochondrial membrane preparations. One-liter cultures of yeast were harvested by centrifugation and washed with 1.2 M sorbitol. Mitochondria were prepared from lysed spheroplasts (6), washed twice with 0.5 M sorbitol, and suspended in 10 ml of 10 mM Tris chloride (pH 8.0)–0.1 mM EDTA (TE) and 1 mM each of the protease inhibitors mentioned above. The mitochondria were sonicated three times for 5 s each, and the sonicate was centrifuged at 81,000 × *g* for 1 h. The pellet was suspended in 1 ml of TE, loaded onto a 30 to 50% linear sucrose gradient, and centrifuged at 53,000 × *g* for 15 h. The outer membrane fraction was collected from the top of the gradient, diluted 10-fold with TE, and sedimented at 118,000 × *g* for 1 h. The

FIG. 1. Overexpression of an 86-kDa protein in a yeast deletion strain. Yeast cells were grown to stationary phase on YEPG (rich glycerol medium), and total cell protein was extracted by vortexing the cells in 20% trichloroacetic acid with glass beads. A 50-µg sample of protein from each extract was neutralized, electrophoresed on a 10% Laemmli gel, and stained with Coomassie brilliant blue. Lanes: 1, wild-type strain LL20; 2, ΔB3 (LL20 with a deletion extending 900 base pairs upstream and 500 base pairs downstream of *CBP1*); 3, CP1L, LL20 with an insertion mutation (*LEU2*) at the *Pst*I site in the *CBP1*-coding sequence.

pellet was suspended in TE containing protease inhibitors and frozen at –80°C. Samples were prepared for electrophoresis from thawed samples.

RESULTS

Overexpression of an 86-kDa protein in a *CBP1* deletion strain. In the course of our studies of the *CBP1* gene (9, 10, 12), we created a deletion-insertion mutation that removed not only the *CBP1*-coding sequence but 900 base pairs of upstream sequence and 500 base pairs of downstream sequence as well. The deleted sequences were replaced with the selectable marker *HIS3*. When a whole-cell protein extract from this *CBP1*⁻ strain (ΔB3) was compared with those of *CBP1*⁺ strains on a Coomassie-stained Laemmli gel, it was observed that an 86-kDa protein was expressed at a level of several percent of total cell protein in the *CBP1*⁻ lane (compare lanes 1 and 2 in Fig. 1).

Further investigation showed that neither insertion mutations in *CBP1*, such as that carried by the CP1L strain (Fig. 1, lane 3), nor a 900-base-pair deletion of the *CBP1*-coding sequence, as in strain ΔKG (data not shown), resulted in the overexpression of the 86-kDa protein. These results suggested that alteration of a region adjacent to *CBP1*, and not

FIG. 2. Expression of the 86-kDa protein is suppressed in *trans*. The haploid parent and diploid product strains were grown and harvested as detailed for Fig. 1. A 50- μ g sample of total cell protein was electrophoresed on a 10% Laemmli gel and stained with Coomassie blue. Lanes: 1, Δ B3; 2, a diploid strain from the cross of Δ B3 to CB11 (a wild-type yeast strain); 3, CB11.

disruption of *CBP1* itself, was responsible for the overexpression of the 86-kDa protein.

Overexpression is complemented in *trans*. To investigate the possibility that the *HIS3* insertion (in the same transcriptional orientation as *CBP1*) might, in conjunction with *CBP1*-flanking sequences, be responsible for the overexpression of the 86-kDa protein, we created two additional strains with deletions equivalent to that in Δ B3; however, the deleted region was replaced with the *LEU2* gene in either orientation. Regardless of insert type or orientation, all three deletion strains overexpressed the 86-kDa protein (data not shown). This result suggested that there were no *cis* effects of the *LEU2* or *HIS3* inserts and indicated that the overexpression of the 86-kDa protein was a direct result of the deletion itself.

To determine whether the deletion was of the *cis*-dominant type (which would suggest that the gene for the 86-kDa protein was adjacent to *CBP1* and was activated by the deletion) or of the *trans*-recessive type (which would suggest that the deletion disturbs a gene encoding a protein that has a negative effect on the expression of the 86-kDa gene), the dominance of the deletion mutation was tested. The Δ B3 strain was crossed to CB11, a wild-type strain of the opposite mating type. Expression of the 86-kDa protein was suppressed in the diploid strain (Fig. 2). This result showed that the deletion mutation is recessive and suggested that a gene adjacent to *CBP1* negatively regulates the expression of the 86-kDa protein encoded at another location in the

FIG. 3. Disruption of a gene downstream of *CBP1* causes overexpression of the 86-kDa protein. Insertion and deletion mutations were constructed and transformed into yeast cells by the method of gene replacement (29). A 50- μ g sample of protein from each strain was prepared as described for Fig. 1 and fractionated on a 10% Laemmli gel. Lanes: 1, wild-type LL20; 2, B2L (strain with insertion mutation in *URF*); 3, B13L (strain with deletion from the 3' end of *URF* through the 5' end of *CBP1*); 4, CP1L (strain with insertion mutation in *CBP1*); 5, CNBL (strain with deletion extending from the 3' end of *CBP1* into the 3' end of *NUC1*); 6, HNUC (strain with insertion mutation in *NUC1*); 7, Δ B3 (strain with original deletion mutation extending from the 3' end of *URF* through all of *CBP1* and including the 3' end of *NUC1*).

genome. We decided to direct our next studies toward defining the gene adjacent to *CBP1* that was responsible for suppressing the production of the 86-kDa protein.

Disruption of a gene downstream of *CBP1* mimics the large deletion. To determine whether the gene controlling the production of the 86-kDa protein was upstream or downstream of *CBP1*, a series of insertion and deletion mutations was created by gene replacement (29). Mutations disturbing the region 3' to *CBP1* resulted in the overexpression of the 86-kDa protein (Fig. 3). As the DNA sequence of this region was not known, the *Pst*I-*Bam*HI fragment spanning the 3' end of *CBP1* and extending to the right endpoint of the large deletion in Δ B3 was sequenced.

The downstream gene is *NUC1*. A continuous open reading frame approaches that of *CBP1* by 230 base pairs on the opposite strand (Fig. 4). At the time this sequence was

+1765 Leu Gln Ile Gly Arg Thr Asp Ile Phe Leu Trp Ala Cys Ala Glu Leu Tyr Gln Asn Gly Met Thr Ile Glu Glu Leu Lys Leu Asp Trp Asn Phe
 CTG CAG ATA GGA AGA ACA GAC ATT TTT CTT TGG GCG TGC GCA GAA CTA TAC CAG AAC GGT ATG ACG ATT GAG GAA TTG AAG TTA GAC TGG AAT TTC
 GAC GTC TAT CCT TCT TGT CTG TAA AAA GAA ACC CCG ACG CGT CTT GAT ATG GTC TTG CCA TAC TGC TAA CTC CTT AAC TTC AAT CTG ACC TTA AAG
 •

+1861 Ile Leu Lys His Gln Ile Arg Asn Ser Glu Phe Lys Thr Asn Lys Glu Ile Ile Gln Asp Ile Lys Lys His Gly Val Ser Ala Val Lys Arg Tyr
 ATC TTA AAA CAT CAA ATT AGA AAT TCA GAG TTC AAA ACA AAC AAG GAG ATC ATA CAA GAT ATT AAA AAG CAT GGT GTG TCG GCT GTC AAA CGT TAC
 TAG AAT TTT GTA GTT TAA TCT TTA AOT CTC AAG TTT TGT TTG TTC CTC TAG TAT GTT CTA TAA TTT TTC GTA CCA CAC AGC CGA CAG TTT GCA ATG

+1957 Leu Arg TER
 TTA AGA TGA ACGGCCAAACGACATTACGATATAAATAACAAAGCAAAATAAACAAAA CAAATTTCTGTAAATATATACTAAATCCAAAGCATTGCATCCTAATCTTTCTTTTGTATC
 AAT TCT ACT TCCCGGTTTGGTGTAAATGCTATATTTATTGTTTCTTTATTATTGTTTT GTTTAAAGACATTATATATGATTAGGTTTGTAAAGCATTAGGATTAAGAAAAAGAAAAAACTAG

+2080 ATCAAAATAAATGATTTATTTATGTTATGCTGCTAGACTTGTATATTCTTCATGCTATTC TATCGTTGTGTTATGAATTAATTTATTTACAGTTTTCAGTACAT TCA ATT CCT TTT
 TAGTTTATTTACATAATAAAATACAAATATACCAATCTGAACAATATAAGCAAGTACGATAAG ATAACAACCAATACTTAATTAATATAAATGTCAAAAAGTCACTATA AOT TAA GGA AAA
 TER Asn Arg Lys

+2202 TTT TGG AGG AGG TAA CAA TTT CAC ATC TTT CGA TTG TTT GAT GCG CCG GTT AGA GAA ATC TCT CAC TAC AAT TTG ACA ATT TAC CTC TTT GCA TAA
 AAA ACC TCC TCC ATT GTT AAA GTG TAG AAA GCT AAC AAA CTA GCG GCG CAA TCT CTT TAG AGA GTG ATG TTA AAC TGT TAA ATG GAG AAA CGT ATT
 Lys Pro Pro Pro Leu Leu Lys Val Asp Lys Ser Gln Lys Ile Ala Ala Asn Ser Phe Asp Arg Val Val Ile Gln Cys Asn Val Glu Lys Cys Leu

+2298 TGC CTT CTT CTT TGA AGG TGG TAC TTT TTG CAG AAG TTC TAG CCC AGT ACT TCT CTC TAA AOC ATC TAT AGG AAC TTC AAA GTC AGT CAA TTT CGT
 ACG GAA GAA GAA ACT TCC ACC ATG AAA AAC GTC TTC AAG ACT GCG TCA TGA AGA GAG ATT TCG TAG ATA TCC TTG AAG TTT CAG TCA GTT AAA GCA
 Ala Lys Lys Lys Ser Pro Pro Val Lys Gln Leu Leu Glu Leu Gly Thr Ser Arg Glu Leu Ala Asp Ile Pro Val Glu Phe Asp Thr Leu Lys Thr

+2394 CTC ATT TGA TAT CCG TTC GTT TGG CAA TAC AAA TCC CCG GAC AOC AAT ATC CTC TCT AOC AGG ATT GGC TGT TCC TCC TTC TCC AAC AAT CAA TTT
 GAG TAA ACT ATA GCC AAG CAA ACC GTT ATG TTT ACG GCG CTC TCG TTA TAG GAG AGA TCG TCC TAA CCG ACA ACG AAG AAG AAG TTT TTA GTT AAA
 Glu Asn Ser Ile Pro Glu Asn Pro Leu Val Phe Ala Ala Val Ala Ile Asp Glu Arg Ala Pro Asn Ala Thr Pro Ala Glu Ala Val Ile Leu Lys

+2490 AAA AAA GTG COT TGG AAC AOC AAT ACT GCG TGG ATT GCC AAT AAC TTC ATA ATT AAC CCT AAA TTT ATT ATC TAT GCG ATC C
 TTT TTT CAC GCA ACC TTG TCG TTA TGA CCC ACC TAA CCG TTA TTG AAG TAT TAA TTG GGA TTT AAA TAA TAG ATA CCC TAG G
 Phe Phe His Thr Pro Val Ala Ile Ser Pro Pro Asn Gly Ile Val Glu Tyr Asn Val Arg Phe Lys Asn Asp Ile Pro Asp

FIG. 4. DNA sequence of the *Pst*I-*Bam*HI fragment containing the convergent 3' ends of *CBP1* and *NUC1*. The *Pst*I-*Bam*HI fragment was subcloned from the T10 plasmid (12) into the multiple cloning site of the plasmids pBS(-) and pBS(+) (Vector Cloning Systems). The sequence was determined in both directions by the Sanger method as modified for the use of Sequenase (United States Biochemical). The sequence from +1765 to +1962 is *CBP1*. The sequence from +2571 to +2193 is *NUC1*. The extent of the *CBP1* sequence previously published by Dieckmann et al. (9) (*) and the extent of overlap by the *NUC1* sequence previously published by Vincent et al. (34) (●) are indicated. The nucleotides are numbered by the method of Dieckmann et al. (9).

obtained it did not match sequences in the Dayhoff bank. However, shortly thereafter the new coding sequence was fortuitously matched to that of *NUC1* (34) when it was observed that the restriction map of the *CBP1* region matched that of *NUC1* (9, 12, 41). *NUC1* encodes the major nonspecific nuclease in the inner mitochondrial membrane (5).

Mitochondrial nuclease- and porin-deficient strains overexpress the 86-kDa yeast dsRNA killer virus coat protein. It was not a complete surprise that the mutation causing the 86-kDa-protein overexpression phenotype was in a gene encoding a mitochondrial function, since it had been reported by Dihanich et al. (14) that an 86-kDa protein was overexpressed in strains that had deletions in the gene for the outer mitochondrial membrane protein porin. A generous gift of antiserum to the 86-kDa protein and a fraction purified from a porin-deficient (*por*⁻) strain was sent to us by Melitta Dihanich. It was determined by Western (immuno-) blotting of equivalent fractions from the *por*⁻ and $\Delta B3$ strains that the 86-kDa proteins were the same. Additionally, digestions with the *Staphylococcus aureus* protease V8 produced peptide products of the same molecular weights on Laemmli gels (data not shown). We concluded that production of the 86-kDa protein is sensitive to some aspect of cellular metabolism which is altered by the *nuc1* and *por* mutations.

As chronicled in a recent publication, Dihanich et al. sought to identify the 86-kDa protein and the gene encoding it (15). They determined that the 86-kDa protein is, surprisingly, the coat protein of the yeast double-stranded RNA viruslike particle, which is a product of the L-A dsRNA itself (31) rather than the product of a yeast nuclear gene. To confirm the identity of the 86-kDa protein in *nuc1* strains, we obtained antiserum to a highly purified preparation of virus coat protein from Reed Wickner of the National Institutes of

Health (19). The 100,000 $\times g$ pellet fraction prepared by differential centrifugation from lysed spheroplasts of the $\Delta B3$ strain and the *por*⁻ strain contained an immunoreactive protein of 86,000 daltons (Fig. 5). (Yeast viruslike particles sediment with the microsomal membrane-enriched fraction, i.e., the 100,000 $\times g$ pellet fraction [23].) The lower-molecular-weight bands appeared to be proteolytic products of the 86-kDa coat protein that accumulated during the differential centrifugation procedure, since even brief incubations of these preparations at higher temperatures resulted in the conversion of the 86-kDa protein to a distinct set of smaller peptide fragments. The $\Delta B3$ sample was somewhat more degraded than the *POR*⁻ sample, which resulted in the detection of the additional 34-kDa fragment. These results led to the conclusion that the 86-kDa protein that is overexpressed in both *por* and *nuc1* strains is the cytoplasmic viruslike particle coat protein. Since the major viral coat protein is encoded by the L-A dsRNA (31), the *POR* and *NUC1* gene products must, directly or indirectly, have a negative effect on coat protein abundance. It is possible that *por* mutations affect the level of the mitochondrial nuclease encoded by *NUC1* or that *nuc1* mutations affect the level of porin in the outer mitochondrial membrane and that one of these two deficiencies has an effect on the abundance of the 86-kDa protein.

Porin levels are normal in *nuc1* mutant strains. To investigate whether the observed phenotype of the *nuc1* mutants could be due to a deficiency in porin, outer membranes from mitochondria of *NUC1* wild-type and *nuc1* mutant strains were prepared (6). Since porin is the most abundant protein in the outer membrane, Coomassie blue staining was sufficient to determine whether the level of porin in *nuc1* strains was different from that in the wild type. The levels of porin were equivalent in the four strains tested, two of which carried *nuc1* mutations (Fig. 6). Therefore, the effect of *nuc1*

FIG. 5. The 86-kDa protein overexpressed in $\Delta B3$ and *por*⁻ is the yeast dsRNA viruslike particle coat protein. The $\Delta B3$ strain was grown to stationary phase in rich glycerol medium, and the cell walls were digested with zymolyase. The cells were lysed with hypotonic buffer and fractionated by differential centrifugation. Supernatant from a $30,000 \times g$ spin was centrifuged at $100,000 \times g$ for 1 h. A 5- μ g sample of protein from the $100,000 \times g$ pellet fraction was electrophoresed on a 10% Laemmli gel adjacent to 5 μ g of a similar $100,000 \times g$ fraction from the *por*⁻ strain obtained from M. Dihanich (14). Duplicate sets of samples were either stained with Coomassie blue or blotted to nitrocellulose. The blot was incubated with rabbit antiserum prepared to a pure preparation of the coat protein of the dsRNA viruslike particle of yeast cells (19). The antibody-protein complexes were visualized by a secondary incubation in ¹²⁵I-labeled protein A and exposure of the blot to X-ray film. Lanes: 2 and 4, $\Delta B3$; 1 and 3, *por*⁻ (strain with a deletion in the gene encoding mitochondrial porin) (14).

mutations on virus coat protein production appears to be independent of porin production. Whether the *por* mutations affect the level of the *nucl* gene product has not yet been tested.

Overexpression of coat protein depends on the absence of M-type dsRNA. To verify our finding that mutations which disrupt the *nucl* gene cause overexpression of the 86-kDa coat protein, we examined the phenotype of the *nucl* strain, A12, sent to us for use in the preparation of nuclease-deficient extracts from yeast mitochondria (5). The surprising result was that this strain, though carrying a knockout mutation in *nucl* similar to that in our HNUC strain, did not overexpress the virus coat protein. To determine whether A12 did not overexpress viral coat protein because of either a difference in its nuclear background or a difference in cytoplasmic factors, 20 tetrads from an A12 $\times$ HNUC cross were analyzed. Whole-cell protein extracts of all 80 *nucl* spores examined on stained Laemmli gels showed that none of the progeny overexpressed the 86-kDa protein. Whole-cell protein extracts from one such tetrad are shown in Fig. 7A. Thus, the overexpression phenotype segregated 0:4, suggesting that the factor suppressing the overexpression in A12 is cytoplasmic.

It is known that there are distinct regulatory relationships between the various yeast dsRNA species; e.g., the M

FIG. 6. Mitochondrial outer membranes of *nucl* mutant strains. Yeast cells were grown to stationary phase on YEPG and mitochondria were prepared as described previously (17). The mitochondria were sonicated, and the membranes were pelleted by centrifugation and fractionated on sucrose gradients. The outer membranes were recovered from the top of a 30 to 50% gradient after 15 h of centrifugation at $53,000 \times g$. A 50- μ g sample of protein from the outer membrane fraction of each strain was electrophoresed on a 10% Laemmli gel. Lanes: 1, B2L (*URF* mutant); 2, ΔKG (*CBP1* mutant); 3, $\Delta B3$; 4, HNUC (insertion mutation in *NUC1*). B2L and ΔKG are *NUC1* strains, and $\Delta B3$ and HNUC are *nucl* strains.

dsRNA negatively regulates L-A copy number, presumably by titrating out factors necessary for replication of both genomes (for a review, see reference 39). We therefore hypothesized that the cytoplasmic factor(s) suppressing coat protein production in A12 would be a dsRNA in the killer system. To investigate differences in the compositions of the dsRNAs in the A12 and HNUC strains and their progeny, viral RNAs were prepared from the parent strains and representative tetrads (18). The HNUC parent carried only the L genome type (4.5 kb) which encodes the virus coat protein, whereas the A12 parent carried both the L and the two molecular weight species of the M type genome (Fig. 7B). The spores of the two tetrads shown all carried L and at least one of the two M-type genomes. The conclusions drawn from this experiment were that the M genomes derived from the A12 parent segregated to all of the progeny spores and that it is likely that the presence of M dsRNA in these strains suppressed the overexpression of the 86-kDa protein caused by the *nucl* mutation.

To investigate further the effect of the *nucl* mutation and the presence of the M dsRNA on overproduction of the coat protein, we examined coat protein and L-A dsRNA abundance in four strains. Shown in Fig. 7C is a comparison of

FIG. 7. The presence of M dsRNA eliminates the overabundance of the L-A genome and the encoded coat protein in *nuc1* strains. (A) Proteins extracted from *nuc1* strains. A 50- μ g sample of protein extracted from whole cells grown on YEPG was electrophoresed on a 10% Laemmli gel. HNUC, α mating-type strain with an insertion mutation (*HIS3*) in *NUC1*; A12, a mating-type strain with an insertion mutation (*LEU2*) in *NUC1*; A through D, four spores of a tetrad from a cross of HNUC to A12. (B) dsRNAs extracted from *nuc1* strains. Viruslike particle RNAs were prepared (18) from cells grown on YEPG, and aliquots normalized to equivalent amounts of total cell protein were electrophoresed on a 1% agarose gel. The gel was stained with ethidium bromide, destained, and photographed on a UV transilluminator (302 nm). 2A through 2D and 4A through 4D, eight spores of two tetrads from the HNUC $\times$ A12 cross. (C) Proteins extracted from *NUC1* and *nuc1* strains that do or do not contain M dsRNA. Methods were as described for panel A. A12WT, strain made from A12 by replacing the disrupted copy of *NUC1* in A12 with the wild-type yeast nuclear sequence; A12, *nuc1* strain; A12-LA, strain derived from A12 by treatment with heat; A12-O, another strain derived from heat-treated A12; LL20, wild-type *NUC1* parent of HNUC; HNUC, *nuc1* strain. (D) dsRNAs from *NUC1* and *nuc1* strains. dsRNAs were extracted from the strains described for panel C and electrophoresed on a 1% agarose gel. The samples are in the same order from left to right as in panel C.

the amount of coat protein in LL20, the wild-type parent of HNUC, with that in HNUC. The amounts of L-A dsRNA in the HNUC and LL20 strains are shown in Fig. 7D. The comparison showed that the overexpression of the 86-kDa protein in the HNUC strain and other *nuc1* strains was correlated with an increased abundance of the L-A genome encoding the coat protein.

We also compared the levels of coat protein and dsRNA in A12 with those in a derivative of A12, A12WT, in which the insertion mutation in *NUC1* was eliminated by gene replacement with the wild-type sequence (see Materials and Methods) (Fig. 7C). A comparison of this *nuc1* strain with the otherwise isogenic *NUC1* strain showed that levels of the L

genome and the smallest M genome are similar in the two strains (Fig. 7D). The *NUC1* derivative appeared to have lost the larger M-type genome on transformation to the wild type. The conclusion drawn from this experiment was that the *nuc1* mutation has no effect on the production of the 86-kDa protein, nor does it result in the increased abundance of the L-A genome in the A12 strain. The only difference of note between HNUC and A12 and between HNUC and the spores from the cross between A12 and HNUC was the presence of at least one M genome in the progeny and the A12 strain. It seems likely that the M genome suppressed the increase in abundance of the L-A genome that was observed in the HNUC strain.

The complement of dsRNAs in a strain can be manipulated by treatment of the strain with heat (37). To strengthen our conclusion about the suppressive effect of the M genome on the expression of the 86-kDa protein in *nuc1* strains, we heat treated the A12 strain to see whether we could recover a strain that had lost the M-type genomes. As shown in Fig. 7C (proteins) and D (dsRNAs), two derivatives which differed in the production of the 86-kDa protein and the dsRNA L-A encoding that protein were recovered from the heat treatment. A12-LA had lost the M genomes and expressed the L-A genome at a level equivalent to that expressed by the HNUC strain. A12-O appeared to have lost both the L-A and M genomes and therefore could not express the coat protein, since there was no genome encoding it. When 10-fold more total viral RNA than was used for Fig. 7D was applied to an agarose gel, A12-O did have a small amount of an L-sized genome (data not shown). We assume that this is the L-(BC)-type genome, as it has been reported that L-(BC) is found at a lower copy number and is resistant to heat curing (31). Furthermore, the L-A coat protein was not present in this strain, as determined by immunodetection (Western blot data not shown). The results of this experiment support the idea that the presence of an M dsRNA genome in a *nuc1* strain completely suppresses the increase in L-A genome abundance that is expected because of the nuclear mutation.

Once we had determined that the phenotype of the *nuc1* mutation with respect to L-A copy number could be attenuated by the presence of M, we wondered about the result which showed that the mutation was nuclear recessive (Fig. 2). We reasoned that if the CB11 strain carried the M genome, then our conclusion that the deletion was a recessive mutation could be wrong. If that was the case, then a strain lacking the M genome would have to be used to repeat the dominance test. Therefore, virus particle RNA was prepared from CB11 and examined on an agarose gel similar to those in Fig. 7B and D. CB11 appeared to be host to none of the dsRNAs, though amounts of the RNA preparation equivalent to 10-fold more than those shown in Fig. 7 were applied to the gel (data not shown). Therefore, we concluded that our original inference that the *nuc1* mutations are nuclear recessive with respect to coat protein overproduction is correct. It was not, therefore, the production of some truncated form of the *NUC1* gene that resulted in the increased abundance of the L-A genome but rather the lack of a functional *NUC1* gene product.

Carbon source regulates coat protein expression in *NUC1* and *nuc1* strains. It was shown by Oliver et al. (23) that the level of dsRNA genome encoding the coat protein is stimulated 10-fold by growth on nonfermentable carbon sources. One hypothesis that could account for the increased production of the coat protein in the *nuc1* strains is that the mutation results in the constitutive production of the 86-kDa

FIG. 8. Carbon source regulation of coat protein expression. Yeast cells were grown to stationary phase on YPD (rich glucose medium) (lanes D) or YEPG (rich glycerol medium) (lanes EG). A 20- μ g sample of total cell protein was electrophoresed on a 10% Laemmli gel and blotted to nitrocellulose as described for Fig. 5. A 3-day exposure of the blot to X-ray film is shown. After autoradiography, the regions of the blot containing the signals were counted by liquid scintillation. Lanes: 1 and 2, strain A12WT (8 ± 8 dpm and 195 ± 4 dpm, respectively); 3 and 4, strain A12WT-LA (17 ± 8 dpm and 195 ± 4 dpm, respectively); 5 and 6, strain A12 (52 ± 6 dpm and 170 ± 4 dpm, respectively); 7 and 8, strain A12-LA (610 ± 2 dpm and 2873 ± 1 dpm, respectively).

protein at levels dictated by growth on nonfermentable media. *nuc1* strains retained the negative response to growth on glucose; coat protein was more abundant when the strains were grown on glycerol than when they were grown on glucose (Fig. 8). Switching both *NUC1* and *nuc1* cells from growth on glucose to growth on glycerol resulted in a 4- to 10-fold increase in coat protein production (Fig. 8) and a concomitant increase in L-A dsRNA (data not shown). The effect of nonfermentable carbon sources on the expression of coat protein in *nuc1* cells was independent of the stimulation by the mutation itself and was not affected by the presence of the M dsRNA genome.

DISCUSSION

The results presented here describe the identification of a new class of nuclear mutants that affect the abundance of the yeast dsRNA genome L-A. These are nuclear recessive mutations and can be suppressed by the presence of the cytoplasmic M dsRNA encoding the killer toxin. We have recently tested the levels of dsRNAs in a *por* mutant and found that the overexpression of L-A and the encoded coat protein in the *por* strain mirrors the effect we have observed in *nuc1* mutants (data not shown). *NUC1* and *POR* encode quite different mitochondrial functions, one a nonspecific nuclease located in the inner mitochondrial membrane (5) and the other a structural polypeptide in the outer mitochondrial membrane (28). The only common features of these two functions are that they are mitochondrial polypeptides and that they are nonessential for respiratory function of the organelle. However, *por* mutations do result initially in strains that are respiratory deficient and thus cannot grow on glycerol medium; they only resume growth after an adapta-

tion period of several days (14). *NUC1* mutations do not appear to inhibit growth on nonfermentable carbon sources (41). Though we have ruled out the possibility that *nuc1* mutations have an effect on porin, it remains a possibility that *NUC1* nuclease levels may be affected by a porin deficiency.

The overabundance of coat protein in both *por* and *nuc1* strains is correlated with an increased copy number of L-A dsRNA genomes. On the one hand, coat protein production is dependent on L-A dsRNA abundance; however, on the other hand, dsRNA propagation and packaging is dependent on the concentration of replicase, which is most likely a product of L-A (19), and the abundance of L-A-encoded coat protein. Therefore, it is difficult to deduce the initial reason for the increase in viruslike particles in these mutant strains. The absence of the *POR* and *NUC1* gene products could have a positive effect (directly or indirectly) on (i) replication of L-A or its stability, (ii) production or stability of plus-strand RNA that acts as mRNA, or (iii) translation of that message. For example, if the *NUC1* gene product was not confined completely to the mitochondrial compartment, the nuclease could affect the abundance of the single-stranded plus-strand mRNA and replicative intermediate. Consistent with this projection is the fact that the *NUC1* nuclease can attack single-stranded RNA but not dsRNA (5). However, to date there is no evidence that the nuclease is in the cytoplasmic compartment. Our current hypothesis is that both *por* and *nuc1* mutations have a similar net effect on mitochondrial metabolism and that this change in organelle function affects one of the three aforementioned aspects of coat protein production. Alternatively, *por* and *nuc1* may each affect different aspects of virus particle production. Measurement of *NUC1* protein in *por* strains, analysis of strains that overproduce either *NUC1* or *POR* protein, characterization of a *nuc1 por* double-mutant strain, and analyses of *ts por* and *nuc1* strains should reveal how mutations in these genes affect viruslike particle abundance and further our knowledge of any relationship which may exist between *NUC1* and *POR*.

That the effect of *nuc1* mutations on dsRNA production is not like that of the *mak* or *ski* class is clear. If they were of the *mak* class, the M genome would not have been maintained in the A12 strain. If they were of the *ski* class, the level of the M genome in A12 would have been elevated relative to the wild-type control strain. Since it is known that the M genome negatively controls the copy number of the L genome by approximately 10-fold (1), it appears that the control mechanism is intact in *nuc1* strains and overrides the 10-fold stimulation of L-A genome copy number by the mutation (for an example, compare the amount of L-A dsRNA in the A12 strain with that in the A12-LA strain [Fig. 7D]). Either the L-A genome is stimulated to replicate more often in the absence of M, or the L-A dsRNAs that are produced are more stable. It is not clear how the M genome would cause a decrease in stability of L-A, and therefore we favor the hypothesis that L-A replication initiation is stimulated in *nuc1* strains lacking M.

As was first described by Oliver et al. (23), viruslike particle coat protein genome production is stimulated by growth of the strain on nonfermentable carbon sources, which requires fully functional mitochondria. The stimulatory effect of growth on glycerol is intact in *nuc1* strains (Fig. 8), and therefore the effect of growth on glycerol appears to be independent of and in addition to the effect of the *nuc1* mutation on coat protein production. Furthermore, the presence or absence of the M dsRNA genome did not affect the

magnitude of the response to growth on glycerol. One wonders why the elimination of mitochondrial functions (albeit unnecessary for respiration) would stimulate production of the viruslike particle to even higher levels when viral genome replication is stimulated by carbon sources requiring a functional respiratory chain for their use. The discovery that altering mitochondrial functions can affect dsRNA copy number is not completely novel, as it has previously been shown that the *mak10* nuclear mutations, which cause the loss of both L and M genomes from a strain, are suppressed in strains lacking mitochondrial DNA (38). The complex relationship between mitochondrial function and dsRNA abundance remains to be dissected by a combination of genetic and molecular genetic approaches.

Before we understood that the 86-kDa protein was encoded by dsRNA, one of our initial goals in beginning the present study was to isolate the powerful promoter associated with the gene. We know now that the gene is encoded by the L-A dsRNA and that the extremely elevated levels of protein production are most likely due to the increased copy number of the RNA genome itself. We are indeed impressed by the potential usefulness of elements of the L-A genome that could be used for the overproduction of proteins other than the coat protein. If the *cis* elements necessary for L-A replication could be separated from the sequences encoding the coat protein, this system might be used for the intracellular production of proteins encoded by nuclear yeast genes or genes from other organisms. Indeed, recently it has been reported that a 530-base-pair deletion genome (X) derived from L-A can be propagated in yeast cells (16). Thus, the shortened genome must contain the *cis*-acting signals for replication. How foreign genes would be engineered into such a dsRNA molecule remains to be determined, but the procedure might include cloning steps in an artificial cDNA intermediate. Additionally, introducing such a chimeric L-A genome into a *nucl* strain lacking an M genome and growing the strain on glycerol medium could result in a very high yield of the protein.

An unexpected result of the studies on the stimulation of coat protein production was the discovery that the *NUC1* gene is adjacent to the *CBP1* gene. In previous work we characterized the *CBP1* gene (9, 10, 12) and showed that the gene product is required for the stabilization of the mitochondrial mRNA for cytochrome *b* by interaction with the 5' end (10, 11). We hypothesized that CBP1 protects the mRNA by binding to the 5' end or by cleavage of the precursor at the 5' maturation site, allowing the untranslated region to fold into a nuclease-resistant structure. It is curious, then, to find the gene for the major nonspecific mitochondrial nuclease adjacent to *CBP1*. *NUC1*, *CBP1*, and *URF* (a reading frame 270 base pairs upstream of *CBP1* of unknown function) each produce at least two different-sized mRNAs, one of which is regulated by the carbon source in the medium (S. Mayer, Y. Liu, and C. Dieckmann, unpublished data). Clustering of genes with related functions, including the divergently transcribed *GAL1* and *GAL10* genes (21) and the genes encoding histones H2A and H2B (26), occurs at other locations in the yeast genome. In both cases cited, clustering has allowed coordinate transcriptional regulation. It remains to be investigated whether the *URF-CBP1-NUC1* cluster has functional significance.

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