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# Organization and expression of the COX6 genetic locus in *Saccharomyces cerevisiae*: multiple mRNAs with different 3′ termini are transcribed from COX6 and regulated differentially

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ABSTRACT

COX6 and its surrounding genetic locus have been characterized for the yeast Saccharomyces cerevisiae. Flanking genes are found closely spaced upstream and downstream of COX6. The upstream gene and COX6 are transcribed from opposite strands and are separated by no more than 300 bp. COX6 is transcribed into three different size classes of mRNA (1000b, 830b, and 700b) differing in length in their 3' untranslated regions. All three classes of mRNAs are found on polysomes and, hence, are most likely translated. The different COX6 mRNAs vary in abundance during growth in rich media and are affected differentially as cells are shifted into media containing high or low glucose concentrations. The largest mRNA is much more susceptible to glucose repression/derepression than are the two smaller mRNAs, whereas the smallest RNA is preferentially accumulated during growth in rich media. These findings demonstrate that COX6 mRNAs with different 3'-termini are either synthesized differentially or differ in stability and suggest the existence of a complex system regulating COX6 expression.

## INTRODUCTION

The assembly and function of holocytochrome c oxidase in eukaryotes requires the protein products of both nuclear and mitochondrial genes (1). In the yeast, Saccharomyces cerevisiae, three mitochondrial genes (COX1, COX2, and COX3) and six nuclear genes (COX4, COX5a or COX5b, COX6, COX7, COX8, and COX9) encode the subunit polypeptides of the holoenzyme (2,3). COX5a and COX5b encode interchangeable isologs of subunit V, designated Va and Vb (4). The other subunits are specified by unique genes present in single copy (3). Although COX1, COX2, and COX3 are linked, by virtue of being on a single circular mitochondrial DNA molecule, each has its own promoter (5) and is capable of being independently regulated. The nuclear genes COX4, COX5a, COX5b, COX6, COX8, and COX9 are unlinked and present on different chromosomes (3). Given that these structural genes specify protein products that are required for a fully functional holoenzyme, their expression raises several interesting questions. For example, are all nine genes coordinately regulated and at what level of gene expression is this regulation effected?

Expression of many yeast genes is regulated at the level of transcription (6, 7) by trans-acting factors that bind to their 5' regions and thereby affect transcription initiation. However, regulation of mRNA 3' end formation in yeast has also been observed. Multiple 3' ends of URA3 mRNA (8) and ALG7 mRNA (9) have been recognized, and in the case of ALG7, have been correlated with changes in gene dosage (9). Previously, we sequenced COX6 and provided evidence that, like URA3 and ALG7, it encodes multiple mRNAs (10). To understand the complexity of RNA associated with the COX6 locus and its variable pattern (10, 11) from cells grown under different conditions, we have characterized the genetic locus around COX6 and have mapped the 5' and 3' ends of the COX6 mRNA. Like the other nuclear COX genes and several other nuclear genes in yeast, COX6 mRNAs exhibit microheterogeneity at their 5' ends. However, unlike the other nuclear COX genes and by analogy to the yeast ALG7 gene (9), the COX6 gene produces at least three different size classes of mRNA that differ in the lengths of their 3' untranslated regions. Surprisingly, the relative abundance of these three classes of mRNA is modulated by growth conditions.

## MATERIALS AND METHODS

### Strains and Media

All plasmids were propagated in Escherichia coli strain HB101 as described (12). All M13 phage were propagated in E. coli strain JM109 (13) and were grown in YT medium (0.8% Bacto-tryptone, 0.5% Bacto yeast extract, 0.5% NaCl). Most work reported here employed the yeast strain, JM43 (MAT , leu 2-3, leu 2-11, ura 3-52, trp 1-289, his 4-550). All yeast strains were grown either in YPD media (1% Difco yeast extract, 2% Difco Bacto-peptone, 2% Dextrose) or SD media (0.67% Bacto yeast nitrogen base without amino acids, 2% Dextrose) supplemented, as needed, with 40 ug/ml of leucine, uracil, tryptophan, or histidine.

### DNA Methods

All procedures for plasmid preparation by alkaline extraction, fragment isolation, Southern blotting, filter hybridization, and probe preparation have been described (10). Restriction endonuclease cleavages were performed as recommended by the suppliers (Bethesda Research Labs, Boehringer Mannheim, or New England Biolabs). DNA sequence determination was conducted as previously indicated (10).

### RNA Methods

RNA was isolated from yeast cells at various points during growth. We used the following isolation procedure modified from Wright et al. (10) for a

500 ml culture. Cells were harvested by centrifugation (5900 g for 6 min), washed once in 50 mls of deionized $H_2O$, collected by centrifugation (as above), and resuspended in 10 mls of 30° C prewarmed sphaeroplasting buffer (50 mM potassium phosphate, pH 7.5, 0.95 M Sorbitol, 14 mM β-mercaptoethanol, 7500 U lyticase, 2 mM aurintricarboxylic acid). Cells were digested for 5 minutes at 30° C and were promptly centrifuged (4° C) at 4350 g for 6 minutes. Cell pellets were lysed in 4 M guanidinium isothiocyanate, 25 mM sodium acetate, pH 5.0, 100 mM β-mercaptoethanol, 2 mM aurintricarboxylic acid. Lysates were immediately extracted with phenol-chloroform-isoamyl alcohol (24:24:1). RNA was purified from the extract by centrifugation on cushions of 5.7 M CsCl, 0.1 M EDTA as described previously (10). Poly $A^+$ RNA fractionation, Northern blotting, probe preparation and hybridization have been described (10), with the exception that all probes were labeled by random primed synthesis, described by the supplier (Pharmacia LKB). The probe for the yeast actin gene was a 3.8 kbp EcoRI fragment derived from plasmid pRB155, kindly provided by Dr. David Botstein.

Primer Extension Analysis

A COX6 synthetic oligonucleotide primer was synthesized on an Applied Biosystems DNA synthesizer. It spans nucleotides +7 through +31 (Fig. 3A) and has the sequence 5'-CTGGATTTCTGAATATGGCCCTTGA-3'. Ten picomoles of primer were kinased with $^{32}$P-ATP. One picomole of labeled oligonucleotide was annealed to 5 ug of poly $A^+$ RNA in 50 mM Tris, pH 8.3, 300 mM NaCl, 1 mM EDTA by heat denaturing the mix at 80° C for three minutes and incubating it for 1 hour at 42° C. Annealing was done in a volume of 10 ul. Templates were extended by avian myeloblastosis virus, AMV, (Life Sciences) or Molony murine leukemia virus, MMLV, (Pharmacia LKB) reverse transcriptases as follows. Six microliters of annealing mix were brought to 50 mM Tris pH 8.3, 10 mM $MgCl_2$, 5 mM dithiothreitol, 50 ug/ml actinomycin D, 0.1 mM of each deoxyribonucleoside triphosphate. One unit of AMV reverse transcriptase or 300 units of MMLV reverse transcriptase were added and samples were incubated at 42° C for 1 hour. To 10 ul of extension reaction 8 ul of 99% deionized formamide, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue were added. Samples were boiled for 3 minutes and loaded on 8% DNA sequencing gels.

$S_1$ Nuclease analysis

The COX6 3' termini were analyzed by $S_1$ nuclease cleavage as follows. The COX6 H2.3 fragment (Fig. 1), spanning the 3' termination region, was isolated from plasmid pVIH2 by HindIII cleavage and fragment preparations on agarose gels. The HindIII restriction sites were filled in with E. coli DNA polymerase large fragment (Klenow fragment, Boehringer Mannheim Biochemicals)

and $^{32}$P-dATP, as indicated (14). This "double headed" probe was cleaved with BglII and the appropriate fragment isolated by gel electrophoresis. This fragment, a "single-headed" probe, is labeled uniquely at the HindIII site in COX6 and spans the entire COX6 termination region. $S_1$ nuclease analyses were conducted as follows: $10^5$ cpm of probe and 50 ug of poly $A^+$ RNA were coprecipitated; precipitates were suspended in 24 ul of deionized formamide and 6 ul of 5x salts were added (5x salts is 2M NaCl, 0.2M Pipes, pH 7.6, 5mM EDTA); the samples were denatured for 15 minutes at 70° C and annealed for 4 hours at 40° C; 235 ul $H_2O$, 30 ul 10X $S_1$ buffer and 5000 U of $S_1$ nuclease were added; digestion was conducted at 37° C for 45 minutes after which products were precipitated in 70% ethanol and analyzed by neutral or alkaline agarose gel electrophoresis. 10x $S_1$ nuclease buffer is 2.5M NaCl, 0.3M sodium acetate, pH 4.6, 10mM $ZnSO_4$, 10 ug/ml sonicated salmon sperm DNA.

Preparation of Yeast Polysomes

Polysomes from cells in mid log phase were prepared as described by Warner et al. (15). Sucrose gradients were fractionated as indicated (15), except that the region from the top of the gradients to the start of the monosome peak was divided in half. This produced five gradient fractions: heavy polysomes, lighter polysomes, monoribosomes, and two fractions from the gradient top. RNA was obtained from the various fractions by extraction in phenol-chloroform isoamyl alcohol and ethanol precipitation (15).

Other Methods

Cell growth has been monitored by change in optical absorbance using a Klett colorimeter with a green filter. Early log phase corresponds to 50 to 100 Klett units, mid log phase corresponds to 100-200 Klett units, and late log phase corresponds to 200-350 Klett units. A Klett of 100 corresponds to $3.0 \times 10^7$ cells per ml.

## RESULTS

### Structure of the COX6 Genetic Locus

Figure 1 summarizes our understanding of the COX6 genetic locus. The region contains three genes; two of these are closely spaced upstream and downstream of COX6. The upstream gene, ORF-U, is transcribed from the opposite DNA strand as COX6. The polarity of the downstream gene, ORF-D, is uncertain, although preliminary data suggest that it is transcribed from the same DNA strand as COX6. Previously, we identified a third open reading frame terminating 65bp upstream of and in frame with COX6 (10). Because we have not

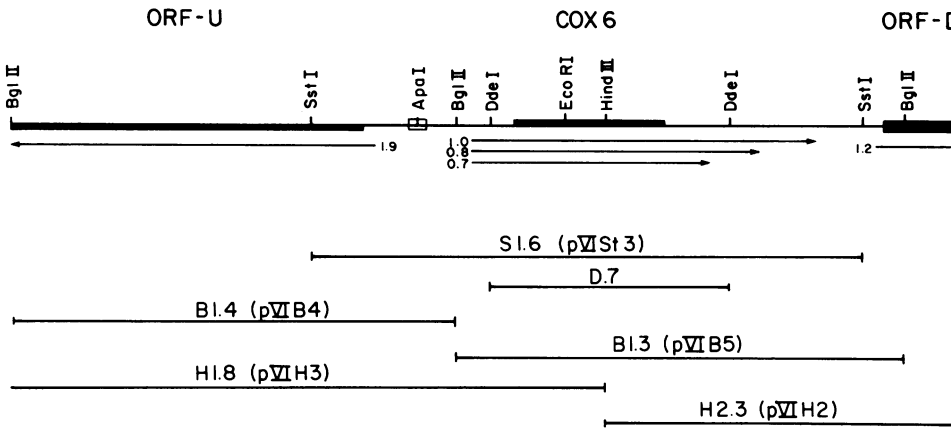



Figure 1. The COX6 genetic locus. Illustrated is COX6 and the genes that flank it. The RNAs produced from each gene are drawn as arrows below each gene; the direction of transcription is indicated by the arrow point. The clones used as sources of probes for Northern blot analysis are indicated by the plasmid name (e.g. pVISt3), and the fragment used as a probe is specified by restriction site (e.g. S for Sst1) and size in Kilobase pairs of DNA. The divergently transcribed genes, COX6 and ORF-U, are drawn as bars on opposite sides of the line. The polarity of ORF-D has not yet been established rigorously but from preliminary experiments appears to be the same as COX6.

detected an RNA from this ORF and because COX6 mRNA 5' termini map within it (see below), it is unlikely that this ORF is expressed.

To arrive at the interpretation shown in Figure 1, we have used extensive subcloning of the COX6 region, Northern blot analysis, and DNA sequence analysis. The DNA fragments used as probes for Northern blots and the RNAs hybridized by three of these are indicated in Figure 2. Table 1 summarizes the behavior of all of the probes diagrammed in Figure 1. We assign a 1.9 kb RNA to ORF-U on the basis of hybridization to probes S1.6, B1.4 and H1.8. DNA sequence of the 5' flanking region of COX6 reveals only one open reading frame (Figure 3). This open reading frame is coded on the opposite DNA strand from COX6. It possesses three in-frame methionine codons and a number of plausible TATAA boxes, between -300 and -470 bp. Preliminary $S_1$ nuclease analyses of this region, using a Klenow polymerase filled-in B1.3 probe (cf. Figure 1) has located ORF-U mRNA 5' termini between nucleotides -400 and -450 bp. A probe labeled on the opposite strand yields no RNA protected signal. Together, these analyses indicate that ORF-U and COX6 are divergently transcribed genes whose coding regions are separated by only 477 bp. We assign a 1.2 kb RNA to

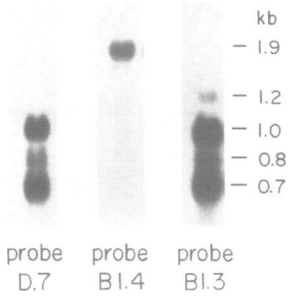



Figure 2. Northern blot analysis of mRNA from COX6 and its neighboring genes. Transcripts from COX6, ORF-U, and ORF-D were detected by hybridization with the probes D.7 or B1.3, B1.4 and B1.3, respectively. Reference to end-labeled $\phi$X174 RF markers was used to define the sizes of each RNA. The poly $A^+$ RNA used in each blot (25 ug per lane) was obtained from strain JM43 grown to mid log phase.

the region immediately downstream of COX6 on the basis of hybridization to probes H2.3 and B1.3. Because the S1.6 probe failed to hybridize this RNA (Table 1), the end of the downstream gene, ORF-D, can be localized to a short region between the BglII and SstI sites (Figure 1).

The three remaining RNAs from this region hybridize to the D.7 probe, and are, therefore, RNAs from COX6. Their sizes are approximately 1000 bases, 830 bases, and 700 bases. We have examined several other laboratory strains of S. cerevisiae (D273-10B, S288C, KL14-4A, and 777-3A/6, data not shown) for COX6 RNAs and find, for each of them, the same pattern of RNAs shown in Figure 2 for strain JM43.

The COX6 Transcriptional Unit Encodes Three Classes of RNA

To determine the organization of COX6 specific RNAs with respect to the COX6 transcriptional unit, we have conducted 5' and 3' end analysis of COX6 RNA. The 5' ends were determined both by primer extension analysis and $S_1$-nuclease protection analysis. By primer extension analysis, we observe three major 5'-ends at -79 bp, -94 bp, and -112 bp (Figure 3) relative to the COX6 initiator ATG. Mapping by $S_1$-nuclease protection produces similar results; three protected fragments are observed. In addition to these three major 5'-ends a large number of minor 5' ends are seen by primer extension analysis; these extend out to -163 bp. We do not observe any consensus nucleotide sequence around each 5' end although most sites are probably adenosine. These results indicate that the overall initiation region for COX6 encompasses 103 nucleotides with the majority of 5'-ends falling within 33 bp. This microheterogeneity of COX6 RNA 5' ends may account for the width of the bands observed by Northern blot analysis (c.f., Figure 2). Whereas it has been fairly easy to obtain a sharp signal for ORF-D RNA, COX6 RNAs invariably produce a broad signal on these blots, and this is independent of the method of RNA preparation.

Table 1. RNA hybridization pattern in the COX6 region.

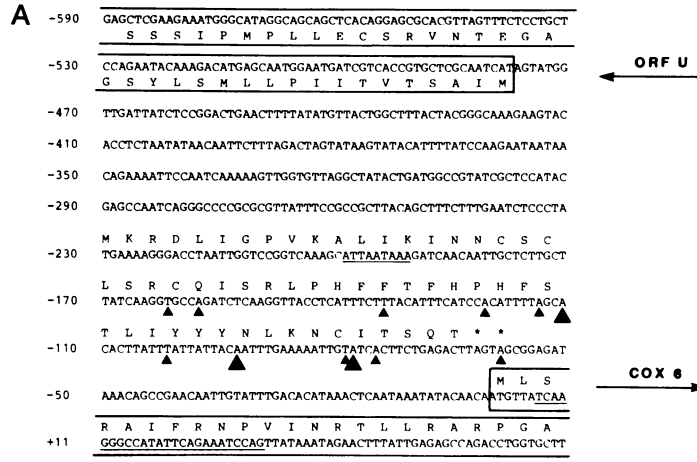
| RNA size (kb) | Transcription Unit | PROBE D.7 | S1.6 | B1.4 | B1.3 | H1.8 | H2.3 |
|---|---|---|---|---|---|---|---|
| 0.7 | (COX6) | + | + | - | + | + | + |
| 0.83 | (COX6) | + | + | - | + | + | + |
| 1.0 | (COX6) | + | + | - | + | + | + |
| 1.2 | (ORF-D) | - | - | - | + | - | + |
| 1.9 | (ORF-U) | - | + | + | - | + | - |

Hybridization probes are indicated as described in Fig. 1. The RNAs hybridized by each probe during Northern blot analysis are indicated by + (hybridizes) or - (does not hybridize). The sizes of each RNA were determined by reference to end labelled Hae III fragments of phage ϕX174 RF DNA.

The location of mature 3' ends for COX6 RNA was determined by $S_1$ nuclease cleavage of DNA-RNA duplexes formed with end-labeled, single-headed probes and poly $A^+$ RNA. Alkaline and native-gel analyses of the cleavage products were able to estimate sites to within approximately 20 bp and, therefore, could resolve polyadenylylation regions only. Some undegraded probe (1100 bp) and three $S_1$ nuclease protected fragments of approximately 320 bp, 430 bp, and 650 bp are observed (Figure 4B). These correspond to three polyadenylylation regions, centered at +590 bp, +740 bp, and +920 bp (Figure 4A). Inasmuch as a) COX6 is transcribed into three classes of mRNA that differ in size by 150 bases (Figure 2), b) the major 5' ends are clustered over a 33 bp region (Figure 3), and c) COX6 RNA has three major 3'-ends that are spaced 150 bp apart (Figure 4), we conclude that the three different RNA classes differ in size primarily at their 3' ends.

All Three Classes of COX6 RNAs are Found in Polysomes

The existence of multiple mRNAs with different 3' untranslated regions led us to consider the possibility that the 3' untranslated regions might render one or another RNA a more efficient substrate for translation. If the different RNA classes vary in their abilities to be translated they should be differentially distributed in polysomes. To examine this we have conducted Northern blot analysis of sucrose gradient fractionated yeast polysomes. In


A
-590
GAGCTCGAAGAAATGGGCATAGGCAGCAGCTCACAGGAGCGCACGTTAGTTTCTCCTGCT
S S S I P M P L L E C S R V N T E G A
-530
CCAGAATACAAAGACATGAGCAATGGAATGATCGTCACCGTGCTCGCAATCATAGTATGG
G S Y L S M L L P I I T V T S A I M
ORF U
-470
TTGATTATCTCCGGACTGAACTTTTATATGTTACTGGCTTTACTACGGGCAAAGAAGTAC
-410
ACCTCTAATATAACAATTCTTTAGACTAGTATAAGTATACATTTTATCCAAGAATAATAA
-350
CAGAAAATTCCAATCAAAAAGTTGGTGTTAGGCTATACTGATGGCCGTATCGCTCCATAC
-290
GAGCCAATCAGGGCCCCGCGCGTTATTTCCGCCGCTTACAGCTTTCTTTGAATCTCCCTA
M K R D L I G P V K A L I K I N N C S C
-230
TGAAAAGGGACCTAATTGGTCCGGTCAAAGCATTAATAAAGATCAACAATTGCTCTTGCT
L S R C Q I S R L P H F F T F H P H F S
-170
TATCAAGGTGCCAGATCTCAAGGTTACCTCATTTCTTTACATTTCATCCACATTTTAGCA
T L I Y Y N L K N C I T S Q T * *
-110
CACTTATTTATTATTACAATTTGAAAAATTGTATCACTTCTGAGACTTAGTAGCGGAGAT
M L S
COX 6
-50
AAACAGCCGAACAATTGTATTTGACACATAAACTCAATAAATATACAACAATGTTATCAA
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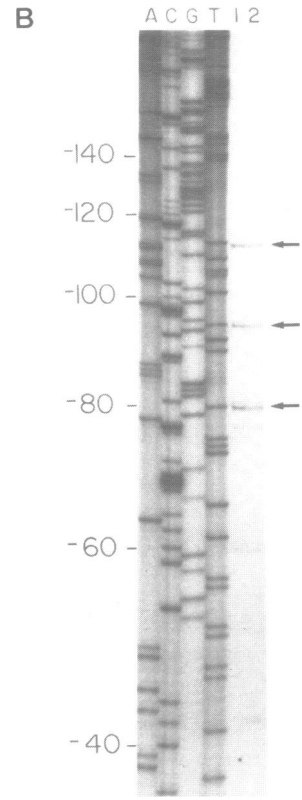
B
A C G T 1 2
-140
-120
-100
-80
-60
-40

Figure 5 it can be seen that all three classes of COX6 RNA are present in the two halves of the polysome peak. Although a slight enrichment of the largest RNA in the heavier polysome fraction can be seen, we do not find selective exclusion from the polysomes of any COX6 RNA, nor any great bias in their distribution. Hence, all three classes of COX6 RNA are polysome bound and most likely translated, and the choice of 3' end does not appear to govern their entry into the polysome population. While the presence of all three COX6 RNAs in the polysomes does not ensure their equivalent translation (16), previous work with yeast suggests that this is likely. For example, deletions and mutations within the 3' untranslated region of the yeast PYK gene have resulted in simultaneous loss of translatability and a severe effect on ribosome loading; this was observed as a shift in RNA position in polysome gradients (17).

Previously we showed that *in vitro* translation of COX6 hybrid selected RNA produced only one protein product (10). Because all three classes of RNA were hybrid-selected and, from the above studies, are most likely translated, they must all produce the same protein.

The Three Classes of COX6 RNAs Accumulate Differentially in Different Growth Conditions

Whereas the nature of the 3' end does not appear to regulate translatability of COX6 RNA or the sequence of the subunit VI polypeptide produced, the choice of 3' end nevertheless is regulated. For example, we have observed significant variability in the abundance of the different RNAs isolated from

Figure 3. The DNA sequence of the intergenic region between COX6 and ORF-U and primer extension mapping of the 5'- ends of COX6 mRNAs. A. Shown is the 5' flanking DNA sequence out to the leftward Sst1 site, at -589 bp. These data extend previous sequence data and correct an error at positions -258 and -259 bp. The location of the 5' ends were determined by primer extension analysis (Materials and Methods) and are shown as triangles, major and minor 5' ends are indicated by large and small triangles, respectively. The region of COX6 that was used in constructing the synthetic oligonucleotide for primer extension analysis (panel B) is underlined. The upstream gene has been reverse translated as indicated. The sequence of this region was determined as indicated previously (10) by the dideoxy chain terminating method. The new sequence data reported here, between -590 and -440 bp, was obtained by sequencing a 200 bp Sst1 to AccI fragment cloned in M13mp18 and M13mp19. We assume that the assignments of 5'-ends are accurate to ±1 nucleotide. A putative TATA box for COX6 has been underlined. B. Primer extension data are shown along with a DNA sequence ladder. Lane 1 shows the extension results using AMV reverse transcriptase. Lane 2 shows results using MMLV reverse transcriptase. The arrows indicate major extension products observed in this and other experiments.

A

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491  CTTTTTTTATTTGTGGAAAATTCCTCAATTTGAAATAAGATTCGTATTTATTTTGTAAAA
551  CGATTTTAGTTTTTATTTTTTTTTTTTCTTCCCTTTTACCTTATTATTTGTTTTACCCTT
                                    I
611  TATGGGATATTCATCCACTCAGAAACTAATATATCAAAATCAACGATAAGATCACAAGAG
671  GTGAAATAAGAAAAAGTAAAAGAAAAAATATTTAAATTTAACCATTATGATTTCCACTTT
731  GTTTTCTCTACTCTATTACTTGAACAAACTTTATTTTTGGTAATGAGGGCTGGTCACAGA
                II
791  TTACTTTAATATCCTTTGTTAGTAGCTTTTTTGTGTTCTATTTCAGGAAAGAAGAATGGG
851  AAAAATAATAAAACAATAAATAACTATATAAAAAACAAAAACCTAGAATATACCATTAAA
911  GAATTACTATTCTATTCTGTGCTTCACCGTTGCGTCAATAAATGTGAAGTTGATGTCACT
                    III
971  TCATACTAAAAAAATTAAAGACAATTTCCTGAAAAAAATTTTTCCACTGGAGCTC
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B

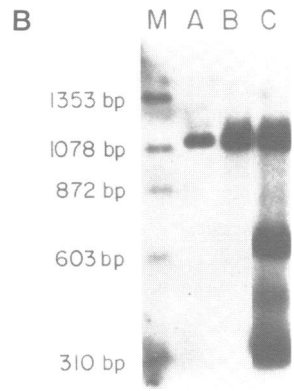
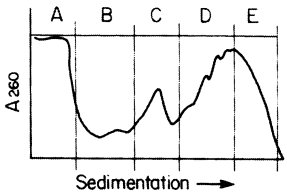



Figure 4. The DNA Sequence downstream of COX6 and the localization of the 3'-ends of COX6 mRNAs. A. The DNA sequence flanking the 3' end of COX6 has been extended to the Sst1 site by sequencing the Dde1 to Sst1 fragment, spanning nucleotides 630 to 1020. Sequence data were obtained as indicated previously (10) using M13mp18 and M13mp19. The broad polyadenylylation sites discussed in the text have been boxed. Each AATAAA sequence has been underlined. B. The 3' ends of COX6 RNA were determined by $S_1$ nuclease cleavage-protection experiments, as described in Materials and Methods. Protected fragments were analyzed on a native agarose gel calibrated with $\phi$X174 RF DNA markers. The lanes shown in the $S_1$ protection assay are: Lane M= $\phi$X174 RF markers; Lane A=1/10 level of probe that was used in lanes B and C; Lane B = $S_1$ cleavage of the H2-3 derived probe; Lane C=$S_1$ cleavage-protection analysis of poly $A^+$ RNA from mid log phase JM43 cells.

cells harvested at different times during growth. Figure 6 shows an example of the changes in composition seen during growth in rich (YPD) medium. As can be seen on the Northern blot (Figure 6A), there is a shift in RNA composition to one predominantly composed of the smallest class of RNA in later phases of growth. From $S_1$ analysis of 3' ends (Figure 6b) it is apparent that this is



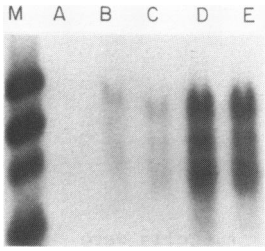




Figure 5. Distribution of COX6 mRNAs on polysomes. A Northern blot of sucrose gradient fractionated yeast polysomes was probed with the D.7 fragment (Figure 1). The sucrose gradients were drip fractionated from bottom to top to yield the pooled fractions: (A), Gradient top, the upper half; (B), Gradient top, the lower half; (C), Monoribosomes; (D), Lighter polysomes; (E), Heavier polysomes. Markers (M) are ϕX174 RF DNA.

due to the predominance of RNAs with the polyadenylylation region closest to the termination codon of COX6, later in growth. The splitting of the smallest band (Figure 6A, lanes D and E) is of variable occurrence; it is most likely due to changes in the initiation of transcription.

Because glucose is consumed and ethanol is produced during yeast cell growth on YPD medium we considered the possibility that the differential accumulation of COX6 RNAs was due to glucose derepression and/or ethanol accumulation. To analyze this, cells were grown to early log phase ($3 \times 10^7$ cells/ml) on YPD medium and shifted into fresh YPD medium containing high (i.e., 2%) glucose or low (i.e., 0.05%) glucose, as described (18). The shift into low glucose leads to an overall increase in COX6 RNA levels (derepression) whereas a shift into high glucose results in an overall decrease in COX6 RNA level (repression). Shifting cells into high glucose favors a population enriched for the smallest RNA class; shifting cells into low glucose yields a population equally enriched for both the smallest and largest RNA classes (Figure 7A). As seen by $S_1$ nuclease analysis the RNA classes that terminate at +740 bp and +920 bp are greatly reduced after the shift into high glucose but are enriched after the shift into low glucose. These observations indicate that glucose repression is exerted on all three RNA classes but, that it has a greater effect on the larger two RNA classes. Conversely, derepression upon shifting into low glucose media is exerted preferentially on the larger two RNA classes. This is readily seen by examining the COX6 RNA that accumulates immediately after a shift into low glucose media (Figure 8). Derepression of the larger RNA is relatively rapid; it is essentially complete 10

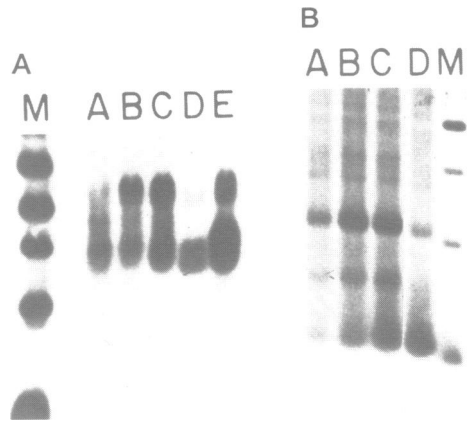



Figure 6. Expression of COX6 during growth in YPD medium. A. Cells were removed at various times during growth in YPD medium and poly $A^+$ RNA was obtained. Northern blots were performed as indicated in Materials and Methods. Lanes A and B used RNA from early log phase culture (cell density = 0.5 and 1.5 x $10^7$ cells/ml, respectively); lane C used RNA from mid log phase culture (cell density = 3 x $10^7$ cells/ml); and lanes D and E used RNA from late log to early stationary phase culture (cell density = 6 x $10^7$ cells/ml). Lane D received 1/5 as much RNA as lane E, allowing us to resolve two bands in the 700b region of the gel. B. $S_1$ analysis of COX6 mRNA 3' ends was conducted as described in Materials and Methods using the RNA from the experiment shown in panel A and the single headed probe H2-3 (Figure 1). Lanes A-D used the same RNA preparation as those used in lanes A, B, C, and D, respectively, in Panel A.

minutes after the shift. A similar derepression of the three COX6 RNA classes is seen when cells are shifted into ethanol (data not shown), a nonfermentable and derepressing carbon source: the longer RNAs are more dramatically derepressed than the smaller.

Overall, the results of these shift studies indicate that glucose repression and derepression affect the level of COX6 RNA but do so by exerting differential affects on RNAs with different 3' ends. Moreover, they indicate that the differential accumulation of the smallest RNA class, as cells enter the late log or early stationary period of growth on YPD medium (Figure 6A) is not due to glucose depletion or ethanol accumulation. Indeed, were this the case the largest and middle size classes of RNA should predominate as cells deplete the glucose in the media and begin to use ethanol as a carbon source. We observe just the opposite: accumulation of the smaller RNAs late in growth phase.

Finally, to evaluate the overall effect of glucose repression on COX6

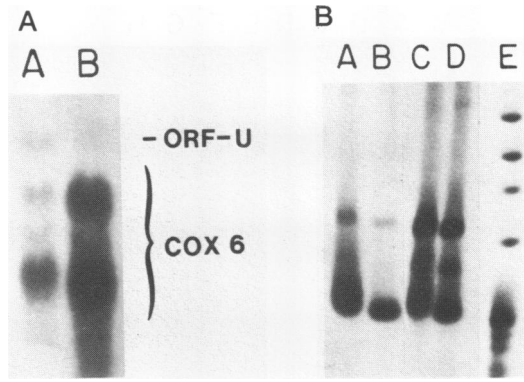



Figure 7. Derepression of COX6 after a glucose shift. A. Cells were grown to early exponential phase (3 x $10^7$ cells/ml) in YPD and then shifted into high (lane A) or low (lane B) glucose media as described in the text (18). After three hours of growth cells were harvested, RNA was prepared, and the poly $A^+$ fraction obtained as indicated in Materials and Methods. Northern blots were run and probed with the B1.3 probe. To ascertain that each lane received an equivalent load of RNA we ran a parallel blot, probed with an actin specific-probe (data not shown). B. $S_1$ analysis of COX6 RNA mRNA 3' ends was conducted as indicated in the legend to Figure 6. COX6 mRNA was obtained as described in panel A. Two different levels of $S_1$ nuclease (500 and 5000 units) were used, as indicated for both high and low dextrose conditions. Lanes A and B = RNA from cells shifted to high glucose, treated with 500 and 5000 units of $S_1$ nuclease, respectively; Lanes C and D = RNA, from cells shifted to low glucose, treated with 500 and 5000 units of $S_1$ nuclease, respeccively.

expression, cells were grown to steady state (16 generations) on YP medium containing either 8% glucose or 2% lactate, a derepressing carbon source, and COX6 RNAs were subjected to Northern blot analysis. Qualitatively, it is clear from Figure 9 that COX6 RNAs are higher in lactate grown than in glucose grown cells. These differences have been quantitated by counting the radioactivity bound to COX6 RNAs (Figure 9, lanes A and B). As an internal control for the RNA loaded, we also determined radioactivity in RNA that corresponds to the actin gene, a gene that is constitutive (19) and unaffected by glucose repression (Figure 9, lanes C and D and ref. 19). After normalization to actin RNA, COX6 RNA levels are 4-fold lower in cells grown to steady state in glucose than they are in cells grown to steady state in lactate.

## DISCUSSION

In this work we have characterized the genetic region around COX6 and have defined the structure of the COX6 transcriptional unit. We have observed

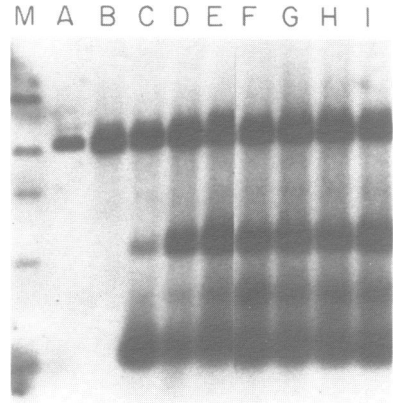



Figure 8. Derepression of COX6 mRNA. Cells were shifted, as indicated in the legend to Figure 7, into low glucose media. At various points following the shift cells were harvested, polyA$^+$ RNA prepared, and 3' $S_1$ nuclease analyses were conducted as indicated in Materials and Methods. Lane M = $\phi$X174 RF markers; Lane A = 1/10 level of probe used in the $S_1$ experiments shown in lanes C-I; Lane B = $S_1$ cleavage of the H2-3 derived probe; Lane C = $S_1$ protection of preshifted cells; Lanes D-I = 5 min, 10 min, 15 min, 30 min, 60 min, and 120 min. post shift.

that COX6 RNA has numerous, closely spaced 5' termini and three widely separated polyadenylylation sites that produce three classes of COX6 RNA. The three classes of RNA are regulated differentially by growth phase and by growth in various derepressing carbon sources.

The COX6 region is similar in organization to many other loci in yeast. Two genes, ORF-U and ORF-D, are closely spaced 5' and 3' of COX6. The 5' region of COX6 exhibits divergent transcription from a very small intergenic domain, approximately 300 bp in length. Some other yeast loci that have this motif include the GAL7-GAL10-GAL1 region (20), the PET56-HISI-DEDI region (21), and the ANBI-CYCI region (22). The 5' intergenic region of COX6 must contain all of the elements necessary for COX6 expression and regulation because a 1.6 kbp Sst1 fragment extending 590 bp upstream of the COX6 ATG is sufficient to complement a COX6 null mutant (Wright and Poyton, unpublished). A large part of this upstream region is, of course, part of the ORF-U promoter and coding region.

In our original characterization of COX6 (10) we observed an open reading frame ending 65 bp upstream of COX6 and in frame with COX6. While sequencing the ORF-U region out to -590 bp, we detected an error in our original determination at -259 bp. The correction reported here (Figure 3) for this GC

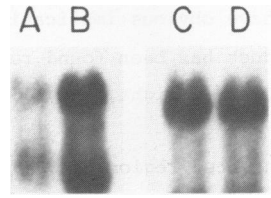



Figure 9. Northern blot analysis of lactate and dextrose grown cells. Cells were grown to mid log phase (cell density = 3 x $10^7$ cells/ml) in YP Lactate or YP Dextrose media. Poly $A^+$ RNA was prepared and Northern blots run as indicated in Materials and Methods. The blot was probed with the COX6 Lanes A and C = YP dextrose grown cells, Lanes B and D = YP lactate grown cells. Lanes A and B were hybridized to the COX6 specific probe D.7. Lanes C and D were hybridized to a 3.8 kbp EcoRI fragment containing the yeast actin gene and 2 kbp of upstream DNA. The COX6 RNA at 830 bp is not well resolved on this gel.

cluster relocates the ATG for the upstream open reading frame to base -231 and reduces the size of the ORF to 56 codons. The correct sequence aound -259 bp is CCGCCGC. All of the COX6 5' termini map within this 56 codon ORF and none map 5' of it. Moreover much of the ORF can be deleted without affecting COX6 expression (Trawick et al., in preparation). Together, these findings strongly suggest that this ORF is not transcribed and is irrelevant to COX6 expression.

COX6 RNAs exhibit a large number of 5' termini that extend over 100 bp with three major termini clustered within a 33 bp region. Multiple 5'-termini have been found for other genes encoding polypeptides of the mitochondrial electron transport chain. For example, CYC1 (23, 24) and COX4 (McEwen et al., pers. comm.; 25), both possess numerous 5' termini with a few of these predominating in the steady state RNA population. Consistent with other determinations for yeast genes, most of the COX6 5' ends map to adenosines.

Other features of the COX6 5' region are noteworthy. A "CAAG" box, identified close to the initiation region of other yeast genes (26), lies between -168 and -164 bp. A "TATA" box is found between -196 and -190, although it is of low homology to the consensus sequence. From -215 to -211 is an oppositely oriented "CCAAT" box, the only such sequence found upstream of COX6 initiation sites. This site possesses near perfect homology to the HAP2-HAP3 responsive site of CYC1 (27) and is included in the sequence TAATTGGT that corresponds to the up-promoter mutant of CYC1, UAS2UP1. In addition to this reverse oriented "CCAAT" box, two forward orientation "CCAAT" boxes are present that contain high similarity to the known HAP2, HAP3 sites of action in the CYC1 promoter (28). These are found in the region from -256

to -341 bp. These findings have obvious implications for the regulation of COX6 since the HAP2 gene product has been found recently to affect COX6 transcription (Trawick, J.D., R.M. Wright, and R.O. Poyton, unpublished observations).

Nowhere in the 3' untranslated region of COX6 do we find sequences analogous to the termination elements proposed for other yeast genes (29). However, two sequence elements in this region are apparent. First, within each polyadenylation region are clusters of the consensus sequence, TAPyPy. The sequence TATT occurs five times in the three sites, TACT occurs twice, and TACC occurs once. Second, a typical, higher eukaryotic polyadenylylation signal (AATAAA) (or the very similar sequence, AATAAG) lies 60 nucleotides upstream from each termination region. The termination site centered at +920 bp is preceded by two tandem polyadenylylation signals (AATAAA) and is followed 20 bp downstream by a third. The existence of these sequences is far more typical of higher eukaryotic genes, where they are found 10-30 nucleotides upstream of the polyadenylylation sites (29), than it is of yeast genes, and may reflect the antiquity of genes encoding this component of the electron transport chain.

The presence of multiple polyadenylylation regions associated with COX6 is a motif often found in higher eukaryotes (30, 31, 32, 33), and one that was reported recently for the yeast gene, ALG7 (9). Central to these complex RNA patterns is the question of whether the choice of 3' terminus regulates expression of the corresponding gene. In higher eukaryotes, variability in polyadenylylation site choice is highly regulated showing either tissue specific or growth state dependence (30, 31, 32, 33). In yeast, the only clearly documented case of differential polyadenylylation site choice that we are aware of is that of the ALG7 gene where gene dosage appears to regulate the abundance of ALG7 RNAs with different 3' termini (9). Here, we have found that the relative levels of the three COX6 RNAs vary with growth phase and are also affected differently by glucose repression and derepression. Hence, the choice of 3' termini for COX6 RNAs is regulated at the level of steady state RNA abundance by biological parameters of cell growth.

The differential accumulation of COX6 RNA during growth could result from the uniform transcription of COX6 and the differential stability of the individual RNAs, or, on the other hand, from differential transcription termination and processing. A similar argument would apply to the differential accumulation of COX6 RNA by growth in different carbon sources. By either scenario, the existence of multiple RNAs whose differential abundance depends

upon the conditions of cell growth defines a complex system of regulation for COX6 expression. Understanding the regulation of COX6 expression will, therefore, entail not only understanding transcriptional initiation but also 3' end formations, RNA stability, and the role played by the different RNAs in the final synthesis of the subunit VI protein.

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REFERENCES

1. Poyton, R.O. (1980) Curr. Topics in Cell. Reg. 17, 231-295.
2. Power, S.D., Lochrie, M.A., Sevarino, K.A., Patterson, T.E., and Poyton, R.O. (1984) J. Biol. Chem. 259, 6564-6570.
3. Wright, R.M., Trawick, J.D., Trueblood, C.E., Patterson, T.E., and Poyton, R.O. (1987) In: Cytochrome Systems, Molecular Biology and Bioenergetics. (ed. S. Papa), Plenum Press, N.Y., pp. 49-56.
4. Trueblood, C.E. and Poyton, R.O. (1987) Mol. Cell. Biol. 7, 3520-3526.
5. Tzagoloff, A. and Meyers, A.M. (1986) Ann. Rev. Biochem. 55, 249-285.
6. Guarente, L. (1987) Ann. Rev. Genet. 21, 425-452.
7. Johnston, M. (1987) Microbiol. Rev. 51, 458-476.
8. Buckholz, R.G., and Cooper, T.G. (1983) Mol. Cell Biol. 3, 1889-1897.
9. Kukuruzinska, M.A., and Robbins, P.W. (1987) Proc. Natl. Acad. Sci. USA 84, 2145-2149.
10. Wright, R.M., Ko, C., Cumsky, M.G., and Poyton, R.O. (1984) J. Biol. Chem. 259, 15401-15407.
11. Parikh, V.S., Morgan, M.M., Scott, R., Clements, L.S., and Butow, R.A. (1987) Sci. 235, 576-580.
12. Miller, J. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
13. Norrander, J., Kempe, T., and Messing, J. (1983) Gene 26, 101-106.
14. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
15. Warner, J.R., Mitra, G., Schwindinger, W.F., Studeny, M. and Fried, H.M. (1985) Mol. Cell. Biol. 5, 1512-1521.
16. Kruys, V., Wathelet, M., Poupart, P., Contreras, R., Fiers, W., Content, J., and Huez, G. (1987) Proc. Natl. Acad. Sci. USA 84, 6030-6034.
17. Purvis, I.J., Bettany, A.J., Loughlin, L., and Brown, A.J.P. (1987) Nucl. Acids Res. 15, 7951-7974.
18. Carlson, M., Osmond, B.C., and Botstein, D. (1981) Genetics 98, 25-40.
19. Szekely, E. and D.L. Montgomery (1984) Mol. Cell. Biol. 4, 939-946.
20. Citron, B.A., and Donelson, J.E. (1984) J. Bacteriol. 158, 269-278.
21. Struhl, K. (1985) Nuc. Acids Res. 13, 8587-8601.
22. Lowry, C.V., and Zitomer, R.S. (1984) Proc. Natl. Acad. Sci. USA 81, 6129-6133.

23. Guarente, L., and Mason, T. (1983) Cell 32, 1279-1286.
24. McNeil, J.B., and Smith, B. (1986) J. Mol. Biol. 187, 363-378.
25. Schneider, J.C., and Guarente, L. (1987) Nuc. Acids Res. 15, 3515-3529.
26. Laughon, A., and Gesteland, R.F. (1984) Mol. Cell. Biol. 4, 260-267.
27. Olesen, J., Hahn, S., and Guarente, L. (1987) Cell 51, 953-961.
28. Forsburg, S.L., and Guarente, L. (1988) Mol. Cell. Biol. 8, 647-654.
29. Birnstiel, M.L., Busslinger, M., and Strub, K. (1985) Cell 41, 349-359.
30. Bond, B.J., and Davidson, N. (1986) Mol. Cell. Biol. 6, 2080-2088.
31. Carroll, S.L., Bergsma, D.J., and Schwartz, R.J. (1986) J. Biol. Chem. 261, 8965-8976.
32. Hickok, N.J., Seppanen, P.J., Kontula, K.K., Janne, P.A., Bardin, C.W., and Janne, O.A. (1986) Proc. Natl. Acad. Sci. USA 83, 594-598.
33. Bilanchone, V., Duester, G., Edwards, Y., and Smith, M. (1986) Nucl. Acids Res. 14, 3911-3926.