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The untranslated leader of nuclear COX4 gene of *Saccharomyces cerevisiae* contains an intron

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ABSTRACT

The nuclear gene for subunit IV of cytochrome oxidase (*COX4*) in *Saccharomyces cerevisiae* contains a 342 bp intron which is contained entirely within the 5' leader of the message. Splicing of the intron results in removal of several small open reading frames; subsequently, the *COX4* AUG becomes the 5' proximal initiation codon. A strain with an *rna2*$^-$ mutation fails to splice mRNA efficiently at restrictive temperature and was used to map the intron splice junctions by RNase protection. Two major mRNA initiation sites were mapped by primer extension of synthetic oligodeoxynucleotides. The splice junctions and internal TACTAAC box conform to consensus sequences previously determined from other yeast introns. One gene for subunit V of cytochrome oxidase (*COX5b*) has also been shown to contain an intron (1). The significance of introns in two nuclear genes encoding subunits of cytochrome oxidase is discussed.

INTRODUCTION

Intervening sequences within coding sequences have been well documented in nuclear genes in higher cells, in genes of the mitochondria and chloroplasts, in nuclear genes of yeast (for review see 2 and 3), and in a gene from the bacteriophage T4 (4). Possible functions for these sequences have been proposed. For example, speculation has been raised that intervening sequences may separate structural or functional domains of a polypeptide chain (5, 6, 7). In the yeast *Saccharomyces cerevisiae* intervening sequences in nuclear genes occur more rarely than in corresponding genes from other yeast or higher cells. Introns in *Saccharomyces cerevisiae* appear to be found in certain classes of genes, for example, many genes encoding tRNA's contain introns (for review see 8). Genes encoding certain cytoskeletal structural proteins such as *ACT1* encoding actin (9, 10), or *TUB1* and *TUB3* encoding α-tubulin (11), contain introns. Also, several genes encoding ribosomal proteins bear introns (12, 13). The product of one of these genes, the large subunit protein L32, appears to regulate its own synthesis by inhibiting splicing of its

mRNA when the protein is in excess (14, 15). This example is the best case yet of splicing serving a regulatory function.

The biochemical mechanism by which splicing of mRNA genes occurs has been worked out recently. In *Saccharomyces cerevisiae* this mechanism appears to be conserved with several notable differences from higher eukaryotes. Most obvious among the differences is a strict conservation within the intron of a sequence that serves as the site of lariat formation during splicing. This sequence, TACTAAC, has been found without variation in all mRNA introns of *Saccharomyces cerevisiae* (12, 13, 16). Evidence has been adduced that the efficient use of the 3' acceptor site, AG, is dependent on the distance of the site from the TACTAAC box (17). In all previous introns studied, the AG lies not further than 53 bp from the TACTAAC box. In addition to the strict conservation of the TACTAAC box, the 5' donor splice sequence GTATGT is more strictly conserved in *Saccharomyces cerevisiae* than in higher cells, although not invariantly (1).

In this report we present data that the nuclear *COX4* gene of *Saccharomyces cerevisiae*, encoding subunit IV of cytochrome oxidase, contains an intron in the untranslated leader region of the message. Strikingly, a second nuclear gene encoding an oxidase subunit, *COX5b*, the minor isolog of subunit V, has recently been shown to contain an intron early in its coding sequence (1). The possible significance of introns in these two genes and other yeast genes is discussed.

RESULTS

Sequence of COX4 5' to the coding region

The DNA sequence of a region extending 919 nucleotides upstream of the *COX4* translational start was determined (see Figure 1). The presence of a TACTAAC sequence 60 nucleotides upstream of the *COX4* ATG codon (position -60 relative to the ATG) led us to suspect that the *COX4* mRNA might initiate upstream of this sequence and contain an intron in its leader. Consistent with this view, a perfect 5' donor sequence, GTATGT, was found 294 nucleotides (position -354) upstream of the TACTAAC box. A site which fits the consensus for a 3' acceptor site derived from ribosomal protein gene introns is located 48 nucleotides downstream of the TACTAAC box (position -13). Another AG is located at position -5. Thus, the possible introns that utilize these signals would be 342 and 350 nucleotides long.

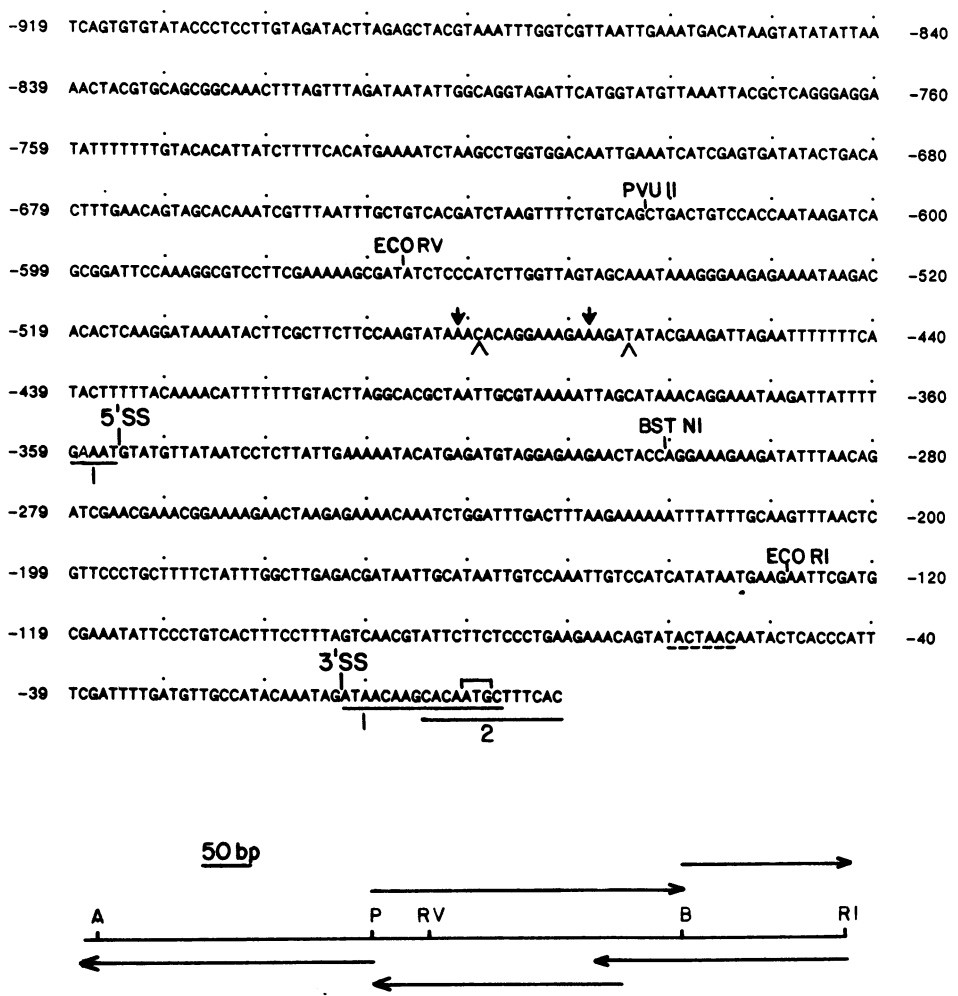


Fig. 1. The sequence of the region upstream of *COX4*. The first codon of the *COX4* reading sequence is indicated by the bracket above the sequence at +1. The region downstream of the *Eco*RI site (-129) has been published by Maarse, *et al.*, (24); part of it is shown here to indicate the 3′ splice site. The 3′ and 5′ sites are indicated above the sequence and the TACTAAC box is underlined with a dotted line. Probes used for primer extension are underlined; probe 1 spans the splice junction. Arrows above the sequence indicate major mRNA initiation sites as determined by primer extension of probe 1 (positions -468 and -481). Carats under the sequence indicate initiation sites determined by RNase protection. The sequencing strategy of the region upstream of the *Eco*RI site is shown at the bottom. Restriction enzyme abbreviations are *Acc*I (A), *Pvu*II (P), *Eco*RV (RV), *Bst*NI (B), *Eco*RI (RI).

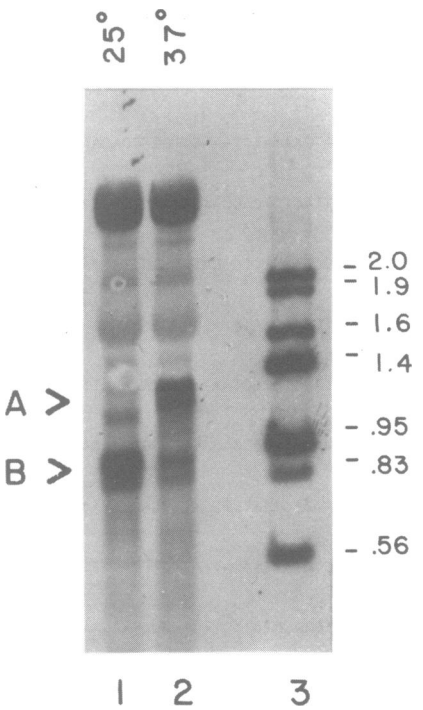


Fig. 2. Northern analysis of DNA from RY426 (*rna2*-1) containing the high copy *COX4* plasmid 19.4. The blots were probed with SPA1 (see Figure 3) and nick-translated λ DNA. Size markers shown in kilobase pairs in **lane 3** were made from a *Hind*III,*Eco*RI digestion of λ DNA. **Lane 1**; RNA from permissively grown cells (25°C). **Lane 2**; RNA from cells after a shift to restrictive temperature (37°C). Note that the group of bands indicated by **B** in **lane 1** decrease in intensity in **lane 2**, while a more slowly migrating group indicated by **A** increases. This probe also hybridizes to ribosomal RNA, seen as a heavy band at the top of the lane.

Analysis of COX4 mRNA in an rna2$^-$ mutant

The *rna2*$^-$ mutant is temperature sensitive for growth and results in a failure to splice efficiently introns from pre-mRNA sequences at the restrictive temperature (37°C) (18). Thus, if the *COX4* message contains an intron, it should accumulate in precursor form at the restrictive temperature in an *rna2*$^-$ strain. Further, if the splicing signals described in the previous section are functional, the precursor should appear about 350 nucleotides larger than the mature message.

To amplify the levels of *COX4* mRNA, we introduced into the *rna2*$^-$ strain a high copy number plasmid (p19.4) containing the *COX4* gene with 1.7 Kb of 5′ flanking DNA and .800 Kb 3′ flanking DNA. RNA prepared from

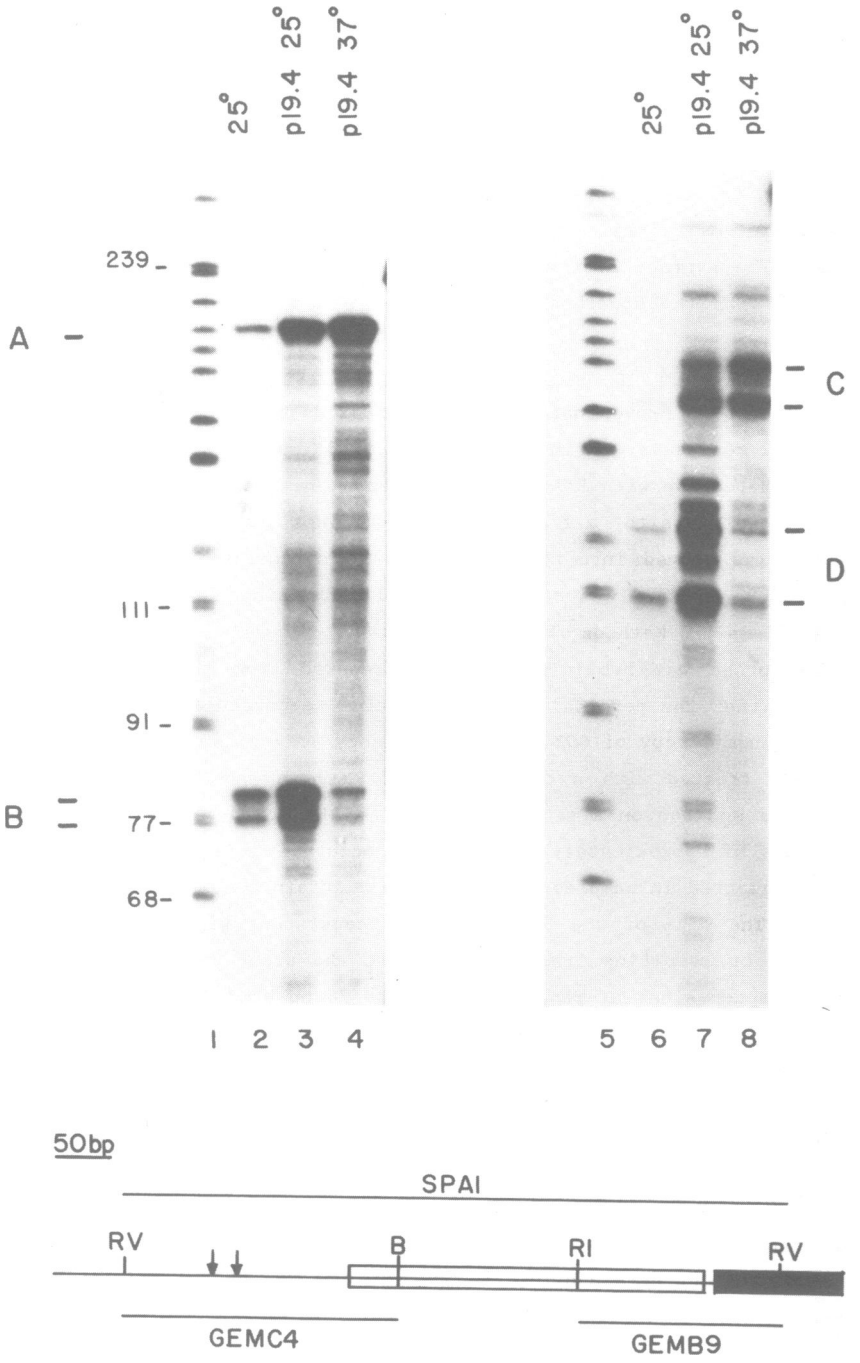
this strain was run on a formaldehyde agarose gel and blotted onto a nylon membrane. The blots were probed with RNA complementary to the message derived from an *Eco*RV fragment containing the 5' end of *COX4* (probe SPA1, Figure 3).

At permissive temperature, a group of bands 0.81-0.87 kbp is seen (see Figure 2, lane 1, B). At restrictive temperature, these bands decrease in intensity while a new group at 1.20-1.27 kbp (lane 2, A) increases. The average difference in size is 390 bp. These results are consistent with the hypothesis that there is a intron in the leader of *COX4*.

Mapping of the acceptor and donor sites of the COX4 intron

In order to prove that the conserved sites described above are actually used to specify an intron, the 5' and 3' splice sites were mapped by RNase protection with RNA probes that span one site or the other. To map the 3' splice site, a fragment which overlaps the site (196 bp *Eco*RI to *Eco*RV) was cloned into pGEM2 in order to synthesize a complementary probe (GEMB9) uniformly labeled with α^{32}P GTP using T7 RNA polymerase (see Materials and Methods; Figure 3).

The probe was hybridized to RNA prepared from the *rna2*$^-$ strain grown at either the permissive or restrictive temperature and containing either a genomic copy of *COX4* or the high copy *COX4* plasmid (p19.4). Hybrids were treated with a combination of RNase A and T1; the products were melted and run on a sequencing gel (see Figure 3, lanes 2-4). Two major bands of approximately 76 and 80 bp occur (indicated by B) and are greatly augmented in samples prepared from a strain bearing the multicopy plasmid. The size of the 80 bp band is consistent with the predicted size of 79 bp resulting from protection of the probe from its 5'end to the 3' acceptor sites at position -13. The 76 bp band is an artifact of the assay (see below). The intensity of these bands is diminished when RNA from cells shifted to the restrictive temperature is employed. In this case, the intensity of a band corresponding to a fragment of 195 nucleotides (indicated by A) is augmented. This fragment represents the entire region of homology between the probe and the unspliced *COX4* mRNA. The difference in quantity of unspliced mRNA isolated at the lower and higher temperature as indicated by the RNAse protection is not as great as that on the Northern hybridization (see figure 2, band A and figure 3, band A). This difference can be attributed to variability in the amount of unspliced mRNA between isolates.


25°
p19.4 25°
p19.4 37°
25°
p19.4 25°
p19.4 37°
239
111
91
77
68
A
B
C
D
1 2 3 4
5 6 7 8
50bp
SPAI
RV
B
RI
RV
GEMC4
GEMB9

In order to map the 5′ donor site, a 265 bp *Bst*N1-*Eco*RV fragment which spans the proposed donor site was cloned into pGEM2 and transcribed by T7 RNA polymerase (see Figure 3). This probe (GEMC4) extends from a site 55 nucleotides (position -301) downstream of the proposed 5′ splice site (i.e., within the proposed intron) to a site 210 nucleotides (position -567) upstream of the proposed site. The probe was used for RNase mapping as above. At permissive temperature, there are two prominent bands of 110 and 125 nucleotides (Figure 3, lanes 6 and 7,band D). These bands correspond to protected fragments extending from the 5′ ends of the mRNA to the 5′ splice site (see below). With RNA prepared from cells grown at the restrictive temperature, these two bands decrease in intensity, while two more slowly migrating bands at 160 nucleotides and 180 nucleotides show an increase (lane 8, band C). The increase of the 160 nucleotide band is not strongly evident in this figure, possibly due to overexposure of the gel. In duplicate experiments the 160 nucleotide band was about two times as dark as the 180 nucleotide band at 25°; both bands increased in intensity upon shift to the higher temperature. These bands correspond to protected fragments extending from the 5′ end

Fig. 3. RNase protection of RNA from RY26 (rna2-1) with probes that span the splice sites. **Lanes 1 and 5** contain size markers of pBR322 digested with *Msp*I and end-labeled. **Lanes 2-4** show RNA fragments protected by probe GEMB9. **Lanes 6-8** show fragments protected by probe GEMC4. **Lanes 2 and 6**: RNA from cells containing only one copy of *COX4* grown at the permissive temperature (25°C). **Lanes 3, 4, 7, and 8**: RNA from cells containing a high copy COX4 plasmid 19.4, grown at permissive (25°C) or restrictive (37°C) temperatures, as indicated. The bands indicated by **B** are digestion products which map the 3′ splice site. Band **A** corresponds to complete protection of the region to which the GEMB9 probe hybridizes. It thus indicates the level of unspliced message. The major bands indicated by **D** correspond to protection of the region from the 5′ splice site to the 5′ end of the message. Bands indicated by **C** correspond to protection of the region from the end of GEMC4 within the intron (see below) to the 5′ end of the message. At the bottom of the figure is shown a representation of the region upstream of COX4. The shaded box is the upstream part of the COX4 coding region. The open box shows the position of the intron. Arrows indicate mRNA initiation sites. The regions covered by probes SPA1, GEMB9, and GEMC4 are indicated. All probes are complementary to the mRNA produced from this region, thus the 5′ end of each probe is the right end of each line. All probes have a small additional, noncomplementary sequence on the 5′ end due to transcription of poly-linker regions in the vectors into which the fragments were cloned. Restriction sites are abbreviated as follows: *Eco*RV (RV), *Bst*N1 (B), *Eco*RI (RI).

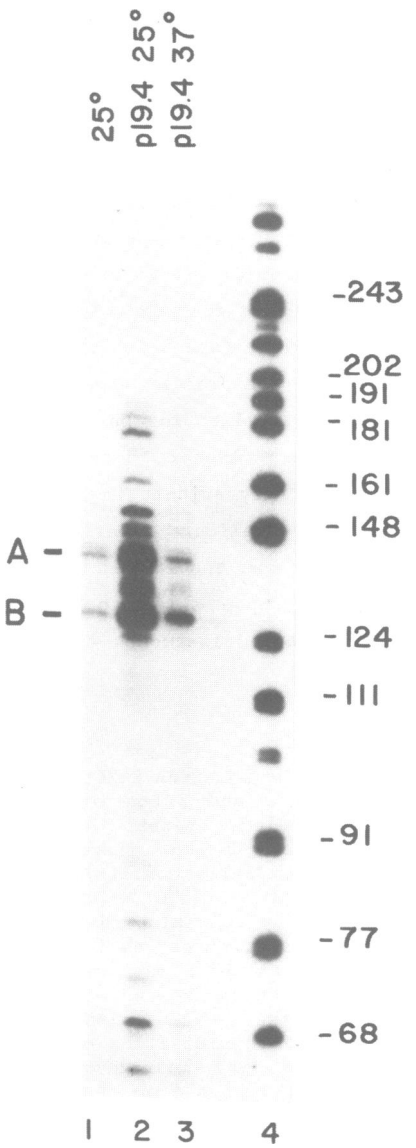


Fig. 4. Mapping of the mRNA initiation sites by primer extension of primer 1. **Lanes 1-3** show reverse transcripts generated when primer 1 is used. **Lane 1**; RNA from RY26 containing one copy of *COX4* grown at the permissive temperature (25°C). **Lanes 2 and 3**; RNA from cells with p19.4 (high copy *COX4*) grown at permissive (25°C) or restrictive (37°C) temperatures, as indicated. **A** and **B** refer to the two bands representing major mRNA start sites. **Lane 4** shows *Msp*I digested, end-labeled pBR322 markers.

of the mRNA to the end of the probe at the *Bst* N1 site. The difference in size of protected fragments yielded by RNA prepared from cells grown at restrictive compared to permissive temperature (50-55 nucleotides) corresponds with the predicted distance of 54 nucleotides from the *Bst*N1 site to the 5' splice site. Thus, we conclude that the sequence GTATGT is actually used as a 5' donor site. Further, we conclude that *COX4* mRNA initiates at two major sites, 110 and 125 nucleotides upstream of the donor site (positions -464 and -479).

Primer extension confirms splice junction and transcript initiation sites

In order to confirm the above conclusions, we synthesized a primer of 21 nucleotides spanning the splice junction which should hybridize and prime synthesis by reverse transcriptase from spliced but not unspliced *COX4* mRNA. The primer spans the junction defined by the upstream of the two possible 3' acceptor sites (see Figure 1, primer 1). When hybridized to RNA prepared from cells grown at the permissive temperatures, primer extension yields two major bands of 130 and 143 nucleotides, indicating initiation sites at positions -468 and -481 (Figure 4, lanes 1 and 2). As expected, the intensity of the two bands is greatly diminished when RNA prepared from cells grown at the restrictive temperature is employed (lane 3). These results confirm that the proposed 5' donor site and the upstream of the two possible acceptor sites are functional, and corroborate the positions of mRNA initiation determined above.

The presence of an additional band at 76bp in mapping of the 3' acceptor site (see Figure 3, lanes 2-4) indicated the possible utilization of another splice site. In order to test this possibility, a primer which hybridized downstream of position -5 was synthesized (see Figure 1, primer 2). If a 3' splice site occurs at position -5 as well as at -13, primer extension should yield four major products. Primer extension from this oligonucleotide with RNA from cells grown at permissive temperature gives rise to two major products of 135 and 150 bp (see Figure 5, lane 2). The pattern obtained is the same as for the previous primer (lane 1), except the bands are about 6bp longer due to the difference in primers and hybridization sites. These results confirm that only the upstream AG at -13 is used as a donor site. Thus, we conclude that the 76bp band resulting from RNAase protection is an artifact of the assay and probably a degradation product of the 80 bp band.

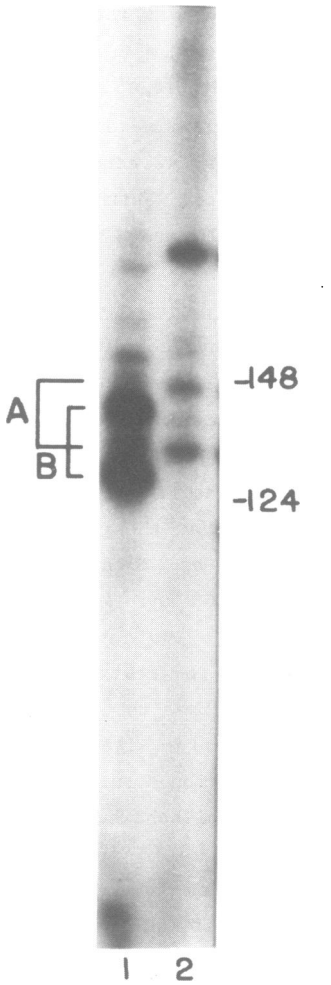


Fig. 5. Primer extension using primer 2 and RNA from p19.4 containing cells grown at 25°C is shown in lane 2. Lane 1 shows primer extension results with the same RNA and primer 1 (as in Figure 4, lane 2) for comparison. B indicates the two major start sites as determined above. A indicates the corresponding bands for oligonucleotide 2.

DISCUSSION

We have shown the existence of an 342 nucleotide intron in the untranslated leader of the *COX4* mRNA, which encodes subunit IV of cytochrome oxidase in *Saccharomyces cerevisiae*. The intron contains the sequence TACTAAC found in all other intron-containing mRNAs of

Saccharomyces cerevisiae. The sequence of the sites at which the intron is spliced conform to consensus sequences derived from other yeast introns: GTATGT for the 5′ splice site and (T/A)T(T/A) t N (a/t)(a/t)(T/C) AG for the 3′ splice site (12). This intron lies 13 nucleotides upstream of the AUG codon at the start of the *COX4* coding sequence; this triplet is the first initiator codon of the spliced *COX4* mRNA. It is unlikely that the intron encodes any *trans*-acting function. The longest open reading frame encoded by the intron is only 34 codons; it appears not to be expressed since fusing it to *lacZ* does not result in synthesis of β-galactosidase (J.C. Schneider, unpublished data).

Our finding along with that of the Poyton laboratory (1) suggest that at least a fraction of the nuclear genes encoding subunits of cytochrome oxidase represent another set of related genes that bear introns. What do these groups of intron-bearing genes have in common? We note that the products of several such groups, e.g. cytochrome oxidase, ribosomal proteins, and tubulin subunits, are required in stoichiometric amounts to form complexes. Although it is tempting to speculate that introns are involved in fine tuning the coordinate expression of these gene products, we have obtained no evidence that splicing of *COX4* mRNA is regulated by physiological conditions. It is conceivable, though, that splicing could be inhibited by a perturbation in the ratio of cytochrome oxidase subunits expressed. Such regulation has been observed for the ribosomal protein L32 mRNA; overexpression of a complementary DNA of the rpL32 gene was shown to inhibit splicing of rp L32 mRNA in *trans* (14).

It is also possible that most introns in genes of *Saccharomyces cerevisiae* represent evolutionary remnants. Recent analysis of intron positions in the triosephosphate isomerase gene in maize and chicken suggests that introns were present in the common ancestor of plants and animals and possibly in earlier cells (19). Evidence from chicken preproinsulin genes supports the notion that intron differences between cells result from intron loss, not gain (20). Thus, introns may have been common in the ancestors of *Saccharomyces cerevisiae*, but lost during evolution.

The positions of introns in *Saccharomyces cerevisiae* show a very strong bias to the beginnings of the coding sequences. The *COX4* intron, along with that of the rp29 gene represent extreme cases in which the intervening sequences lie in untranslated mRNA leaders (21). It is possible that introns could have been lost in evolution through the combined action of reverse transcription and gene conversion (G. Fink, personal

communication). This mechanism would favor intron loss at the promoter distal end of the gene since reverse transcription could generate products that are primed at the 3' end of the mRNA and terminate in the body of the message. Such products may result in the removal of introns in promoter distal portions of genes by recombination with the genome. There are several candidates for such a reverse transcriptase activity in yeast. Ribonuclease H(70) has been found to display reverse transcriptase activity under conditions that inactivate the RNAase activity (22). In addition the Ty element encodes a reverse transcriptase activity allowing it to transpose via an RNA intermediate (23).

MATERIALS AND METHODS

Strains and plasmids

Strain RY26 (*rna*2-1 (ts368), *ura*3-1, *ura*3-2, *ade*-1, *ade*-2, *tyr*1, *his*7, *can*R) was provided by R. Young. M13mp18, M13mp19, and λ DNA were from New England Biolabs, Inc. Plasmid SP65 was purchased from Promega Biotec. Plasmid GEM2 was a gift from J. Fridovitch-Keil. The plasmid 19.4, containing the COX4 clone and a 2μ origin of replication was a gift from H. Riezman (see 24).

Enzymes and biochemicals

Restriction enzymes, polynucleotide kinase and DNA polymerase I (Klenow fragment) were obtained from New England Biolabs, Inc. T4 DNA ligase was from Collaborative Research. AMV reverse transcriptase was purchased from Life Sciences, Inc. T7 RNA polymerase, SP6 RNA polymerase and RNasin were from Promega Biotec. α^{32}P-GTP (400Ci/mmole) was obtained from Amersham. γ^{32}-ATP (7000 Ci/mmole) was from ICN. The upstream synthetic deoxyoligonucleotide was made on an Applied Biosystems model 380A DNA synthesizer. The downstream oligonucleotide was purchased from ChemGenes Corp.

Sequencing

Fragments were cloned into M13mp18 and M13mp19 and sequenced by the dideoxy chain-termination method (25).

RNA isolation

RNA was prepared from cells by glass bead lysis as in Teem and Rosbash (26). For isolation of RNA from cells grown at the restrictive temperature, cells grown at 25°C were added to an equal volume of media at 49°C. Incubation was continued for 1 hour at 37°C.

RNA transfer and hybridization

Electrophoresis of RNA in a formaldehyde agarose gel, capillary

transfer to filter paper and stock solutions used below are as described in Maniatis (27). The filter was baked at 80°C for two hours under vacuum and prehybridized in a solution of 0.5% SDS, 100 μg/ml sonicated calf thymus DNA, 10X Denhardt's solution and 4X SSC overnight at 55°C. Hybridization was carried out in a solution of 4X SSC, 10mM Tris pH7, 0.1% SDS, 10X Denhardt's solution to which the SPA1 probe was added; this mix was incubated overnight at 55°C. The filter was washed twice with 2X SSC for 5 min at 25°C, twice with 2X SSC, 1% SDS for 15 min at 55°C and twice with 0.1X SSC for 15 min at 25°C.

Plasmid construction and probe synthesis

Plasmid SPA1 was made by ligating the 495bp *Eco*RV fragment into pSP65 which had been digested at the *Hinc*II site. Plasmid GEMB9 was constructed by ligating the 196bp *Eco*RV - *Eco*RI fragment into pGEM2 which had been digested at *Hinc*II and *Eco*RI. The fragment cloned into pGEM2 in order to make pGEMC4 was derived from a plasmid which has a 12-mer *Bam*HI linker inserted at the upstream *Eco*RV site. The fragment was made by digesting the linker containing plasmid with *Bst*NI, filling in with the Klenow fragment of DNA polymerase I and dATP, then digesting with *Bam*HI. This fragment was ligated into pGEM2 which had been digested with *Hinc*II and *Bam*HI, to give pGEMC4.

The vectors were linearized with an appropriate restriction enzyme (pSPA1 with *Hind*III, pGEMB9 with *Eco*RI and pGEMC4 with *Bam*HI). Continuously labeled RNA probes were synthesized with α^{32}P-GTP and SP6 RNA polymerase (for pSPA1) or T7 RNA polymerase (for pGEMB9 and pGEMC4), using a modification of a procedure described by Melton (28). The modification is described by Promega Biotec (transcription protocol #1 in Technical Bulletin 2). Probes synthesized from pGEMB9 and pGEMC4 were purified on a 8% acrylamide-8M urea gel. The gel slice containing the probe was cut from the gel; the probe was eluted in 0.5 M sodium acetate, 1mM EDTA and 0.1% SDS and concentrated by ethanol precipitation.

RNase mapping

RNA probes GEMB9 and GEMC4 were hybridized to RNA and digested with a combination of RNase A and RNAse T1 as described in Melton (28), except that the hybridization of RNA and riboprobe at 45°C was carried out for only two hours, and the temperature of the RNase digestion was 15°C. The products were resuspended in 80% formamide loading dye and electrophoresed on an 8% acrylamide-8M urea sequencing gel. The size marker, pBR322 digested with *Msp*I, was end-labeled with α^{32}P-dCTP and DNA poly-

merase I (Klenow fragment) . The sizes of fragments produced were deduced by referring to a semi-log graph of basepairs versus mobility of the pBR322/ *Msp*I digest.

Primer extension

The primers 5'GCATTGTGCTTGTTATATTCC3' and 5'GTGAAAGCATTGTG3' were labeled at the 5' end with γ^{32}P-ATP and polynucleotide kinase. Hybridization to RNA and extension of the primers were carried out as described by Hahn (29). Products were analyzed on a 8% acrylamide-8M urea gel.

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