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Modulator sequences mediate oxygen regulation of *CYC1* and a neighboring gene in yeast

(yeast transformation vector/*in vivo* plasmid transcription/regulatory mutation)

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ABSTRACT **Three transcripts from *Saccharomyces cerevisiae*—*CYC1* mRNA (transcribed from the iso-1 cytochrome *c* gene) and two RNAs of unknown function, designated *tr-1* and *tr-2*—were identified by reverse Southern blot analysis and found to be regulated in response to oxygen. *CYC1* mRNA and *tr-1* accumulation occurred only in the presence of oxygen while *tr-2* appeared only under anaerobic conditions. *tr-2* was transcribed from a region approximately 1 kilobase 5′ from the *CYC1* coding sequence and in the opposite direction. *tr-1* showed homology to the same region as *tr-2* but was transcribed from elsewhere in the genome. Expression of *tr-2* and *CYC1* was observed to be normal in cells transformed with centromeric plasmids carrying the two genes. Mutant transforming plasmids were constructed in which a 400-base-pair region between *tr-2* and *CYC1* was either deleted or inverted. The deletion led to low-level nearly unregulated expression of both the *CYC1* and *tr-2* genes, suggesting that sequences upstream from both genes are important for their expression and regulation. The inversion mutation produced a reversed pattern of *CYC1* regulation in which the mRNA was present in anaerobically grown cells but absent in the presence of oxygen, mimicking wild-type *tr-2* regulation and suggesting that the *CYC1* transcription unit is under the control of the translocated *tr-2* modulator sequences. Models for the function of these modulators are discussed.**

There are a number of examples in eukaryotes of upstream modulators, regions of DNA that influence gene expression and are located some distance from the start of transcription (1–4). Guarante and Ptashne (2) presented evidence suggesting that there is such a region located at least 190 base pairs (bp) from the start of transcription that is essential for normal expression of the *CYC1* gene of yeast. A number of questions can be asked about these modulating sequences and the mechanisms by which they exert their effect. Among these are whether they are directly involved in the regulation of transcription and whether they are functionally specific for their genes or promiscuous—i.e., capable of controlling and regulating other genes if put in proximity to them.

To answer such questions, it was desirable to extend our observations of regulatory phenomena affecting *CYC1* gene expression and the expression of neighboring genes. Catabolite repression causes only partial repression of *CYC1* mRNA synthesis under aerobic conditions (5), but it was thought that yeast grown anaerobically might show total repression, because cytochrome *c* is known to be absent in such cells (6, 7). In this paper, we report that RNA blot analysis confirmed this expectation, indicating complete absence of *CYC1* mRNA in anaerobically grown cultures. This method also permitted the identification of a regulated transcript designated *tr-2*, of unknown function, that is transcribed from a region approximately 1 kilobase (kb) upstream from the *CYC1* gene. It is synthesized only in the absence of oxygen and is thus regulated in a manner opposite to that of *CYC1* mRNA.

Our purpose was to study mutations affecting these phenomena to learn more about modulators and their function. A deletion and an inversion of a region between the two genes were constructed *in vitro* in yeast transforming plasmids containing a centromere, CEN3 (8). The centromere was included so that transcription in transformants would not be affected by variations in copy number and would reflect the effects of mutations on regulation of expression accurately. The results obtained through the use of these plasmids show (*i*) that both genes are controlled by upstream modulators and (*ii*) that the *tr-2* modulator can function promiscuously, because the inversion mutation puts the *CYC1* gene under oxygen-linked repression. This suggests that modulators are functionally discrete and nonspecific—capable of retaining intrinsic regulatory properties when juxtaposed to new transcription units.

MATERIALS AND METHODS

Strains and Transformation. The yeast diploid strain dRZ1 (9) is wild type for all genes tested. The strain used for transformation GM-3C-2 [*trp1-1, leu2-3, leu2-112, his4-519, cyc1-1, cyp3-1,* (*cyc7*$^-$), *gal*$^-$ (10)] carries a total deletion of the *CYC1* gene and a region at least 1.8 kb on the 5′ side (unpublished data). GM-3C-2 cells were transformed essentially as described by Hinnen *et al.* (11) except that spheroplasts were generated with the enzyme mixture lyticase prepared from *Oerskovia xanthinolytica* (obtained from Randy Shekman, University of California, Berkeley) and purified through the CM-cellulose step (12). Transformation of *Escherichia coli* strain HB101 (13) was carried out as described (14). Transfections with M13 derivatives were carried out as described (15).

To assay for respiratory competence of yeast cells containing *CYC1* mutations, glycerol and lactate plates were used (16). Cells completely respiratory deficient (such as GM-3C-2) are unable to grow on glycerol or lactate plates. Cells containing reduced levels of cytochrome *c* can grow on glycerol plates but not on lactate plates. Wild-type yeast can grow on both.

Plasmids. The plasmids used for the construction of YCp*CYC1*(2.4) and as probes in RNA blot analysis are diagrammed in Fig. 2*A*. pYe*CYC1*(2.4), pYe*CYC1*(0.6), and pYe*CYC1*(4.5) were obtained from Hall and Montgomery (17). M13 mp8 was obtained from Bethesda Research Laboratories. pACYC177 was obtained from S. N. Cohen (18). YRp7 was obtained from Davis (19). pYe(*CEN3*)41 containing *CEN3* on a 2.2 kb *Bgl* II/*Bam*HI fragment was obtained from J. Carbon (8).

RNA Preparation and Analysis. Cells were grown at 30°C in YPD (2% peptone/1% yeast extract/3% glucose) or YPR (2% peptone/1% yeast extract/3% raffinose) medium and shaken

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Abbreviations: kb, kilobase(s); bp, base pair(s).

vigorously (aerobic growth) or shaken and bubbled with nitrogen in sealed flasks (anaerobic growth). Before harvesting, the anaerobic cultures were chilled by continued shaking and bubbling on ice for 15 min. Cells were harvested and RNA was prepared as described (9). Agarose gel electrophoresis in the presence of formaldehyde and RNA blotting onto nitrocellulose were carried out as described (21, 22) with 20 mM 4-morpholinepropanesulfonic acid, pH 7.0/5 mM sodium acetate/1 mM EDTA as the running buffer. Nick-translated plasmid and restriction fragment DNA probes were prepared as described (23).

The single-stranded M13 mp8 phage DNA was labeled with [^{32}P]dATP by a primer extension method provided by Bethesda Research Laboratories with the M13 mp8 vector. The primer used was a 21-bp *Eco*RI fragment excised from the plasmid pHM235 provided by Bethesda Research Laboratories and purified by polyethylene glycol precipitation. The primer was heat denatured, hybridized to the single-stranded phage DNA, and extended under the same conditions as those for nick-translations but without DNase. The primer hybridized to a region adjacent to the insert and primed synthesis in a direction away from the insert. Because the extension rarely proceeds the full length of the template under the condition used, the insert remains single stranded, and the complex serves as a strand-specific probe, with the specificity determined by the orientation of the inserted DNA in the M13 mp8 vector. In the hybridization reactions, these partially double-stranded probes are not denatured so that, if the insert hybridizes to a RNA molecule fixed to nitrocellulose, the hybrid complex is visualized by connection to the labeled extended primer.

For hybridization, the blots were first soaked in hybridization buffer (100 mM Tris·HCl, pH 7.8/300 mM NaCl/2 mM EDTA/50 mM sodium phosphate, pH 6.5) and then prehybridized for 4 hr at 65°C in hybridization buffer supplemented with 5-fold concentrated Denhardt's solution (24), sheared and denatured salmon sperm DNA at 100 μg/ml, and 0.1% NaDodSO$_4$. The blots were then hybridized for 18 hr at 65°C in the same solution supplemented with 10% dextran sulfate and denatured probe ($1–5 \times 10^6$ cpm). The filters were rinsed and washed at 65°C with two changes of 2 mM Tris·HCl, pH 7.8/60 mM NaCl/0.4 mM EDTA/0.2% NaDodSO$_4$. About 1 liter of wash solution per blot was used.

RESULTS

Oxygen-Linked Regulation of *CYC1* mRNA and Two Other Transcripts. RNA blot analysis is a useful tool for the identification and relative quantification of transcripts synthesized from specific regions of a genome. As shown in Fig. 1*A*, blots probed with plasmid pYe*CYC1*(2.4) (Fig. 2) revealed the presence in normal diploid cells (dRZ1) of three regulated transcripts homologous to regions of a 2.4-kb fragment containing the 0.6-kb *CYC1* coding region and a 1.8-kb segment 5′ from that sequence. One of these transcripts was identified as the 700-nucleotide *CYC1* mRNA by comparison with a parallel blot probed with pYe*CYC1*(0.6) (Fig. 2). The other two were the transcripts designated *tr-1* and *tr-2* of approximately 900 and 800 bp, respectively, that, like *CYC1* mRNA, were strongly regulated in response to growth conditions. *CYC1* mRNA accumulated maximally during growth on raffinose, was partly repressed on glucose, and was absent under anaerobic conditions (Fig. 1*A*). The *tr-1* transcript paralleled *CYC1* mRNA in being reduced under anaerobic conditions. The *tr-2* transcript levels were regulated in a manner opposite to those of *CYC1* mRNA, being present only in anaerobically grown cells and absent in aerobically grown cells whether grown on raffinose or glucose.

Site of Transcription of *tr-1* and *tr-2*. To further localize these two transcripts, hybridization probes were prepared from

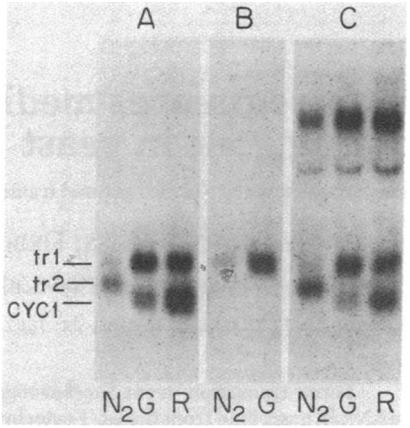

FIG. 1. RNA blot analysis of RNA probed with pYe*CYC1*(2.4). Ten-microgram samples of total cellular RNA were electrophoresed on 1.5% agarose gels, blotted, and hybridized with nick-translated plasmid pYe*CYC1*(2.4). RNA was from dRZ1 (*A*), GM-3C-2 (*B*), and GM-3C-2 transformed with YCp*CYC1*(2.4) (*C*). Cells were grown anaerobically in YPD medium (lanes N_2) or aerobically in YPD (lanes G) or YPR (lanes R) medium. RNA bands are, as indicated, at the approximate positions of 900 nucleotides, *tr-1*; 800 nucleotides, *tr-2*; and 700 nucleotides, *CYC1* mRNA.

plasmid pYeJLW(2.8) and the two single-stranded phages M13mp8DAW(0.8)A and -B. These experiments localized the regions to which both transcripts hybridized to a 1.2-kb segment between the *Sal* I site (−1,000) and the *Bam*HI site (−1,800). In addition, the hybridization to M13mp8DAW(0.8)A but not to M13mp8DAW(0.8)B showed that *tr-1* and *tr-2* were complementary to the same strand, which was the one opposite to the *CYC1* template strand. The directions of transcription (arrows) are shown in Fig. 2.

Even though *tr-1* and *tr-2* hybridized to closely linked or overlapping segments of the same strand of DNA, the results with the *CYC1* deletion strain GM-3C-2 showed that they are transcribed from different locations in the genome. Southern blots of restriction digests of DNA from GM-3C-2 showed that the entire 2.4-kb region is deleted in this strain. Nonetheless, as shown in Fig. 1*B*, GM-3C-2 cells synthesize *tr-1* in a manner dependent on oxygen, just as wild-type cells do. These results indicate that *tr-1* is transcribed from a site outside the deleted region even though it is homologous to a segment within it. On the other hand, *tr-2* is missing in GM-3C-2, indicating that, like *CYC1* mRNA, it is in fact transcribed from the 2.4-kb region in the wild-type. The results described below confirm this conclusion.

Transcription in Transformed Cells. To study the effects of mutations constructed *in vitro* on regulation of transcription, a plasmid was constructed [YCp*CYC1*(2.4)] that was capable of transforming yeast cells with the *CYC1* gene region or its mutant derivatives (Fig. 2). The cloned centromere of yeast chromosome II, *CEN3*, was included in the construction to confer mitotic stability and single copy number onto the plasmid. This permitted analysis of transcription of plasmid-borne genes without interference from variations in copy number or plasmid loss in the nonselective media used. YCp*CYC1*(2.4) transformants grew normally on lactate plates, showing that the plasmid *CYC1* gene is transcribed at a sufficient rate to permit normal respiration in cells (GM-3C-2) whose phenotype is respiratory negative before transformation. When RNA from transformed cells grown under the three conditions described above was analyzed on RNA blots, the wild-type regulatory pattern for both *CYC1* and *tr-2* transcripts was revealed (Fig. 1*C*). Two additional transcripts appeared in these blots that were seen in yeast cells

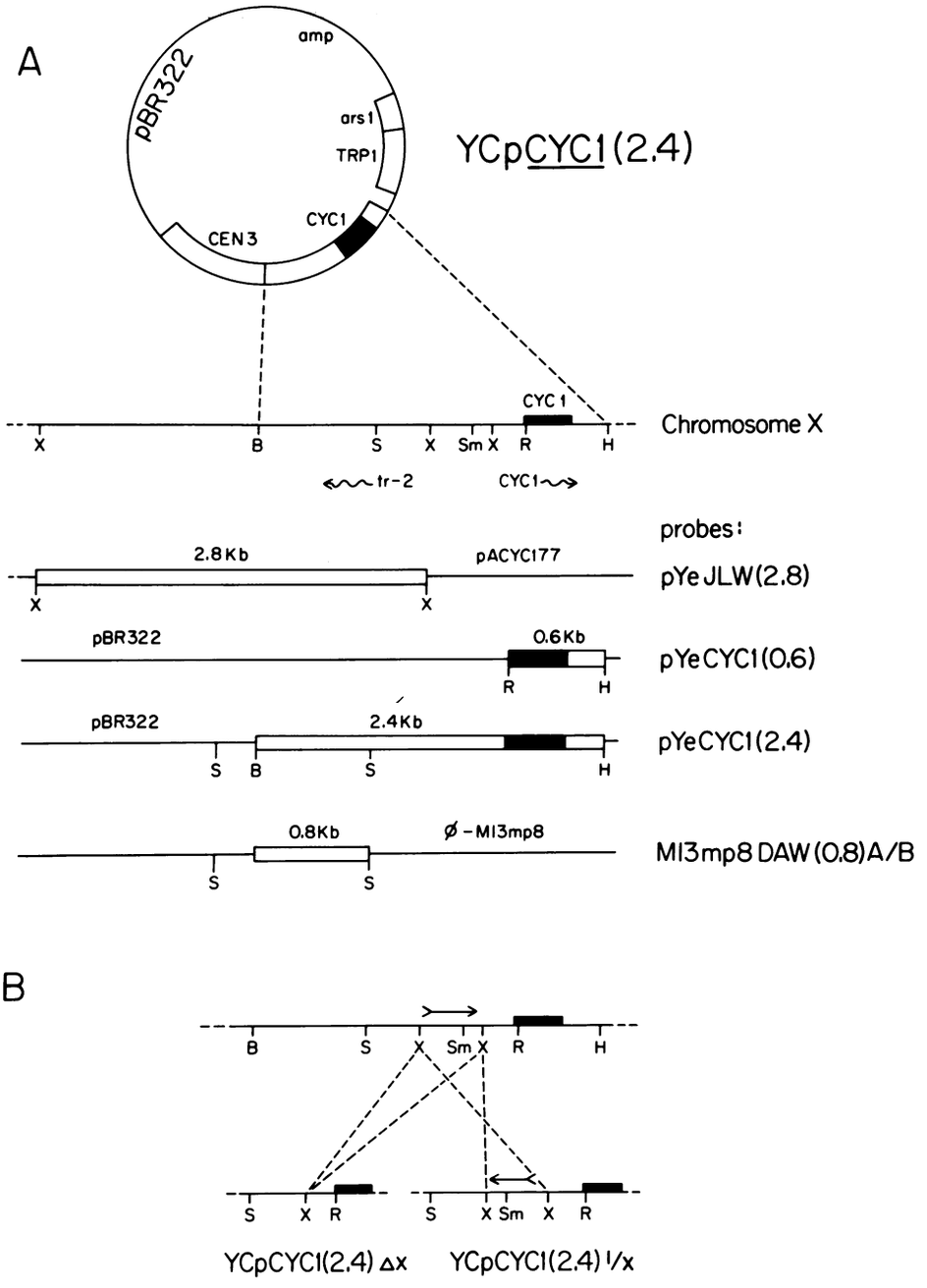

FIG. 2. Plasmids used for transformation or probing blots. (*A*) Transforming plasmids. □, DNA segments derived from yeast chromosomal DNA; ■, coding sequence of the *CYC1* gene; —, DNA derived from *E. coli* on the single-stranded coliphage M13. The *CYC1* transforming plasmid YCp*CYC1*(2.4) was derived from plasmid YRp7 by cutting with *Eco*RI, blunting with DNA polymerase I, and religating to destroy the two *Eco*RI sites followed by insertion of the 2.4-kb *Cla* I/*Bam*HI fragment containing the *CYC1* gene obtained from plasmid pYe*CYC1*(2.4). Into the resulting intermediate, YRp*CYC1*(2.4), was then inserted at the *Bam*HI site, the 2.2-kb *Bgl* II/*Bam*HI fragment that contains *CEN3* from plasmid pYeCEN41. The same intermediate was also converted into a deletion mutant, pCp*CYC1*(2.4)ΔX, and an inversion mutant, YCp*CYC1*(2.4)I/X, both of which were then supplied with the 2.2-kb *CEN3* fragment as above. The deletion was constructed by cutting YRp*CYC1*(2.4) with *Xho* I and ligating under dilute conditions so as to recircularize the plasmid with the 400-bp fragment excluded. The inversion was constructed by ligating the *Xho* I digest and screening transformant clones for plasmids containing the 400-bp fragment in an inverted orientation, shown by digestion with *Sma* I. The results shown are for the wild type and ΔX plasmid with the orientation of the centromere shown above and for the 1/X plasmid in the opposite orientation. In other experiments (data not shown), indistinguishable transformant phenotype and RNA gel blot analyses were obtained by using 1/X plasmids with the centromere in either orientation. (*B*) Plasmids used as probes. Plasmid pYeJLW(2.8) was constructed by inserting the 2.8-kb *Xho* I fragment from plasmid pYe*CYC1*(4.5) into the *Xho* I site of pACYC177. Single-stranded phages M13mp8DAW(0.8) A and B were constructed by inserting the 1.1-kb *Sal* I fragment from pYe*CYC1*(2.4) into the purified replicative form of phage M13mp8 and isolating replicative form derivatives having inserts in both orientations (A and B; A is shown). Restriction enzyme sites: B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; S, *Sal* I; Sm, *Sma* I; X, *Xho* I.

transformed with a variety of vectors containing pBR322 sequences and shown to be plasmid specific (unpublished data).

Transcription from Mutant Plasmids. Because normal regulation of expression was observed in plasmid-borne genes, it was possible to study the effects of mutations in regions regulating transcription. Two mutations were constructed involving the 400-bp region bounded by the *Xho* I sites at −250 and −650 (250 and 650 bp 5′ from the *CYC1* translation initiation

codon). Therefore, this sequence lies 190 bp upstream from the start of the *CYC1* message at −61 (61 nucleotides 5′ from the ATG initiation codon; ref. 25). In one mutant plasmid, YCp*CYC1*(2.4)ΔX, this region was deleted and, in the other, YCp*CYC1*(2.4)1/X, it was inverted (Fig. 2*B*). The deletion mutation was similar to the one constructed by Guarante and Ptashne (2). Cells transformed with the deletion plasmid were found to have impaired respiration, growing slowly on lactate. Cells transformed with the inversion plasmid were almost respiratory negative, growing slowly on glycerol and not at all on lactate. It was inferred from the phenotypes that transcription of *CYC1* mRNA would be reduced in the deletion plasmid and null in the inversion plasmid. RNA blot analysis of RNA extracted from transformants confirmed this expectation and showed an altered regulatory pattern for both genes (Fig. 3).

In cells carrying the deletion plasmid, expression of both genes was much less sensitive to growth conditions than in wild-type cells. *CYC1* mRNA was present at approximately the same decreased levels in cells grown anaerobically and aerobically in glucose medium (Fig. 3, lanes 1 and 2 vs. lanes 3 and 4). Thus, it seems that *CYC1* expression had been reduced and that there was an impairment of the shut-off mechanism that normally prevents mRNA accumulation in the absence of oxygen. Possible differences due to catabolite repression could not be determined reliably at this low level of expression. The deletion affected expression of the *tr-2* gene in a similar way. The levels observed were lower than maximal under all conditions but did not show the shut-off normally observed in aerobic cultures (Fig. 4, lanes 1–3 vs. lanes 4–6). This change was apparently

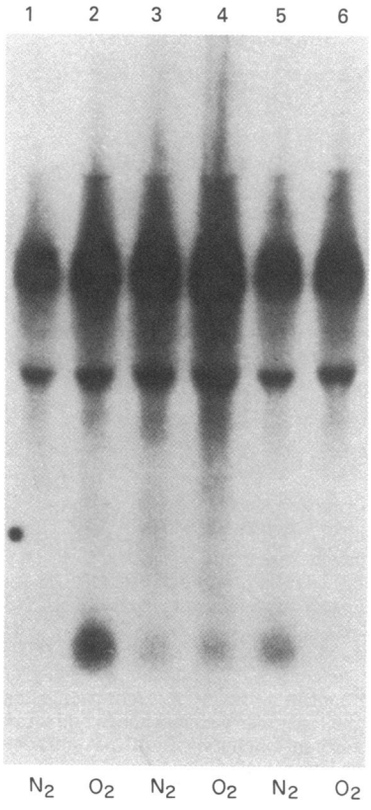

FIG. 3. *CYC1* mRNA in cells transformed with wild-type and mutant plasmids. RNA blots were probed with nick-translated pYe*CYC1*(0.6), which is specific for pBR322 plasmid transcripts and *CYC1* mRNA. RNA was prepared from cultures of transformed GM-3C-2 cells grown in YPD anaerobically (N_2) or aerobically (O_2). Transforming plasmids were YCp*CYC1*(2.4) (lanes 1 and 2), YCp*CYC1*(2.4)ΔX (lanes 3 and 4), and YCp*CYC1*(2.4)1/X (lanes 5 and 6).

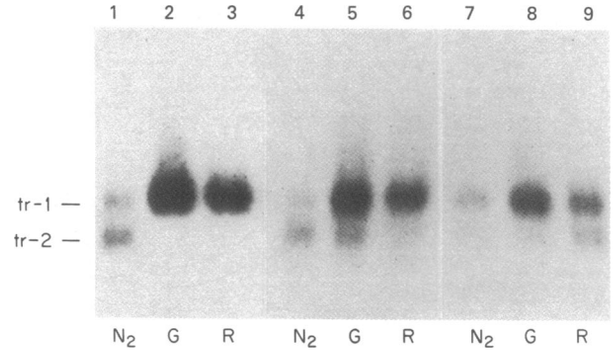

FIG. 4. *tr-1* and *tr-2* transcripts in cells transformed with mutant plasmids. RNA blots were probed with the nick-translated 1.2-kb *Xho* I/*Bam*HI fragment prepared from pYe*CYC1*(2.4), which is specific for *tr-1* and *tr-2*. Lanes: 1–3, GM-3C-2 cells transformed with YCpCYC(2.4); 4–6 and 7–9, GM-3C-2 cells transformed with YCp*CYC1*(2.4)ΔX and YCp*CYC1*(2.4)1/X, respectively. Cells were grown anaerobically in YPD (N_2), aerobically in YPD (G), or aerobically in YPR (R).

accompanied by catabolite activation of expression of *tr-2*, as levels were somewhat higher in aerobic cultures containing glucose than in those containing raffinose. It is difficult to interpret this last observation because, in aerobic wild-type cultures, *tr-2* expression is too low to allow detection of any catabolite effect.

Regulation of expression was also markedly affected in cells transformed with the 1/X inversion plasmid. *CYC1* mRNA was now absent under aerobic conditions but appeared at higher than the constitutive level in cells grown under nitrogen (Fig. 3, lanes 5 and 6), mimicking the regulatory pattern of the wild-type expression of *tr-2*. The effect of the inversion on the regulation of *tr-2* expression was similar to the effect of the deletion except that there was a moderate but consistent increase in expression in cells grown on raffinose compared with those grown on glucose (Fig. 4, lanes 5 and 6). In addition, in a majority of analyses, a weak stimulation by oxygen would be observed (Fig. 4, lanes 4 and 5). These effects parallel the normal regulation of *CYC1* transcription, but they are rather weak and may be due to other less-specific effects on RNA metabolism. Hence, it appears that, in the inversion mutant *CYC1*, transcription has come under control of the *tr-2* regulation system and possibly that *tr-2* is partially under control of the *CYC1* regulation system.

DISCUSSION

The results presented here show that *CYC1* mRNA accumulates only during growth in aerated media. In contrast, the transcript *tr-2* was shown to be present only in anaerobically grown cells. The *tr-2* transcription site was localized to a region of DNA 5′ from the *CYC1* gene and on the opposite strand. Thus, the two neighboring genes are transcribed in opposite directions and are regulated in an opposite manner. The spatial arrangement of the two transcription sites allowed the construction of mutations in the intervening region that simultaneously affect expression of both genes.

The deletion of a 400-bp region produced a phenotype in which expression of both genes was weakly constitutive—well below normal levels, though not absent, and much less stringently regulated. This indicated that modulator sequences affecting both genes had been wholly or partially deleted. The inversion of the same 400-bp region also disrupted the regulation of the two genes. The most dramatic effect was that now *CYC1* mRNA accumulated only in the absence of oxygen, indicating that the *CYC1* transcription unit had been put under a new control mechanism, presumably that of the *tr-2* modu-

lator. Expression of the *tr-2* gene in the inversion mutant was weakly constitutive, similar to that in the deletion, but it consistently showed a slight catabolite repression. Although this may be due to other factors, such as differences in growth rate, it is consistent with the possibility that the inversion has brought the *CYC1* regulatory region into proximity to the *tr-2* transcription unit where it exerts a weak regulatory control.

The existence of a *CYC1* modulator sequence and the fact that it functions at a minimum distance of 190 bp from the start of *CYC1* transcription [at −61 (25)] was evident from the reduction of transcription from mutant plasmids, because in these a region 190 bp from the start of transcription had been deleted or inverted (Fig. 2*B*). The length of the transcript was the same in the wild type and in the two mutants, within the limits of resolution of the gel blot analysis, indicating that transcription in the mutants started at the normal position. Thus, by the same reasoning, when the *tr-2* modulator was brought into control over the *CYC1* transcription unit in the inversion mutant, it must also have been controlling *CYC1* expression at a distance of at least 190 bp. The minimum distance between the *tr-2* modulator and the start of its own gene is less precise, since the *tr-2* transcript is not fully characterized. However, judging from the boundary of the two mutations affecting *tr-2* expression (−650) and the position of the start of the one long open reading frame in the *tr-2* region [−980; Michael Smith (University of British Columbia), personal communication], the modulator is probably at least 230 bp from the start of transcription, assuming a 100-nucleotide leader sequence on the *tr-2* mRNA.

The oxygen-linked regulation of the mutant and wild-type genes studied here is most easily explained by assuming that the mechanism is bimodal—i.e., alternately enhancing or repressing transcription in response to the physiological state of the cell. This would take into account the observation that in the deletion mutant expression of both the *tr-2* and *CYC1* genes was weakly constitutive under both aerobic and anaerobic conditions, intermediate between the maximal and undetectable levels present in the wild type at the two physiological extremes. A simple activation mechanism would require that, when the activator site was removed, expression could not take place. A simple repression mechanism would require that, when the repressor site was removed, expression would be maximal. However, removal of an upstream region by deletion neither abolished nor maximized expression but instead reduced it and rendered it almost constitutive. This suggests that sequences with both enhancing and repressing functions have been removed, exposing an intrinsic activity for the two unmodulated transcription units. Another explanation of the constitutive expression in the deletion mutant is that it is an artifact due to a disturbance of DNA spacing (e.g., nucleosome phasing) or the result of the juxtaposition of weakly activating sequences (pseudomodulators) at the ends of the deletion. More detailed modifications are required to eliminate the possibility of such topological effects, including smaller deletions and point mutagenesis.

The translocation of the *tr-2* modulator in the 1/X inversion has apparently placed the *CYC1* transcription unit under oxygen repression. This raises the possibility of further investigating the function of the *tr-2* modulator by mutagenesis. As proposed here, the inverted *tr-2* modulator represses *CYC1* mRNA synthesis, producing a nonrespiring phenotype, and it may be possible to select for plasmid and host mutations that suppress the 1/X mutation by inactivating the *tr-2* shut-off mechanism. Such suppressor mutations would be expected to permit weak constitutive production of *CYC1* mRNA and, consequently, growth on lactate plates.

Other questions that may be resolved by further DNA manipulation and mutagenesis experiments concern the number of modulator sites. It is not known whether the catabolite repression mechanism overlaps the oxygen-linked regulation of *CYC1*. Small plasmid deletions affecting one regulatory function but not the other could be studied to determine whether one or more sites are involved. Similarly, although our interpretation of the pattern of oxygen-linked regulation of the *tr-2* and *CYC1* genes assumes the existence of two modulators, other explanations are possible. For example, there may be a single *oriented* modulator that directs transcription toward *CYC1* in the presence of oxygen and toward *tr-2* in its absence. The isolation of plasmid mutations simultaneously affecting oxygen-linked regulation of both genes would favor such a hypothesis.

It is not clear why the *tr-2* modulator showed a greater regulatory effect over *CYC1* than did the *CYC1* modulator over *tr-2*. The spacing of the various sequences may happen to favor the *tr-2* modulator in the inversion studied here, but it is possible that the two modulators function in a different way. The *CYC1* modulator may have some specificity for its own gene or require sequences spanning the *Xho* I site at −250 that have been interrupted by the inversion.

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