Regulatory interactions between mitochondrial genes: Interactions between two mosaic genes

(mitochondrial genetics/intervening sequences/gene expression/"box" phenomenon)

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ABSTRACT We have studied the mitochondrial DNA and the phenotypes of strains of Saccharomyces cerevisiae with specific intervening sequences in two mosaic genes: cob (the gene for apocytochrome b) and oxi3 (the gene for subunit I of cytochrome oxidase). The results suggest the following. (i) The presence of an intervening sequence downstream encompassing the intron box7 is sufficient for the regulation of oxi3 by cob (BOX phenotype); two sequences (containing intron loci box3 and box10) upstream in cob and two in oxi3 are dispensable. (ii) Strains without the two sequences upstream still contain the downstream sequence and the competence to specify a functional trans-acting element. Mutational lesions in this segment are phenotypically indistinguishable from box7 mutants, including the accumulation of polypeptides with homologous amino acid sequences. (iii) A catabolite-sensitive BOX phenotype, characteristic of mutants in the first exon, requires the simultaneous presence of an adjacent intervening sequence. A model is presented in which a hypothetical product specified by an intron (locus box7) of the cob gene controls the expression of a second mosaic gene (oxi3).

Recent studies on the organization and expression of the mitochondrial gene for apocytochrome b of the bc1 complex in Saccharomyces cerevisiae (cob gene) have revealed some remarkable properties (reviewed in refs. 1–3). First, the gene exhibits the mosaic organization characteristic of mutants with a "short" or "long"—where D273-10b ("D273") contains both genes in their short forms (cob5 and oxi33) and ID41.6/161 ("161") contains both in their long form (cob8 and oxi31) (Fig. 1).

We will demonstrate that the BOX phenotype does not require any of these inserts, singly or in combination, and that, among the three introns present in cob8, only the third (the site of box7) is necessary for regulating the normal expression of the oxi3 gene.

MATERIALS AND METHODS

Most of the genetic and biochemical techniques have been described (10, 12, 18, 21). The cob mutants in strain D273 were generated and characterized (26) by methods described for strain 161 (10). Hybrids containing defined box alleles of 161 in the mitochondrial genomic background of D273 were constructed by converting them to rho− (2) with ethidium bromide. Such strains retaining the desired allele were then mated to mit− rho− kar1 derivative of D273. Several mit− recombinants (nuclear background of 161) were isolated and shown to retain the desired cob mutations and to be mit− elsewhere in the genome including the cob region. These and all other strains used in this study are described in Table 1. With the exception of tr-1 and M8-219, all are isonuclear with 161.

RESULTS

Rationale. These studies were based on the observation (23) that cob mutants in D273, a strain now known to be cob5 and oxi33, failed to exhibit the BOX phenotype. Thus, the BOX phenomenon, a paradigmatic example of intron(s) in one mosaic gene controlling the expression of a second mosaic gene, could be caused by the presence of inserts IIa and IIIb in oxi33. This hypothesis was tested by determining the phenotype of hybrid mitochondrial genomes combining different mutations that

The above description, apparently valid for a number of strains including three (ID41-6/161, 777-3A, and KL14/4A) used in the construction of a coherent, self-consistent map of the cob/box gene (3), may not be universal. Fourby and Tzagoloff (23) reported that none of their cob mutants with a single lesion, derived from strain D273-10b, exhibited the BOX phenotype and that those that did all bore a second lesion in one of the oxi genes. Furthermore, this and other similar strains (e.g., JSI-3D) harbor two deletions [IIa, 1900 base pairs (bp), and IIIb, 1400 bp] within the cob region which are the sites of intron loci box3 and box10, respectively (7, 8, 24, 25). The same strains also carry two deletions (IIa and IIb), 7400 bp long, between old2 and the proximal segment of oxi3. We shall refer to these polymorphic forms of cob and oxi3 as "short" and "long"—where D273-10b ("D273") contains both genes in their short forms (cob5 and oxi33) and ID41.6/161 ("161") contains both in their long form (cob8 and oxi31) (Fig. 1).

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Abbreviation: bp, base pair(s).  §To whom reprint requests should be addressed.

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generate the stringent or conditional BOX phenotype in their original ox13 context, but now with an ox13* gene. If the hypothesis is wrong, the BOX phenomenon may reflect a role of the intervening sequences in cob and be susceptible to analysis in these terms.

Are Inserts Required for Expression of BOX Phenotypes? Although the constructions described (Table 1) define the mitochondrial genomes of the hybrids in genetic terms, the retention or deletion of the desired segments in their mtDNA can be probed more rigorously with restriction nucleases. Fig. 2 Left displays typical HincII digests of mtDNA from the parental and hybrid strains. As shown earlier (2, 9, 17, 24), most of the cob region is contained within fragments H2-9 of D273 and H2-5 of 161, and the difference of 3300 bp between them reflects the presence of inserts IIIa and IIIb (Fig. 1). The hybrid construct D273/A103 and the mit+ strain tr-1 (not shown in Fig. 2) contain cob*, D273/F21 and D273/EM25 contain cobb*. Because the ox13 gene is contained within the same large HincII fragment in both forms, we also used EcoRI digests (Fig. 2 Right). There is a difference of 2700 bp between fragment R1-4 from D273 and fragment R1-5 from 161, consistent with the presence of the combined inserts IIa plus IIb in the ox13 region of the latter. The three hybrid strains clearly contain ox13*, in contrast, tr-1 (not shown) contains ox13-*. These digests also confirm the two alternate forms of cob: fragment R1-3 is larger in strains containing cob* than in those with cobb*. Fragments R1-5 of D273 and R1-6 of 161, which contain the remaining sequences of the cob region—i.e., the third intron (box7) and all sequences downstream from it—a total now known (27) to extend over 2495 bp, are clearly of the same size. Location and spacing of four Hae III cleavage sites confirm this conclusion (P. Zassenhaus, personal communication).

The polypeptides accumulating in various intron (box3 or box7) mutants are shown in Fig. 3A and B. The patterns (Fig. 3A) were identical, whether or not the mutation is in its original (161, ox13*) or hybrid (D273, ox13*) context: The stringent BOX phenotype and the intron products are independent of the length of ox13 and of the presence or absence of the preceding introns singly or in combination.

Expression of the catabolite-sensitive BOX phenotype in certain mutants (such as EM25) in the first exon (box5/4) is equally independent of the form of ox13 (Fig. 3B). However, in contrast to the box4 hybrid (D273/EM25), which remains cobb*, mutations that differ from it only by being in cobb* did not exhibit this BOX phenotype.

These results prove conclusively that the BOX phenotypes exhibited by mutants in box3, -4, and -7 are not due to a second mutation in an ox region (33) or elsewhere. Because they are indistinguishable in all respects from those described for mutations at the same box loci in the original cob* ox13* context, we conclude that a single lesion at an appropriate position in the cob gene is both necessary and sufficient for the generation of the BOX phenotype.

Does D273 Contain a Functional Intron? The restriction analysis presented in Fig. 2B—in particular, the properties of

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### Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Mutations at locus</th>
<th>cob</th>
<th>ox13</th>
</tr>
</thead>
<tbody>
<tr>
<td>161*</td>
<td>None</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D273</td>
<td>None</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>tr-1</td>
<td>None</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A103*</td>
<td>box7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D273/A103*</td>
<td>box7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M8-219*</td>
<td>box7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PZ1*</td>
<td>box3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D273/PZ1*</td>
<td>box3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EM25*</td>
<td>box4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D273/EM25*</td>
<td>box4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S8*, S10*</td>
<td>box4</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Wild type (ID41-6/161) and mutants derived from it (10).
+ Wild type (D273-10B); its mutants have been described by Foury and Taqgold (23).
+ Wild-type recombinant from a cross of PZ1 (a) (box3) x S9 (a) (box8) containing cob* (from D273) and ox13*.
 Derived from crosses of rho* (box3n) x D273.
 Map location defined by deletion mapping with a library of defined rho* strains (3, 28).
 Mutants in the mitochondrial genome of D273 placed in the nuclear context of 161. Map location as for %.
Hind for well (D273) differing by 1). mology two strains. This now strain so, this maps In fact, this locus at 35, 1168 bp Mr with box7. gies with the box7 (box7-it the Lamouroux showed (28) known-action element the cobS containing (see text) the cobS gene is contained (see text) in fragments R1-5 (161) and R1-4 (D273) differing by 2700 bp, corresponding to insert II.

DISCUSSION

The following interpretations rest on three assumptions: (i) any difference in the cob genes of D273 and 161 are confined entirely to the region upstream from box7 (Fig. 1) and, specifically, the segment corresponding to box7 in 161 closely resembles and is functionally equivalent to the same sequence in D273; (ii) the construction of the D273/A103 hybrid has not affected this homology; and (iii) the phenotype of the cob5 mutant M8-219 is due entirely to an alteration in its cob region. Assumption i is based on published data (17, 24), together with our observations (Fig. 2B), which also serve as a verification of ii. Assumption iii is verified by the finding that this mutant recombines readily with rho- strains containing the cob+ region but lacking sequences outside it (including all nine mitochondrial markers). In contrast, as a control, M9-229, a second mutant in D273, which harbors two mutations (23) (one near box1 and the other in an oxi gene) fails to recombine with any of these cob+ specific rho- strains, the behavior expected for a double mutant.

Regulation of oxi3. The inhibition of expression of oxi3 by certain cob mutants (the BOX phenotype) is independent of the presence of the inserts (IIa plus IIIb) in the oxi3 gene characteristic of the long-form strains in which this phenomenon was first documented. The hypothesis advanced above in Rationale is thus invalidated.

Furthermore, the stringent BOX phenomenon is not confined to cob5—i.e., it is independent of the two intervening sequences in the upstream half of the cob gene (Fig. 1). Therefore, we can rule out all models that assign a separate and specific function to each of the three introns of cob5 in controlling fragments R1-5 of D273 and R1-6 of 161—indicates a close homology of the “downstream” portions of the cob region in these two strains. This segment contains two intervening sequences. The first is the site of the intron locus box7 in cob5 strains and is now known to contain an open reading frame extending over 1168 bp out of a total of 1414 bp (8). Therefore, the same intron function might be expressed by either form of the cob gene. If so, this suggests the possible existence of mutations in the cob5 strain D273 with the phenotype characteristic of box7 in cob5. In fact, we find that strain M8-219 is not a double mutant (23) but maps exclusively in this region and exhibits the multiple-novel polypeptides (Fig. 3A; ref. 26) characteristic of mutants at this locus (3, 12, 21). Three of the proteins (apparent Mr = 54, 35, and 23 × 103) exhibit virtually complete—and one (apparent Mr = 43 × 103) exhibits extensive—sequence homologies with the analogous polypeptides and accumulating in long form box7 mutants (Fig. 3C) (26).

Complementation tests add strong, independent evidence. Lamouroux showed (28) that exon mutants of D273 complement box7 mutants in cob5, so cob5 contains the trans-acting element associated with box7. We have confirmed this result with A103 (box7 in cob5) and D273/A103 (box7 in cob5) and have extended it to the cob5 box7 mutant M8-219 which can be complemented by exon mutants in either form of the gene. Although heteroplasmone between PZ1 and A103 (box3 and -7), respectively, both in cob5 exhibit complementation, PZ1 and the cob5 version of box7 fails to do so. Thus, even though PZ1 contains the mit* sequence in box7, its mit* lesion upstream in box3 prevents the expression of box7* to produce the trans-acting element required to complement the mit* form of box7.
The expression of \( \text{oxi3} \) is difficult to process in its normal form in a particular strain, and therefore polyproteins with \( \text{oxi3} \) intron are not present in \( \text{cob}^b \). The observation that mutants in \( \text{box3} \) also exhibit the BOX phenotype is probably due to their inability to generate the trans-acting "box7" product. Their reported (15, 16, 17, 25) failure to process the box7 intron normally is consistent with this supposition. The converse does not appear to hold: box7 mutants experience difficulty in processing the first (box3) intron. Finally, because mutants with large deletions in \( \text{cob} \) exhibit the BOX phenomenon (2, 12, 14) whereas similar deletions in \( \text{oxi3} \) have no effect on the synthesis of cytochrome \( b \) (16, 29), the regulation of COX I synthesis must involve a unidirectional, positive effect of the hypothetical "box7" product at some stage of the expression of the \( \text{oxi3} \) gene (also see ref. 25).

Intron Functions. All strains of \( S. \) \( \text{cerevisiae} \) examined so far appear to share in and require the expression of at least a part of the intervening sequence containing, but not necessarily coterminal with, the box7 loci in 161 and related \( \text{cob}^b \) strains. Their wild-type equivalents are required for successful excision of the intervening sequences and for the regulation of \( \text{oxi3} \).

These inferences are equally compatible with either of the models currently favored to account for intron function—i.e., specification of RNA guides or of a maturase protein (1, 3, 7, 15, 16). In this context, we examined the sets of apparently similar proteins that accumulate in box7 mutants of both \( \text{cob}^b \) and \( \text{cob}^b \) strains and found that polypeptides with apparent \( M_r \) of 43, 35, and \( 23 \times 10^3 \) exhibited extensive sequence homologies. The first of these contained a \( 20,000 \) fragment (Fig. 3C) that, upon digestion with a second protease, yielded identical peptides unrelated to cytochrome \( b \) and therefore probably originated from intron sequences (26). The results obtained are sufficient to rule out a supposition that the box7 polypeptides, which also exhibit significant homologies (unpublished data) with others of similar mobility in box3 mutants (3, 10, 12), are in fact due to the presence of the same "box3 product". Because M8-219 does not contain box3, this interpretation is untenable.

The conditional version of the BOX phenomenon differs from the stringent one by a requirement for the presence of one or two of the intervening sequences downstream from the first exon(s): mutants in this segment (box5/4 in \( \text{cob}^b \)) of an isochromosomal \( \text{cob}^b \) strain, which lacks them, do not exhibit this phenotype (Fig. 3B). We have extended these observations to five additional, nonallelic mutants of the same \( \text{cob}^b \) strain; it is therefore unlikely that we have missed the phenomenon due to the particular mutant alleles selected for the initial study. Our tentative interpretation is that, under conditions of catabolite
repression, susceptible mutants in box3/4 in cob− cannot process the immediately adjacent downstream intron(s) and thereby generate or exacerbate a block in the normal splicing sequence (15, 17, 25) required for the ultimate expression of the "box7 product" (13). The same lesion in a cob5 strain does not produce this result for two reasons. First, processing of sequences around box7 may obey different rules, depending on whether they are in a cob5− or cob5− context. Second, because in cob5− strains exon 5/4 is fused to exons 8 and 1/9, mutations in the former are far removed from the first intron downstream (Fig. 1). The observed accumulation of large transcripts of cob and α3 in some box3/4 mutants (15–17, 25) is consistent with the hypothesis. So is the reported polarity of complementation of box3/4 mutants with downstream introns (13).

There are precedents for dispensability of intervening sequences in closely related genes. Some strains of S. cerevisiae contain, and others lack, such an insert in the mitochondrial gene for the 215 ribosomal RNA (3, 29, 30). Rat preproinsulin is specified by two genes (5), only one of which contains an intervening sequence in the structural part; the common intron is upstream from the initiator codon (G1, 32). The more complex form appears to represent the more ancestral version (33). Finally, Nishioka et al. (34) have determined the sequence of a mouse α-globin-like gene in which the two intervening sequences found in all other globin genes have been cleanly excised. These results constitute the closest analogy to the existence of alternate forms of the cob gene differing in two introns. However, so far as we know, the mitochondrial system in yeast is the only one currently available that permits the controlled combination and study of mosaic genes and their expression in various alternative and mutant forms.

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