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Construction of a yeast mutant lacking the mitochondrial nuclease

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ABSTRACT

The nuclear gene from Saccharomyces cerevisiae that encodes the major mitochondrial nuclease was cloned. Gene sequences were identified from a λgt11 library by antibodies specific to the mitochondrial nuclease. DNA from the phage recombinant was used to isolate the entire nuclease gene from a plasmid library. Yeast strains containing the nuclease gene on a multicopy plasmid vector overproduced mitochondrial nuclease 20-40 times relative to a wild-type strain. Strains containing a null allele of the nuclease gene lacked all traces of mitochondrial nuclease. Both cell types, however, were phenotypically wild-type indicating that the nuclease is not an essential enzyme for mitochondrial function. The locus encoding the mitochondrial nuclease is termed NUC1.

INTRODUCTION

The mitochondria of Saccharomyces cerevisiae contain a very active nuclease which is responsible for more than 50% of all nuclease activity detected in a yeast cell (1,2). This enzyme is bound to the mitochondrial inner membrane and degrades single stranded RNA and DNA as well as double stranded DNA, on which it shows both an endonucleolytic and a 5'-3' exonucleolytic activity. Mitochondria from other fungi (e.g. Neurospora crassa [3]) and higher eukaryotes (4) display similar potent nuclease activities. However, only the enzymes from yeast and Neurospora have been purified sufficiently to show that a single enzyme displays all of those nuclease activities. We have also demonstrated by biochemical, immunological, and genetic criteria that almost all mitochondrial nuclease activity in yeast is due to only this one enzyme (5).

The function of the mitochondrial nuclease in yeast or in other eukaryotes is completely unknown despite the extensive biochemical and genetic analyses of mitochondrial function in both yeast and mammalian cells. In vitro these mitochondrial nucleases appear to lack specificity (but see [6]) yet in vivo mitochondrial nucleic acids are protected against their action. In yeast,

isolated, intact mitochondria can be incubated for hours without extensive degradation of their RNA or DNA; disruption of the mitochondria, however, releases the latent activity. It is this release which has made experiments on nucleic acid metabolism using mitochondrial extracts so difficult.

We report here on the cloning of the nuclear gene encoding the mitochondrial nuclease in yeast. The clone has been used to disrupt the chromosomal gene creating a strain lacking mitochondrial nuclease activity. Surprisingly this strain is phenotypically rho$^+$ as is a strain we constructed which contains 20-40 times the amount of mitochondrial nuclease found in a wild-type strain. In the accompanying report (7) we present the sequence of the nuclease gene and data on its expression.

MATERIALS AND METHODS

Affinity Purification of the Antibody

The preparation of antiserum raised against the yeast mitochondrial nuclease and its affinity purification have been described previously (5). Briefly, a partially purified preparation containing mitochondrial nuclease was fractionated by SDS-PAGE and the gel lightly stained with Coomassie Brilliant Blue to identify the band containing nuclease. This band was excised, the nuclease electroeluted from the gel (8), and the purified protein coupled to Affigel 10 (Bio Rad, Richmond, California). IgG from crude antiserum (prepared by protein A agarose affinity purification [9]) was then applied to the nuclease affinity column and anti-nuclease antibodies were prepared. In Western blots this antibody preparation detected three other mitochondrial proteins in addition to the nuclease (see Fig. 1).

In order to make an antibody preparation affinity purified against the mitochondrial nuclease fusion protein specified by a recombinant λgt11 phage, a standard (100 x 15mm) petri dish was first plated with 10^4 pfu of the purified phage. Induction with IPTG and transfer of the proteins in the plaques onto nitrocellulose were as described by Benton and Davis (10). Following the plaque lift, the nitrocellulose circle was blocked with Blotto (11) and incubated overnight at 4°C with anti-nuclease antibodies. The antibody solution was then removed and the nitrocellulose filter was washed three times with 20 ml Blotto at 4°C and three times with 0.15M NaCl, 20mM Tris-HCl, pH 7.5 at room temperature. Bound antibody was eluted twice with 1 ml of a solution containing 0.1M glycine-HCL, pH 2.9, 0.1M NaCl, 1% (w/v) BSA, 0.1% (v/v) NP-40 and 10% (v/v) ethylene glycol. Immediately following

elution, the eluates were pooled, neutralized to pH 7.5 with 1M Tris base, and stored at 4°C until use.

Western Blots

Gradient purified mitochondria were prepared as described previously (5), solubilized in SDS and fractionated by SDS-PAGE (12), and the proteins electrophoretically transferred onto nitrocellulose (13). The blots were probed overnight at 4°C with antibody using Blotto as a blocking agent. The bound primary antibody was detected using horseradish-peroxidase conjugated second antibody, and the bound complex was visualized according to the manufacturer's directions (Cooper Biomedical Inc.).

Nuclease Assays

Assays of the RNase and DNase activities of the mitochondrial nuclease have been described previously (5). RNase was assayed at 30°C using poly(rU) as a substrate; DNase was assayed using ^{32}P-labeled pBR322 DNA. One unit of enzyme activity is defined as that amount of enzyme which degrades 1 µg of poly(rU) in ten minutes at 30°C.

Southern Blots

Total yeast DNA was isolated (14) and analyzed by the method of Southern (15). ^{32}P-labeled probe DNA was prepared by random oligomer (Pharmacia, Piscataway, New Jersey) priming (16).

Materials

A library of yeast genomic fragments cloned into λgt11 was obtained from ClonTek, Palo Alto, California. The library was probed with affinity purified antibody as described by Benton and Davis (10), except that Blotto was used as the blocking agent. The library of partial Sau3AI fragments of yeast genomic fragments cloned into YEp13 has been described (17).

RESULTS

Previously, we described the preparation of an antibody which reacted primarily with the 38,000 dalton yeast mitochondrial nuclease in Western blots (5). This antiserum was raised in rabbits and was affinity purified using isolated nuclease coupled to an agarose column. The resulting antibody, though polyclonal, was nearly monospecific for the nuclease. We used this antibody to screen a library of yeast genomic DNA fragments cloned into λgt11 for expression of yeast mitochondrial nuclease in phage plaques; four positive plaques were obtained from a screening of over 2×10^6 plaques.

The four positive clones were plaque purified. Polypeptides expressed by these recombinant phage were then tested for their specificity to

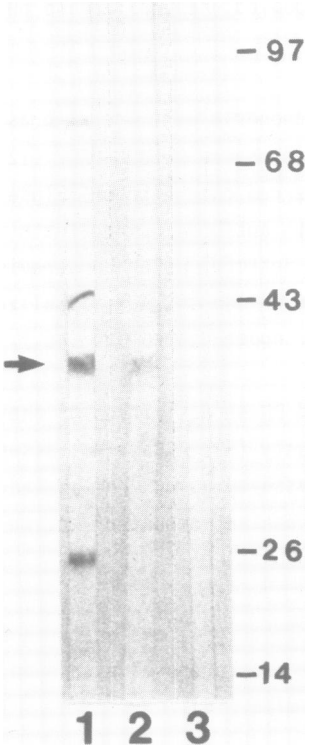

Fig. 1. Western blots of mitochondrial proteins probed with plaque purified antibody. Mitochondrial proteins were fractionated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and strips from this blot probed with antibody affinity purified by isolated plaques (see Methods). Reactive bands were visualized by horseradish-peroxidase conjugated second antibody. Lane 1: unfractionated anti-nuclease antibody; the nuclease band is indicated by the arrow; the other bands seen in the blot are unrelated to nuclease and are visualized due to contaminating antibodies in this preparation. Lane 2: antibody affinity purified by the positive recombinant phage. Lane 3: antibody affinity purified by a negative recombinant phage. Molecular weights (in kilodaltons) for standards run in parallel are indicated to the right.

anti-nuclease antibodies rather than to minor non-nuclease antibodies that could have contaminated our affinity purified IgG fraction. The antibodies reacting with polypeptides in the recombinant plaques were affinity purified as described in Methods. These purified antibodies were then used to probe a Western blot of mitochondrial proteins fractionated by SDS-PAGE (Fig. 1) to determine if the antibodies also reacted with mitochondrial nuclease. By this method only one of the four phage recombinants was shown to express a

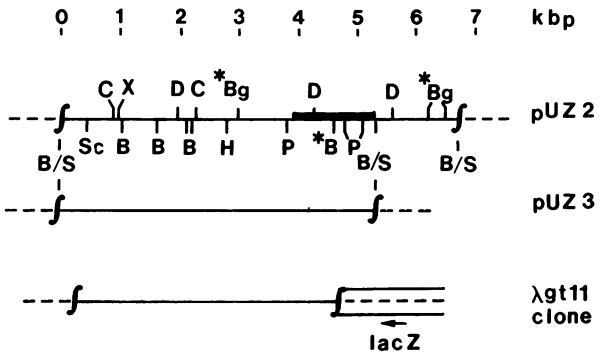

Fig. 2. Restriction maps of plasmid clones homologous to λgt11 recombinant. The restriction map of the insert in the longest plasmid clone (pUZ2) is shown; the inserts in pUZ3 and the λgt11 clone had identical maps for the overlapping portion except for the absence of the HindIII site in the lambda clone. B: BamHI; Bg: BglII; C: ClaI; D: DraI; H: HindIII; P: PstI; Sc: SacI; X: XhoI; B/S: BamHI-Sau3AI junction. The solid bar on the map indicates the approximate position of the gene encoding nuclease. The boundary between vector and insert sequences is indicated by the ∫ symbol, and asterisks indicate sites used for the disruption experiment described in the text.

polypeptide antigenically related to the nuclease.

Attempts to demonstrate directly that this phage recombinant contained nuclease gene sequences failed when tested by the usual protocols of in vitro translation of yeast mRNA that had been hybrid selected by DNA prepared from the recombinant. Because of this failure we instead used the DNA from the phage recombinant as a probe to identify homologous DNA sequences contained in a library of Sau3AI partial digest fragments of yeast genomic DNA inserted into YEp13 (17). Several positive clones were isolated and two (pUZ2 and pUZ3) were analyzed in more detail. The restriction maps of the yeast genomic DNA inserts in these two clones, relative to the phage insert, are shown in Fig. 2. Both plasmids contained sequences homologous to the entire phage insert but pUZ2 contained an additional 1.5 kb of yeast genomic DNA not found in pUZ3. Previous analysis of the nuclease related polypeptide expressed by the phage recombinant had shown that it was synthesized as a lacZ fusion protein which had an apparent molecular weight 15,000 daltons larger than the lacZ product from wild-type λgt11. Thus the direction of transcription and approximate limits of the nuclease gene within the clones could be estimated (assuming no introns within the nuclease gene); the estimates suggested that both plasmid clones could possibly encode the entire nuclease gene. The

Table I
RNase activity in plasmid transformants

Strain[1]	Mitochondrial RNase activity (units/mg protein) Minimal Media[2]	Rich Media[2]
MY54:pUZ2	1.8×10^5	3.5×10^4
MY54:pUZ3	3.1×10^3	1.9×10^3
MY54:YEp13	3.9×10^3	1.3×10^3

[1]MY54 is a, leu2-3, trp1, ura3-52, rho$^+$.
[2]Cultures were grown 24 hours in either rich media (1% Bacto Yeast Extract, 2% Bacto Peptone, 2% glucose) or minimal media (24) supplemented only with uracil and tryptophan in order to maintain the plasmid (carrying the LEU2 gene) within the cells. At the time of harvest, approximately 50-80% of the cells grown in rich media still retained the plasmid.

additional DNA in pUZ2 was located upstream of the sequences it shared with pUZ3.

To test whether one or both of the plasmids harbored the full-length nuclease gene, a yeast strain (MY54) was transformed separately with each of the plasmids and the resulting transformants assayed for mitochondrial nuclease activity. Since YEp13 is a multicopy plasmid in yeast, a higher level of mitochondrial nuclease would be expected in transformants compared to wild-type strains if the recombinant plasmids contained a functional nuclease gene. Table I shows that only the pUZ2 transformant gave a higher level of mitochondrial nuclease activity than wild-type; pUZ3 transformants could not be distinguished from wild-type with respect to nuclease activity. The nuclease activity in the pUZ2 transformant was at least 20-40 times greater than either those found in a control transformed with the vector alone or in a pUZ3 transformant. Western blot analysis of mitochondrial proteins from the pUZ2 transformant that were fractionated by SDS-PAGE and probed with the anti-nuclease antibody revealed that the increase in nuclease protein (molecular weight 38,000 daltons) was approximately correlated with the increase in nuclease activity (Fig. 3).

Those Western blots did not indicate an increased level of any mitochondrial protein larger in molecular weight than the nuclease. Despite the 20-40 fold elevation in nuclease levels in the pUZ2 transformant, no difference was seen in the pattern of Coomassie stained protein bands (analysed by SDS-PAGE) when comparing the transformant to a wild-type (data not shown). This result reinforces our previous finding that the

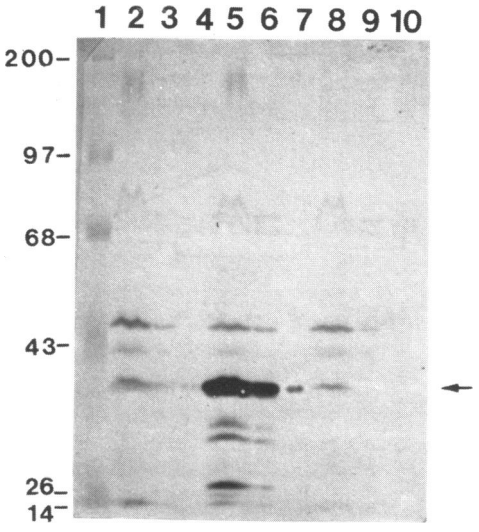

Fig. 3. Western blot of mitochondrial proteins prepared from plasmid transformants. A yeast strain was transformed with the plasmid vector, YEp13 (lanes 2-4), pUZ2 (lanes 5-7), or pUZ3 (lanes 8-10). Transformants were grown in rich medium, mitochondria prepared, and proteins analyzed by Western blotting. For each transformant 0.5mg of mitochondrial protein or serial 1:10 dilutions (left to right) were loaded per lane. The blot was probed with antibody affinity purified against a partially purified preparation of nuclease. Lane 1 contains BRL pre-stained protein standards with molecular weights indicated. The arrow points to the 38,000 dalton nuclease.

mitochondrial nuclease, though enzymatically potent, represents only a very small fraction of the total mitochondrial proteins (5).

In order to confirm that the pUZ2 clone contained the nuclease gene we used this clone to disrupt the chromosomal copy of the gene by integrative transformation (19). From an estimate of gene size, based upon the apparent molecular weight of the nuclease and assuming no introns, we concluded that the BamHI site indicated in Fig. 2 would be located within the nuclease gene. A new plasmid was constructed in which the LEU2 gene, carried on a BglII cartridge, was inserted into pUZ2 at that BamHI site. From this new plasmid the presumably disrupted nuclease gene and flanking sequences were excised as a BglII fragment (Fig. 2) and used to transform MY54, a haploid strain. The resulting yeast transformants were assayed for mitochondrial nuclease activity and analyzed by Southern blotting to verify that homologous integration had occurred at the correct chromosomal locus. Fig. 4 shows the Southern analysis for one such clone. A homologous integration event would be indicated by the

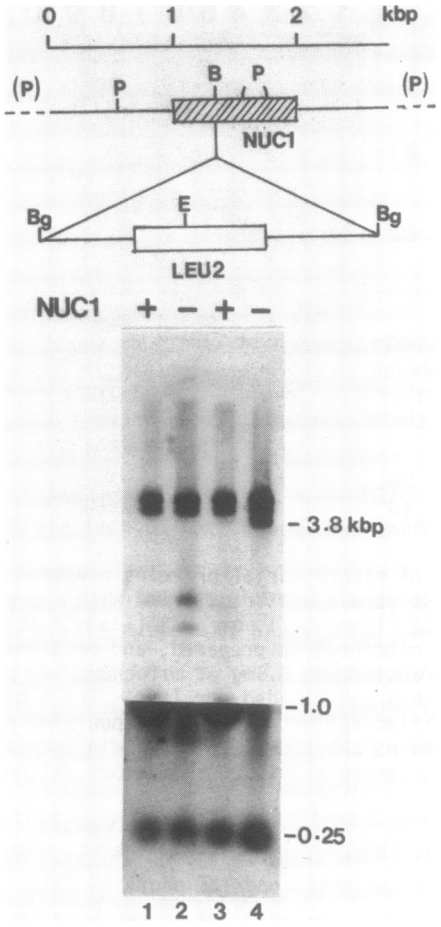

Fig. 4. Southern blot of disruptant. Total yeast DNA was prepared from the disruptant (NUC1$^-$) or its wild-type parent (NUC1$^+$), digested with PstI and EcoRI (lanes 1 and 2) or PstI (lanes 3 and 4), and 10 µg DNA per lane analyzed by Southern blotting. The probe was made from plasmid DNA which contained the entire insert present in pUZ2, subcloned into pBR322. The bottom third of the nitrocellulose blot was overexposed in order to more clearly visualize in the autoradiograph the 0.25 Kb PstI fragment from the NUC1 gene that remains in the gene after disruption since it flanks the point of insertion. Above the autoradiograph is diagrammed the restriction map of the disruptant relative to the map of the wild-type; the dotted lines indicate flanking genomic DNA containing unmapped PstI sites not present in pUZ2. B, BamHI; Bg, BglII; E, EcoRI; P, PstI.

elimination from the nuclease region of the 1.0 kb PstI gene fragment and its replacement by a 3.8 kb PstI fragment containing the LEU2 gene and its unique EcoRI site. As Fig. 4 shows, the Southern analysis of DNA isolated from this

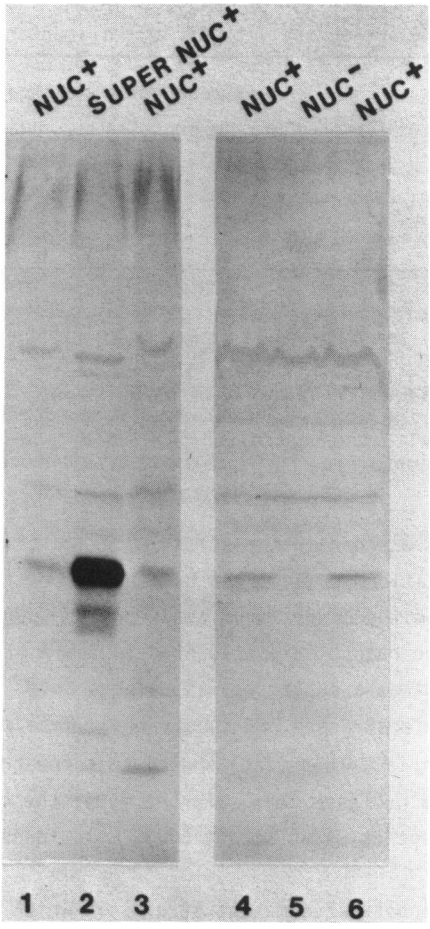

Fig. 5. Amount of nuclease in mitochondria from disruptant versus control and overproducer. Western blots of mitochondrial proteins (0.5mg/lane) prepared from the nuclease overproducer, controls, and the disruptant were probed with anti-nuclease antibody as in Fig. 3. Strains used were: Lane 1, MY54 transformed with YEp13; Lane 2, MY54 transformed with pUZ2; Lane 3, MY54 transformed with pUZ3; Lane 4, MY54; Lane 5, MY54 containing a disrupted NUC1 gene; Lane 6, MY54:REV, a leu^+ revertant selected from MY54 and thus phenotypically identical with respect to growth requirements as the MY54 derivative containing the disrupted NUC1 gene.

transformant met these expectations.

Fig. 5 shows the Western blot analysis of mitochondrial proteins isolated from the transformant, which revealed that the 38,000 dalton nuclease polypeptide was absent (cf. lanes 5 to lanes 4 and 6). We have not tested by

Table II
Nuclease activity in NUC1 disruptant

	Mitochondrial Nuclease Activity	
Strain[1]	RNase (units/mg protein)	DNase[2] (% of control)
MY54	1.3×10^3	100
MY54:REV	1.5×10^3	108
MY54 NUC1: LEU2	< 0.3	< 0.02

[1]MY54 is the parental strain used for the disruption of the NUC1 allele. MY54:REV is a revertant phenotypically leu^+ isolated from MY54 so as to have a strain phenotypically identical, with regards to growth requirements, to the NUC1 disruptant.

[2]Single stranded DNase activity was assayed using 2×10^4 cpm of ^{32}P-labeled pBR322 DNA and measuring the rate of acid-soluble material liberated. The 100% value measured with the control (MY54) was a rate of 4×10^2 acid-soluble cpm/min in an assay containing 20 µg mitochondrial protein.

Western blot analysis whether in the strain with a disrupted NUC1 gene any truncated NUC1 translation products can be found extramitochondrially. Nuclease activity in mitochondria from the transformant was assayed on RNA and DNA substrates and the results are presented in Table II. RNase activity as assayed using poly(rU) as a substrate was undetectable. Similarly, using either single or double stranded DNA substrates, DNase activity could not be detected in mitochondria. These data showed that the transformant indeed contained a disrupted nuclease gene. Furthermore, the mitochondrial nuclease activity in the disruptant was at least 5,000 fold lower than in the wild-type control.

We subsequently constructed mutant strains in which sequences located between the BamHI and StuI sites (Fig. 2) were eliminated and replaced with the LEU2 gene. These deletion/insertion constructions were phenotypically identical (data not shown) to the insertion mutation described above. Taken together, these results demonstrate that we have cloned the gene encoding yeast mitochondrial nuclease. We designate the locus encoding the mitochondrial nuclease NUC1.

DISCUSSION

The evidence that we have identified and cloned the nuclear gene (NUC1) that encodes the mitochondrial nuclease in Saccharomyces cerevisiae can be summarized as follows:

1) A carboxy-terminal portion of the gene (approximately the last third),

cloned and expressed in E. coli as a lacZ fusion protein, encodes a polypeptide antigenically homologous to the mitochondrial nuclease.

2) In yeast, placing the entire gene on a multicopy plasmid vector results in the overproduction of mitochondrial nuclease in transformants, as assayed by enzymatic activity of nuclease and amount of nuclease protein found in mitochondria.

3) Disruption of the chromosomal NUC1 gene in yeast eliminates nuclease activity and detectable nuclease protein in mitochondria.

Furthermore, as will be presented in the accompanying report (7), the molecular weight (37,209 daltons) of the protein predicted from the nucleic acid sequence is compatable with the apparent molecular weight of the enzyme (38,000 daltons by SDS-PAGE).

That the proposed coding region indeed indentifies NUC1 is shown most strongly by the results of the disruption experiment. Insertion of leu2 at the BamHI site within that coding region (Fig. 2) completely eliminates nuclease activity indicating that expression of that coding region is necessary for mitochondrial nuclease activity. From the location of the fusion point in the λgt11 clone (Fig. 2), we know that the sequences immediately downstream of the BamHI site encode a polypeptide antigenically homologous to the nuclease. Thus, the simplest conclusion is that the coding region in fact identifies NUC1 rather than, perhaps, a regulatory gene of nuclease expression. The location of NUC1 within the yeast genomic insert contained in pUZ2 is also shown by the failure of pUZ3 transformants to show elevated nuclease levels. Since pUZ3 differs from pUZ2 only by the absence of sequences upstream of the Sau3AI site (Fig. 2), those missing sequences must be essential for nuclease expression. As we show in the accompaning report (7), those sequences include the promoter region and, indeed, mapping of the vector-insert fusion point in pUZ3 reveals that pUZ3 contains intact the entire coding region of NUC1.

In a previous report, we demonstrated that the yeast mitochondrial nuclease reacted with an antibody to a similar nuclease found in the mitochondria of N. crassa (5). Chow and Resnick have shown that the same antibody also reacts with a 70,000 dalton RNase/DNase isolated from post-mitochondrial supernatants from yeast (20). This antigenic similarity raises the question of whether the mitochondrial nuclease may be derived from a much higher molecular weight precursor which is located and functions outside the mitochondria. Two further arguments make this possibility worth considering. First, Ramotar et al. have shown that the very similar

mitochondrial nuclease in N. crassa is located not only in that organelle but also in vacuoles and in the nucleus (21). In both vacuoles and mitochondria, the enzyme apparently is found as a 70-80 kilodalton membrane bound precursor which is liberated as a 31 kilodalton soluble form by proteolytic cleavage. In the nucleus antigenically cross-reactive forms of even higher molecular weight can be found. Second, biochemical and genetic evidence have shown that a number of mitochondrial enzymes are also found and function outside of the mitochondria (22).

Our data from the cloning and disruption of the NUC1 gene suggest that the yeast mitochondrial nuclease is neither derived from a much higher molecular weight precursor nor is it located within other compartments of the yeast cell. As shown in Fig. 3, the Western blot of proteins from isolated mitochondria of the nuclease overproducing transformant shows no evidence of a higher molecular weight precursor. Higher (or lower) molecular weight forms of the nuclease are also absent from Western blots of proteins from the overproducer, whether using fractions enriched for vacuoles and nuclei, post mitochondrial high-speed supernatants, or whole spheroplasts lysed in SDS. Other cellular fractions were not enriched for nuclease beyond levels due to normal contamination by mitochondrial fragments during isolation. In Western blots of proteins from mitochondria prepared from the overproducer, we do see three lower molecular weight forms (Fig. 3) which we had previously observed during the isolation of the enzyme from wild-types. We attributed these forms to proteolytic degradation during isolation (5). Whether these forms indeed are due to artifacts of isolation or are present and functional in vivo, we do not know at present. It seems clear, however, that in contrast to the situation in Neurospora, the yeast mitochondrial nuclease is not derived from a higher molecular weight precursor. Further, as is presented in the accompanying report (7), the sequence of the gene shows that its coding capacity rules out the possibility of a much higher molecular weight precursor as observed for the Neurospora enzyme.

Our data do not rule out the possibility that the mitochondrial nuclease might be found in other cellular compartments. Since most enzymes imported into mitochondria have their signal sequences cleaved by an intramitochondrial signal peptidase (23), we have taken as evidence of a possible extramitochondrial location of the nuclease the detection of a form of the nuclease with a higher molecular weight than that seen within mitochondria. No such higher molecular weight forms were detected in our Western blots of various cell fractions. It is possible, however, that extramitochondrial

nuclease is either present in amounts below detection, even in the overproducer, or that similar processing of the enzyme occurs as in the mitochondria. Rather than by criteria derived from cell fractionation, we are screening the overproducer and the disruptant for genetic evidence of a role for the nuclease in nuclear gene expression, DNA repair or recombination.

With regards to the extramitochondrial yeast nuclease described by Chow and Resnick (20), our evidence suggests that despite its antigenic similarity to the yeast mitochondrial nuclease the two enzymes are distinct gene products. Foremost, the molecular weights of the enzymes (extramitochondrial: 70,000 daltons, mitochondrial: 38,000 daltons) are very different. Recently, Chow has cloned a genomic fragment containing the gene for the extramitochondrial nuclease and comparison of the respective restriction maps of the two yeast genes shows quite clearly that they are not identical (Chow, personal communication). It will be interesting, however, to compare the two gene sequences since their antigenic relatedness at the protein level may indicate that they have diverged from a common precursor by gene duplication. If so, perhaps the fact that apparent higher molecular weight forms of the mitochondrial nuclease in Neurospora are located extramitochondrially may indicate that in this fungus a single gene encodes both intra- and extra-mitochondrial nucleases, and that this single gene may show homology to both nuclease genes from yeast.

As Fig. 4 and Table I indicate, the strain with NUC1 disrupted shows no detectable nonspecific DNase or RNase activity within mitocondria. We will present elsewhere a more detailed characterization of that strain, but we note here that the absence of nuclease seems to have no profound phenotypic effect. The strain is rho^+ and grows as well as the wild-type on fermentable and nonfermentable carbon sources. Similarly, the overproducer also evidences no profound effect on mitochondrial function although it contains up to 40 times as much nuclease as the wild-type. These data imply that the nuclease is not essential for mitochondrial function, and show that no other general nuclease within the mitochondria has increased in activity to compensate for the function provided by the NUC1 gene product. The observation that the overproducer is also phenotypically rho^+ further suggests that within the mitochondrion the nuclease activity is tightly regulated to prevent degradation of mitochondrial nucleic acids. We are currently testing whether a mitochondrially synthesized protein or nucleic acid may be involved in such regulation and thus be overproduced to compensate for elevated nuclease levels in the mutant strains.

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