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Pleiotropic Mutations within Two Yeast Mitochondrial Cytochrome Genes Block mRNA Processing

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Summary

The mRNAs from two yeast mitochondrial genes *cob-box* (cytochrome b) and *oxi-3* (cytochrome oxidase 40,000 dalton subunit) are processed from large (7–10 kb) precursors. Certain mutations in each gene block the maturation of the RNAs from both genes at a variety of specific steps. The pleiotropic cytochrome b mutants seem to lack a functional trans-acting RNA required for the processing of both messengers. In contrast, the *oxi-3* mutants may act by producing an activity that inhibits specific steps.

Introduction

The cytochrome b gene of yeast mitochondria is a mosaic gene with an intron-exon structure typical of many genes of higher cells (see Gilbert, 1978; Crick, 1979; David and Wahli, 1979). The characterization (Kotylak and Slonimski, 1976, 1977; Slonimski and Tzagoloff, 1976; Pajot et al., 1976; Claisse et al., 1978; Slonimski et al., 1978a, 1978b; Alexander et al., 1979; Haid et al., 1979; Hanson et al., 1979) of a large number of mutations in this gene by genetic and physical mapping, complementation, cytochrome spectra and gel electrophoresis of the mitochondrial proteins have produced evidence for a split gene whose 900 nucleotides of coding sequence are spread over 7–9 kb of DNA. Nonconditional mutants in this mitochondrial DNA region (the *cob-box* region) which affect the chain length of the 30,000 dalton cytochrome b gene product have a map order colinear with the protein but fall into clusters. Mutations in these clusters, *box*-5, -4, -8, -1, -9, -2 and -6, fail to complement each other as expected for structural lesions in a single cistron. Physical mapping of these loci relative to rho-petite deletions (Slonimski et al., 1978b; C. Jacq and J. Lazowska, unpublished data) and R loop mapping of the wild-type (Borst and Grivell, 1978; A. C. Arnberg, L. Grivell and G.-J. B. van Ommen, unpublished data) suggest the structure for this gene illustrated in Figure 1. Many other mutations in this gene, however, exhibit unusual properties. Interspersed between the structural loci are three clusters of mutations (*box*-3, -10 and -7) that block the synthesis of the cytochrome b protein but complement each other and most of the structural mutants fully (Slonimski et al., 1978b; A. Lamouroux et al., unpublished results). The complementation shows that these mutations lie outside the structural cistron, but genetic and physical mapping locate these mutations within that cistron, presumably in introns. The complementation occurs in trans and expresses the message in cis: all the cytochrome b exons from the parent carrying the deficient intron are translated in the hybrid (A. Kochko et al., unpublished results). Furthermore, these mutations are pleiotropic and block the synthesis of the 40,000 dalton subunit of cytochrome oxidase, from the *oxi-3* locus. Thus these mutations are recessive but affect some function that normally acts in trans on both the *box* and the *oxi-3* genes. We conjectured that these mutations might block the processing of the messenger RNAs.

Results

Cytochrome b mRNA Precursors

We examined the messenger RNA of the cytochrome b gene to determine the pattern of precursors that would appear if that RNA were to be spliced. To examine these messengers we purified yeast mitochondria, isolated their RNAs with phenol and separated the RNAs by size on agarose gels in the presence of methylmercury. After transferring the RNAs to the diazotized paper of Alwine, Kemp and Stark (1977), we hybridized to these immobilized RNAs a probe, *ob*, corresponding to a fragment of the carboxy terminal exon, *box*-6, of the cytochrome b gene. This *ob* probe is the DNA of a petite mutant corresponding to about 200 bp in sequence, and is capable of restoring most *box*-6 mutants but no others (Slonimski et al., 1978b; C. Jacq, unpublished observations; Figure 1 shows the position of this probe). Figure 2 shows some of the results. In wild-type mitochondria we see a pattern of RNA sizes ranging from the largest precursor (about 8.5 kb) down to the mature message (which accumulates at 2.3 kb). We often observe weak hybridization to about seven precursors of 3.0, 3.9, 4.9, 5.4, 6.6, 7.5 and 8.5 kb in length. Lanes (a) and (b) in Figure 2 show the patterns observed after growth in galactose (partially induced state for the cytochromes) and glucose (catabolite-repressed state), respectively. There are slight differences in the patterns. Lane (f) in Figure 2 shows an ethidium bromide staining of one of these gels. Three major bands appear. The lowest and highest bands are the two mitochondrial rRNA species. The second band from the bottom represents cytoplasmic contamination, since it is the small cytoplasmic rRNA; the two large rRNAs co-migrate. The ribosomal RNAs dominate the pattern totally, and the mature messengers are not always visible in the fluorescent pattern. The isolations and analyses of the RNA are variable, and the precur-

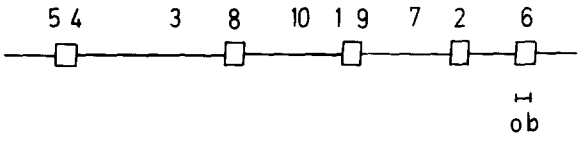

Figure 1. Cytochrome b Gene Structure

The exons are depicted schematically as rectangles. The entire gene stretches over about 8 kb. The coding sequence need only be 0.9 kb, however, so the mature messenger and hence the sum of the exons is 2.3 kb. The mutations in cytochrome b map to one of the ten *box* loci indicated above the line. The petite *ob* used as probe in Figures 2 and 5B maps genetically and physically as shown.

sors do not always appear (Figure 2e).

The complementable intron mutants in *box*-3 and *box*-7 block the processing of the messenger RNA. Figure 2 shows that *box*-3 mutations located in the first long intron block the processing at an early step and lead to the accumulation of the two longest precursors (lane c). The *box*-7 mutant, which lies within a later intron, fails in a late step in the processing since no mature message appears (lane d). The rest of the precursor pattern, however, is similar to that of the wild-type.

Mutations in *box*-1, *box*-2 and *box*-6, exon mutations, do not affect this precursor pattern. Mutant alleles in *box*-4 and *box*-5, however, which are presumably located in the first exon, produce a variety of RNA patterns, some resembling the wild-type and some dominated by the large precursor. Figure 3 shows that allele 4-2 makes virtually no mature messenger whereas 4–3 resembles the wild-type; 4-1 is intermediate. The different alleles of *box*-4 complement weakly and generally at late times. Table 1 summarizes the RNA patterns.

Pleiotropic Effects

The *box*-3 and -7 intron loci were originally characterized as showing pleiotropic effects, specifically blocking the synthesis of the 40,000 dalton subunit of cytochrome oxidase (the product of the *oxi-3* locus) as well as the synthesis of cytochrome b, but not affecting the other two mitochondrially encoded oxidase subunits. Genetic analysis, however, showed that these mutations lay completely within the cytochrome b locus, which is well separated from *oxi-3* on the mitochondrial DNA (Figure 4). Since these mutations block the processing of cytochrome b messages, we hypothesized that the *oxi-3* gene might also be split and have a processed messenger so that the *box*-3 and -7 mutations could exert their effects by blocking that processing. Unfortunately, little is known about the genetic structure of the *oxi-3* locus. To study these RNAs we used an Eco RI fragment probe (Figure 4) that corresponds to sequences removed by deletions that lie within *oxi-3*. Figure 5A shows the patterns of RNAs from various mutants hybridized to the cytochrome oxidase probe. The wild-type mitochondria have two dominant RNA species of 2.6 and

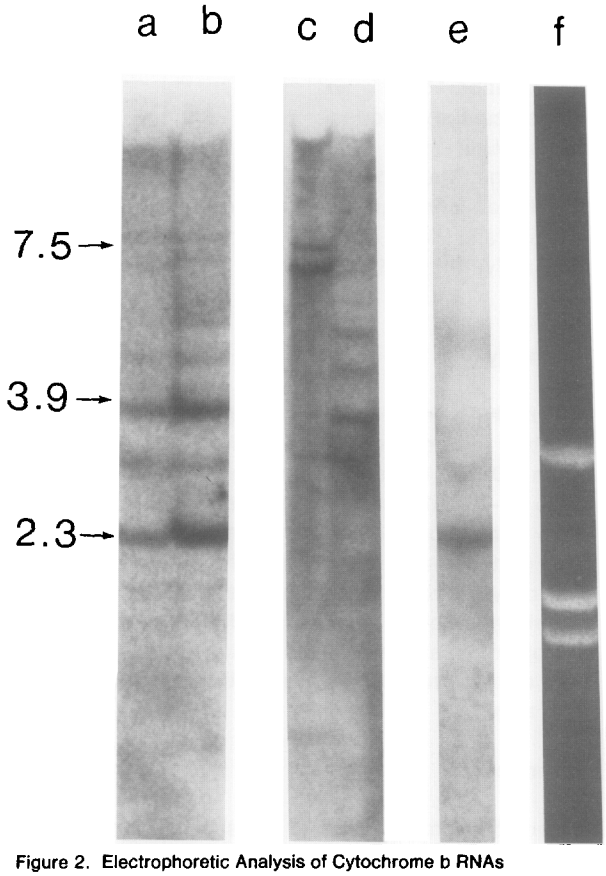

Figure 2. Electrophoretic Analysis of Cytochrome b RNAs

Yeast mitochondrial RNA was isolated by standard procedures (Casey et al., 1972). 800 ml cultures were grown in 2% galactose, 1% yeast extract, 1% bactopeptone (unless otherwise indicated) to mid log-phase. Glusulase- or zymolyase-produced spheroplasts were lysed in 0.6 M mannitol, 20 mM Tris, 1 mM EDTA (pH 7.5); washed 3 times; pelleted for 15 min at 15,000 × g; clarified for 5 min at 2000 × g and extracted with phenol-chloroform, and the RNA was precipitated with ethanol. The dry pellets were resuspended in 6 mM methylmercuric hydroxide, 10 mM sodium sulfate, 0.2 mM EDTA and 50 mM sodium borate (pH 8.2). Each lane contains 5–20 μg of total RNA electrophoresed through 1.4% agarose in the same buffer (Bailey and Davidson, 1976) at 2 V/cm. The RNA in these gels was transferred to diazotized paper according to the method of Alwine et al. (1977), except that the partial alkaline hydrolysis of RNA in situ step was normally omitted. The preincubations and hybridizations were performed as described by Alwine et al. (1977) and Wahl, Stern and Stark (1979). In lanes (a–e) a nick-translated (Rigby et al., 1977) mitochondrial DNA from the petite *ob*, (KL14-4A/RP617A1B1/RD1E13), was hybridized to the immobilized RNA. Lane (f) is the pattern of total RNA observed with ethidium bromide staining, after methylmercury neutralization with mercaptoethanol just prior to transfer. The ribosomal RNA bands dominate the pattern at 1.7, 2.0 and 3.3 kb.

(Lane a) Sacchromyces cerevisiae 777-3A (α ade1 op1) wild-type for mitochondrial genes; (lane b) 777-3A in 2% glucose; (lane c) *box*-3-4 = G55; (lane d) *box*-7 = G1659; (lane e) 777-3A; (lane f) same RNA as in lane (a).

2.3 kb. Both the *box*-3 and *box*-7 cytochrome b mutations affect this pattern and fail to synthesize the 2.3 kb messenger. Furthermore, there are two alleles of *oxi-3* which have pleiotropic effects on cytochrome b. Figure 5A shows that these mutations, W264 and

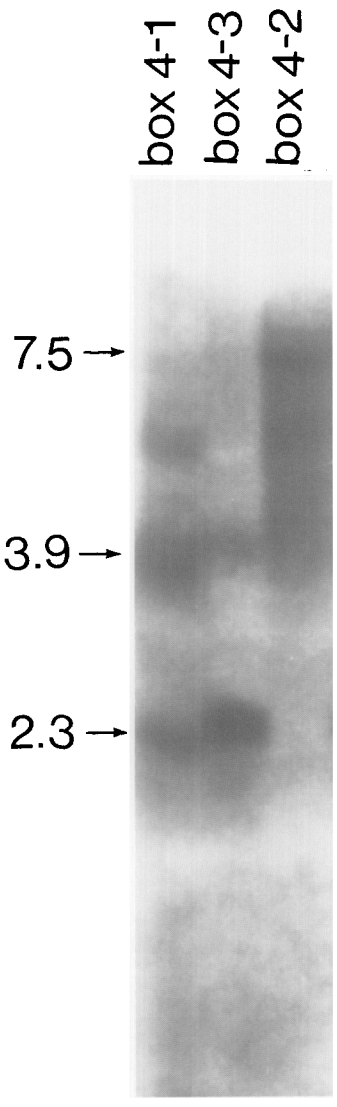

Figure 3. Effects of *box*-4 Mutations on Cytochrome b RNA

RNA was isolated, electrophoresed, immobilized and hybridized with probe *ob* as in Figure 2. The lanes contain RNA from *box* 4-1 (G1334), *box* 4-3 (W7) and *box* 4-2 (G171).

G341, make no mature cytochrome oxidase message but lead to the accumulation of very large precursors with bands at 5.0, 5.8, 9 and 11 kb, which have a size heterogeneity that may be due to selective degradation (not observed when the same size range is reprobed for cytochrome b RNA). Figure 5B shows the same immobilized RNA molecules, now hybridized to the cytochrome b exon DNA from *box*-6. These pleiotropic mutations in cytochrome oxidase also alter the processing pattern of cytochrome b messengers. W264 mimics *box*-3 and accumulates the longest precursors; G341 blocks processing analogously to *box*-7 and accumulates intermediate size precursors. There is, however, a difference: *box*-3 and *box*-7 mutations completely prevent the formation of cytochrome b message, while G341 and W264 only decrease it. No cytochrome b is formed by the former mutants, while a small amount is synthesized by the latter under derepressing conditions (Pajot et al., 1976, and our unpublished data).

Discussion

These experiments demonstrate that the two yeast mitochondrial genes *cob-box* (the cytochrome b gene) and *oxi-3* (the gene for the 40,000 dalton subunit of cytochrome oxidase) produce a series of messenger precursors. The cytochrome b gene shows a processing pattern that proceeds from an 8.5 kb molecule to a 2.3 kb mRNA. We infer that these patterns correspond to the splicing out of intron sequences and conclude that both of these genes have a mosaic structure. Not all mitochondrial genes have this structure, however; the coding sequence of the *oli-1* locus for subunit 9 of the ATPase is not split (Macino and Tzagoloff, 1979; Hensgens et al., 1979).

Mutations that affect only cytochrome b—that is, exon mutations in *box*-1, -2, and -6—do not alter the splicing patterns, although pleiotropic mutants that block synthesis of both cytochrome b and *oxi-3* proteins interfere with both splicing patterns. This is true for mutations that lie in three regions of cytochrome b as well as for two mutations that lie in *oxi-3*. The interference with splicing provides a mechanism for the pleiotropic effect, but what accounts for the effects on splicing? A mutation in a splicing signal, or that RNA sequence in the messenger that must be identified for the splice to occur, would be expected to alter the splicing pattern and disrupt the synthesis of the protein in question. Such lesions, however, should exercise their effects in cis, and should be noncomplementable. The 4-2 allele of *box*-4 (Figure 3) might seem to be a candidate for such a mutation since a large messenger accumulates and the allele does not complement well. The striking phenomenon is that mutants that complement and apparently lie well within introns produce defective splicing patterns. The complementation means that the defects in splicing can be repaired if the mitochondria contain a good RNA sequence; the wild alleles of these mutations must clearly provide some function necessary in trans for the splicing of cytochrome b RNA.

The mutations in the cytochrome b locus that block processing are recessive since they complement. The pleiotropic mutations in this locus are rather common; in one search (Kotylak and Slonimski, 1977) 11 of 20 mutants in cytochrome b affected cytochrome oxidase as well. Thus these mutations are candidates for the loss of function: the *box*-3$^+$ and *box*-7$^+$ alleles could correspond respectively to functions involved in early and later splicing steps for cytochrome b RNA. If the same splicing functions are involved in the processing of the *oxi-3* messenger, we would understand the pleiotropy. This interpretation is strongly supported

Table 1. Summary of RNA Patterns Observed with Petite DNA Probe from *ob* (*box*-6 Region)

RNA Size (kb)	5-1	5-2	4-1	4-2	4-3	3-2	3-4	1-3	7-1	2-1	6-1	wt	G341	W264
8.5	w	w	–	w	–	w	w	–	–	–	–	w	w	w
7.5	+	+	–	+	–	+	+	–	–	–	–	w	w	+
6.6	w	–	–	–	–	–	–	–	w	–	–	w	w	–
5.4	w	–	w	–	–	–	–	–	w	–	–	w	w	–
4.9	–	–	w	–	–	–	–	–	w	–	–	w	w	–
3.9	+	–	+	–	w	–	–	+	+	w	w	+	+	w
3.0	–	–	–	w	–	–	–	–	–	–	w	w	–	–
2.3	+	w	+	–	+	–	–	+	–	+	+	+	w	w
with R5 probe (*oxi-3* region)														
11	–	–	–	–	–	–	–	–	–	–	–	–	w	w
9	–	–	–	–	–	–	–	–	–	–	–	–	w	w
5.8	w	w	–	w	–	–	–	–	–	–	–	–	+	+
5.0	–	–	–	–	–	–	–	–	–	–	w	–	+	+
3.3	–	–	–	–	–	w	w	–	w	–	–	–	–	–
2.6	+	+	+	+	+	+	+	+	+	+	+	+	–	–
2.3	–	–	–	–	+	–	–	+	–	+	+	+	–	–

Data are derived from autoradiograms such as those in Figures 2 and 4. Strong hybridization to a band is indicated by +, weaker hybridization by w and undetectable by –. Variations in large rRNA masking of diazo sites may weaken 3.0, 3.3 and 3.9 kb band intensities and selective degradation may do the same for *oxi-3* bands in the 5–10 kb range. Molecular sizes are calibrated using E. coli 16S and 23S RNAs (1541 and 2904 nucleotides; J. Brosius, personal communication) and denatured phage lambda and pBR322 DNA restriction fragments; these could be off by 10% or more in the high molecular weight range.

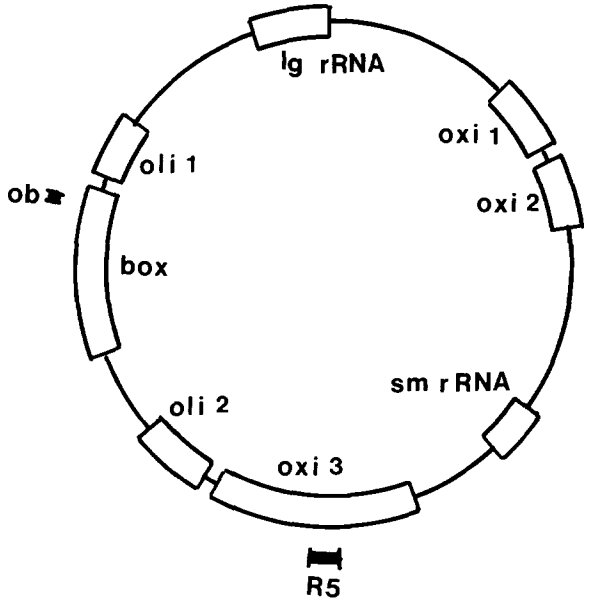

Figure 4. Map of the Yeast Mitochondrial Genome

The locations of *box* and *oxi-3* genes are indicated relative to other known mitochondrial genes on the inner circle. The genome is a 68–76 kb circle (Borst and Grivell, 1978). The positions and approximate sizes of the *box* and *oxi-3* DNA hybridization probes used in Figure 5 are indicated as the outermost dark curves ob and R5, respectively.

by the fact that a deletion mutant in cytochrome b (G625, which spans the entire *box* gene) also eliminates *oxi-3* function. However, there are other strains in which the cytochrome b gene lacks a region corresponding to *box*-3 (but not *box*-7). These strains (D273-10B) are not single site derivatives of the strains we have studied and could have moved the *box*-3 locus or altered the requirement of *oxi-3* for this trans-acting function.

Pleiotropic mutants in *oxi-3* are rare; only 3 of 72 oxidase mutants affected cytochrome b formation (Kotylak and Slonimski, 1977). The two mutations that we have characterized interfere with the processing of cytochrome b at the same two types of steps, early and late, defined by the *box*-3 and *box*-7 patterns. Their effect on the *oxi-3* RNA itself, however, is different from that of the *box* mutants. We think it improbable that these *oxi-3* mutants represent the loss of functions, but suggest rather that they interfere with normal function. The 7300 bp deletion (Morimoto, Lewin and Rabinowitz, 1979a) mutant M10-150, which covers 11 of 13 *oxi-3* restoration groups (Slonimski and Tzagoloff, 1976) and also covers the mutant G341 (Carignani, Dujardin and Slonimski, 1979), makes fully functional cytochrome b. Thus the wild allele of G341 cannot provide a trans function for splicing. These mutations might lead directly to a dominant interfering function or might lead to the accumulation of RNA precursors which themselves might interfere with other splicing. The fundamental difference between the two genes is that deletions of cytochrome b block *oxi-3* expression while deletions of *oxi-3* do not affect cytochrome b.

The pleiotropic effects, however, are quite specific: the large rRNA precursor which contains a 1000 bp

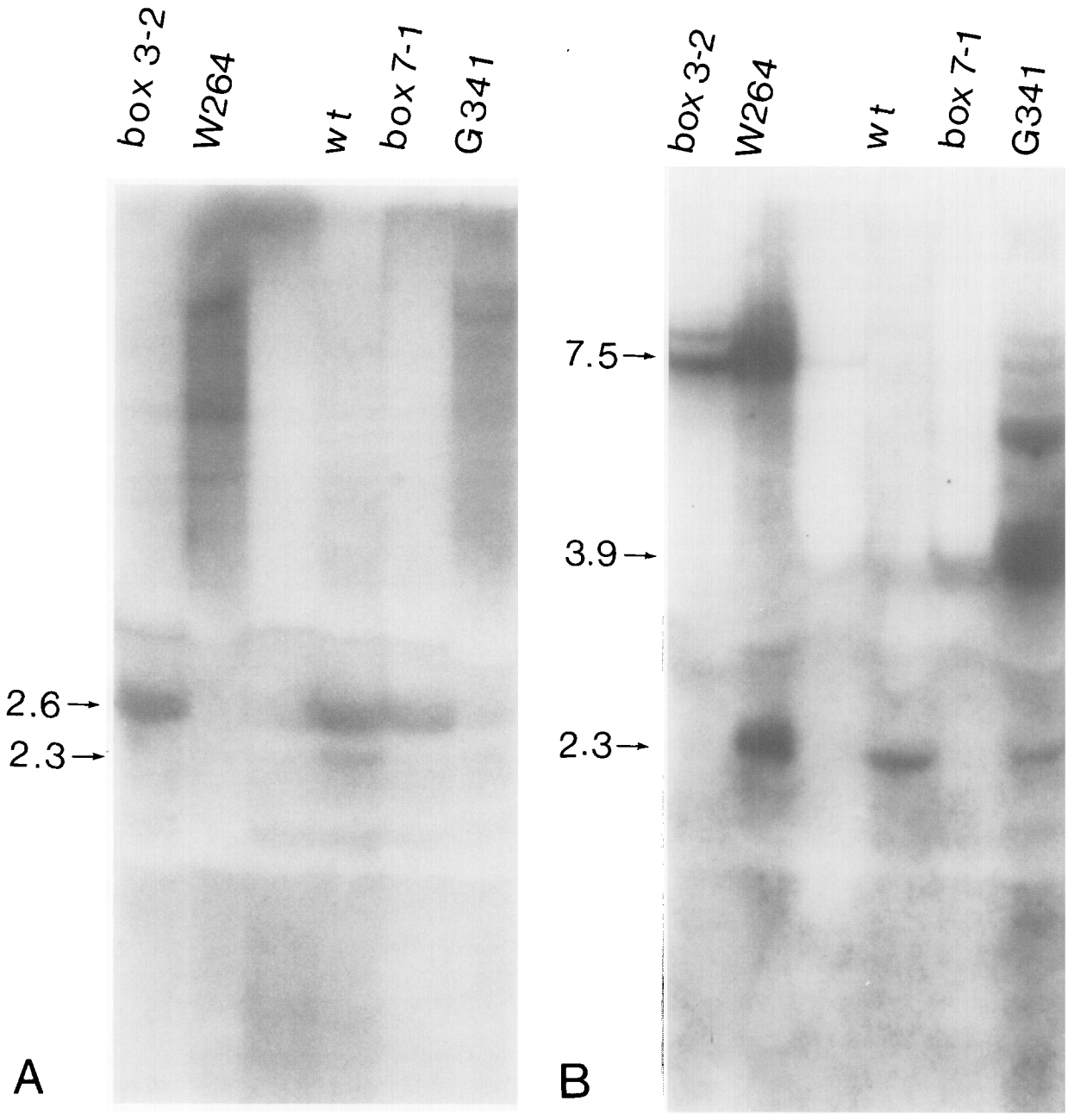

Figure 5. Pleiotropic Effects of Mutants on the RNA Precursors for the *oxi-3* (A) and *box* (B) Genes

RNA was isolated, electrophoresed, immobilized and hybridized as in Figure 2. In (A) the probe is nick-translated DNA from the petite *ob*, an exon of cytochrome b. In (B) the probe is the nick-translated plasmid pBLTB8107 (Berg et al., 1979) (pMB9 plus a 3 kb piece of Eco RI fragment #5 of MH 41-7B mitochondrial DNA, from the *oxi-3* region). The lanes contain RNA from the *box* 3-2 (G1909) and *box* 7-1 (G1659) mutants of cytochrome b, and the *oxi-3* mutants W264 and G341 of cytochrome oxidase.

intron (Jacq et al., 1977; Bos et al., 1978; Heyting and Menke, 1979; Faye et al., 1979) is processed normally in all mutants (since the ethidium rRNA pattern and protein synthesis are unaffected). The other two mitochondrial oxidase genes function normally. Mutant G341 interferes with splicing of *box* RNA only at a final step. Mutants in *box*-3 and -7 do not affect processing of the *oxi-3* 2.6 kb RNA, while this RNA is clearly capable of being held up in high molecular weight forms as observed in the W264 and G341 patterns.

Mutations influencing splicing patterns that have been observed before have been either defects in an intron junction of SV40 (Lai and Khoury, 1979) or lesions in nuclear functions that influence the splicing of mitochondrial rRNAs in Neurospora (Manella et al., 1979) or of tRNAs in yeast (Hopper, Banks and Evangelidis, 1978).

What function might be provided by the *box*-3$^+$ or *box*-7$^+$ alleles? In principle, since these mutations lie in long and otherwise silent regions, these areas could code for a protein product needed for splicing. We think that this is improbable, since in petite mitochondria containing only the cytochrome b region (which are unable to synthesize any mitochondrially encoded protein), RNA the size of mature cytochrome b mRNA is visible on ethidium-stained gels (Morimoto, Locker and Rabinowitz, 1979b). Other arguments against the involvement of proteins, based on complementation by petites, are given by A. Lamouroux et al. (unpublished data). Thus the RNA itself, from the *box*-3 and -7 regions, is likely to be the active participant. We propose that these regions of RNA, either in the precursors or as free introns, serve a guide function to direct a splicing enzyme correctly. The simplest such model would be that a guide RNA serves by hybridization to align the points to be spliced by an enzyme. Multiple and more subtle uses of RNA base pairing might be involved in pulling out the twisted skein of messenger RNA to make substrates for splicing. The enzymes involved are presumably all nuclear; thus there is no problem with the splicing occurring in petites. In the nucleus the splicing enzymes are presumably always associated with general guide RNAs. These RNAs, however, would not function in the mitochondria since the mitochondrial membrane seems to be impermeable to nucleic acids. All mitochondrial RNAs are synthesized inside (ribosomal, transfer and messenger), while most of the proteins, the polymerases and the components of the biosynthetic machinery are produced on the outside. Thus if guide RNAs are needed for splicing, they too must be encoded in the mitochondrial DNA.

The mutations block the cytochrome b splicing pattern in two ways, suggesting that there is some definite order of steps involving more than two splicing elements. The *box*-3 mutant completes one processing step, but then the 7.5 kb intermediate accumulates even in the presence of the *box*-7$^+$ sequence. When *box*-3$^+$ is provided in *box*-7 mutants, the next series of splices takes place but does not go to completion to form a mature message. Thus the *box*-7 guide behaves as though it determines an ultimate step; the *box*-3 guide either participates in all the intermediate steps or triggers a critical splice required before others can be made. In general, in such RNA guide models the actual guiding element for a given splice might be the product of some earlier splicing event. The *box*-3 mutations, in blocking an early event, might in so doing eliminate a whole set of guide elements. This interpretation may apply to the behavior of the 4.2 allele: a cis defect in splicing, possibly at an exon-intron junction, may block the later release of a critical component.

We point out that the critical tests of this model have not been performed. Such experiments should demonstrate that the splicing pattern is normal in a petite mutation that carries only the cytochrome b gene, that the mutations affect the pattern appropriately and that complementation between petites restores the splicing by supplying only the RNA molecules.

In any case, the phenomena described here show that there can be functions in introns that affect the splicing of other RNAs in trans. In principle, the production of a new RNA can result in the encouragement or discouragement of splicing of other sets of RNA molecules and thus serve as a general regulatory mechanism.

Experimental Procedures

Hybridization Probes

The petite strain ob (KL14-4A/RP617A1B1/RDE13) contains DNA localized to the *box*-6 locus (Figure 1) by restoration of only *box*-6 mutants (Slonimski et al., 1978b; G. Jacq, unpublished data) and gives hybridization patterns similar to that of a 400 bp fragment containing only the *box*-1 region (Berg et al., 1979; G. Church, unpublished observations). The plasmid pBLTB8107 (containing a fragment of Eco RI fragment #5), referred to as R5 in Figure 4 and Table 1, hybridizes uniquely to single Eco RI, Hha I and Xba I restriction fragments overlapping within the *oxi-3* region (Berg et al., 1979).

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