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Deletion mapping of sequences essential for *in vivo* transcription of the iso-1-cytochrome *c* gene

(yeast RNA polymerase II promoter mapping/multiple mRNA 5′ ends/*in vitro* mutation–yeast transformation)

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ABSTRACT **The 5′ termini of yeast *CYC1* RNA molecules have been mapped, by nuclease S1 digestion of mRNA·DNA duplexes, to seven locations from 29 to 93 base pairs upstream from the initiating ATG codon. When the *CYC1* gene is introduced into yeast in plasmid YEp13, substantially the same transcripts are made. Using this system to study *in vivo* gene expression, we measured the capacity of enzymatically produced DNA deletions to form the normal set of RNAs. Four regions of 5′-flanking DNA were identified as functional. Sequences within the region −242 to −139 are required for maximal *CYC1* transcript formation; their deletion reduces transcription by a factor of 15 but does not change the pattern of 5′ ends observed. Deletion of the sequence between −242 and −99 does not further change the overall transcript level but does affect the specificity of *CYC1* mRNA starting. A deletion that extends from −242 to −75 causes both an additional shift in the pattern of 5′ ends observed and a further large decrease (factor of 10–20) in *CYC1* RNA level. Deletions that extend from −242 to −43, and particularly two deletions that extend still closer to the initiating ATG, cause the appearance of an abundant transcript which starts upstream of position −1078 and of minor transcripts starting in the region −325 to −245.**

The DNA sequences essential for promoter recognition by *Escherichia coli* RNA polymerase have been identified by combining physiological studies of mutant organisms, genetic mapping, DNA sequence determinations and a variety of tests of RNA polymerase-binding specificity (1, 2). Molecular cloning and DNA sequence determinations of genes transcribed by eukaryotic RNA polymerases I, II, and III (3) has stimulated investigation of the recognition signals for these enzymes (3). Most of such studies of eukaryotic genes have used an approach analogous to the mutant analysis of *E. coli* promoters but with *in vivo* mutagenesis, genetic mapping, and physiological study replaced by *in vitro* experiments to change the DNA and to characterize the resulting "mutant" genes both structurally and functionally (4–7).

In *Saccharomyces cerevisiae*, questions about the transcriptional role of sequences flanking genes can be answered by testing the capacity for *in vivo* function of genes that have been modified by *in vitro* manipulations (8, 9). By partial digestion of the cloned *CYC1* gene (10, 11) with *E. coli* exonuclease III and subsequent nuclease S1 treatment, we have constructed a series of deletion mutations, each of which begins at base pair (bp) −242 (before ATG) and extends for a varying distance toward the yeast *CYC1* coding region. By use of a yeast transformation system (8) and a plasmid vector that can replicate both in *E. coli* and yeast (12), each mutant *CYC1* gene was cloned and then introduced into a yeast recipient strain lacking *CYC1*. Each of the mutant genes is capable of *CYC1* functional complementation, even with deletions approaching as closely as nine bp to the initiating AUG. The deletion mutations have large effects upon the level and distribution of starting points of physically observable *CYC1* transcripts, suggesting the existence of four distinct functional regions within the *CYC1* promoter.

MATERIALS AND METHODS

Yeast Strains. Strain GM-3C (*α*, *leu2*-3, *leu2*-112, *trp1*-1, *his4*-519, *cyc1*-1, *cyp3*-1) carries the mutation *cyc1*-1 which deletes the entire iso-1-cytochrome *c* gene (13, 14) and the point mutation *cyp3*-1 in the iso-2-cytochrome *c* gene (15). GM-3C was unable to grow on the 1.0 M sorbitol medium used in yeast transformation because the deletion *cyc1*-1 eliminates the *osm1* gene (13). However GM-3C-2, a spontaneous "revertant" able to grow on hypertonic medium, has been isolated.

***E. coli* and *S. cerevisiae* Transformation.** DNA preparation, restriction analysis, and cloning in *E. coli* were carried out as described (10, 16). Yeast transformations were done essentially as described (17).

Deletion at the 5′ End of Iso-1-cytochrome *c* Gene. From yeast strain D311-3A, we recloned the iso-1-cytochrome *c* gene as a 5.2-kilobase *Hin*dIII–*Hin*dIII fragment; from this we transferred a 2.5-kilobase *Bam*HI–*Hin*dIII fragment to pBR322 to make recombinant plasmid pYe*CYC1*(2.5), to YRp7 (18) to make pTC1, and to YEp13 (12) to make YEp13*CYC1*(2.5). To generate deletions at the 5′ end of iso-1-cytochrome *c*, the large *Xho* I–*Xho* I fragment of pYE*CYC1* (lacking *CYC1* 5′ flanking sequences from −670 to −247) was subjected to limited exonuclease III digestion followed by nuclease S1 treatment to generate deletions (19). After attachment of *Xho* I molecular recombination linkers and subsequent *Xho* I and *Hin*dIII nuclease digestion, the pool of deleted *CYC1* genes was used to replace the original *Xho* I-*Hin*dIII *CYC1* fragment in recombinant plasmid pTC1. The exact end points of deletions in individual mutant clones were determined by DNA sequence determination by using the terminator method (20) with a synthetic oligodeoxyribonucleotide primer (11). Six hybrid plasmids were chosen for further study. For each of these, the *Xho* I–*Xho* I fragment (lost during mutagenesis) was reintroduced at the *Xho* I site in the original orientation. Then the yeast *Bam*HI–*Hin*dIII DNA fragments of these plasmids were transferred to the YEp13 vector. These new hybrid plasmids, named ΔX-139, ΔX-

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Abbreviation: bp, base pair(s).

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99, ΔX-75, ΔX-43, ΔX-24, and ΔX-9, were used to transform yeast strain GM-3C-2 to *LEU2*$^+$.

Deletion of *Eco*RI-*Kpn* I Fragment. The plasmid pYe*CYC*1(2.5) was digested with *Eco*RI and then with *Kpn* I. The fragment was purified, repaired with Klenow-modified DNA polymerase, and then nuclease S1-digested and recircularized by blunt-end ligation. Then the *Bam*HI–*Hin*dIII yeast fragment was transferred to the YEp13 vector. The hybrid plasmid thus obtained was named "KR4701."

Nuclease S1 Mapping. *RNA.* GM-3C-2 transformed with each of the hybrid plasmids bearing *CYC*1 deletions was grown in minimal medium (without leucine) with 2% (wt/vol) glucose (cell concentration, OD_{600} = 3–4). Total RNA was extracted and polyA$^+$mRNA was purified on an oligo(dT)-cellulose column.

DNA probe. Hybrid plasmid DNAs were digested with appropriate restriction enzymes and were 5′ end-labeled with T4 polynucleotide kinase. Relevant restriction fragments were isolated, and their strands were separated as described (21). Nuclease S1 mapping was as described (22) with the modifications introduced by Nasmyth *et al.* (23). For quantitative comparisons of bands, the autoradiograms were scanned with a Joyce–Loebl densitometer.

Cytochrome Absorption Spectra of Yeast Cells. Yeast cells were grown in 1% yeast extract/1% bactopeptone/2.5% (wt/vol) glycerol or in minimal medium (without leucine) with 1% glucose. Spectra were measured in liquid nitrogen (24) with an Aminco DW-2 apparatus.

RESULTS

Complementation of Cytochrome *c* Function from a Yeast Plasmid. As the recipient for transformation experiments, a yeast strain (GM3C2) was constructed that carried both the *trp*1 and *leu*2 markers, a deletion mutation (*cyc*1-1) that eliminated the gene coding for iso-1-cytochrome *c*, and a point mutation (*cyp*3-1) in the structural gene for iso-2-cytochrome *c*. The *CYC*1 gene, as a 2535-bp *Bam*HI–*Hin*dIII fragment (Fig. 1*A*) of known sequence (ref. 11; unpublished data), was inserted into the yeast cloning vectors YEp13 and YRp7, which carry *LEU*2 and *TRP*1, respectively. Each of the resulting plasmids was introduced into the recipient yeast strain by transformation, with selection for growth either on leucine-free or tryptophan-free agar and with glucose as the carbon source. In subsequent growth tests, both the *LEU*$^+$ transformants produced by YEp13*CYC*1(2.5) and the *TRP*$^+$ transformants produced by pTC1 were capable of growth with either lactate or glycerol as the sole carbon source. Therefore, the gene is functional when present on either plasmid.

As an independent test of cytochrome *c* function in the YEp13*CYC*1(2.5) transformants, spectroscopic assays for cytochrome *c* hemoprotein were carried out on the frozen yeast cells. The results (Fig. 2) show an enhanced cytochrome *c* absorption band as compared to that observed for a *CYC*1$^+$ yeast strain (D311-3A). By comparison, the recipient strain used in transformation shows no discrete band at 547 nm. These data indicate that cells carrying a cytochrome *c* gene on the high-copy number plasmid YEp13 overproduce cytochrome *c* by a factor of 3–4 compared to the level of chromosomal *CYC*1 gene expression.

5′ Termini of *in Vivo* Transcripts from Chromosomal and Plasmid-Borne *CYC*1 Genes. The ability of the *CYC*1 gene in plasmid YEp13*CYC*1(2.5) to code for a functional cytochrome *c* in transformant yeast cells demonstrates that translatable mRNA is made from this gene. To determine whether the transcription signals that mediate synthesis of *CYC*1 mRNA from the plasmid are the same ones involved in transcription of the *CYC*1 gene in its normal chromosomal context, the 5′ ends of *CYC*1 mRNA molecules were mapped and compared for the two cases. For the *CYC*1$^+$ strain D311-3A, polyA$^+$RNA was prepared from cells grown either with glucose, glycerol, or galactose as the carbon source. The 5′ ends of the *CYC*1 mRNA molecules present were mapped by hybridizing the total cellular polyA$^+$RNA with a 5′ end-labeled *Xho* I–*Eco*RI complementary DNA strand (covering the initial 11 bp of the *CYC*1

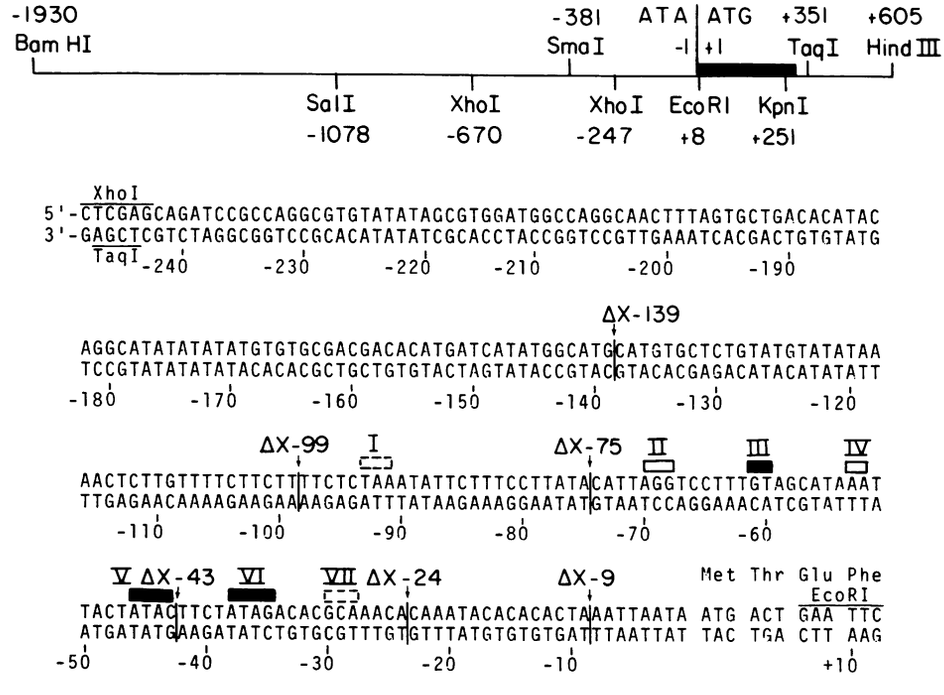

FIG. 1. (*A*) Restriction diagram of the *Bam*HI–*Hin*dIII restriction fragment carrying the iso-1-cytochrome *c* gene. (*B*) The 5′ termini of different transcripts of this gene shown on the *Xho* I–*Eco*RI DNA sequence (11). ■, □, and ⊏⊐ indicate the approximate positions of 5′ ends of high, medium, and low abundance, respectively. These have been determined by nuclease S1 mapping. The end points of the six deletions studied are indicated.

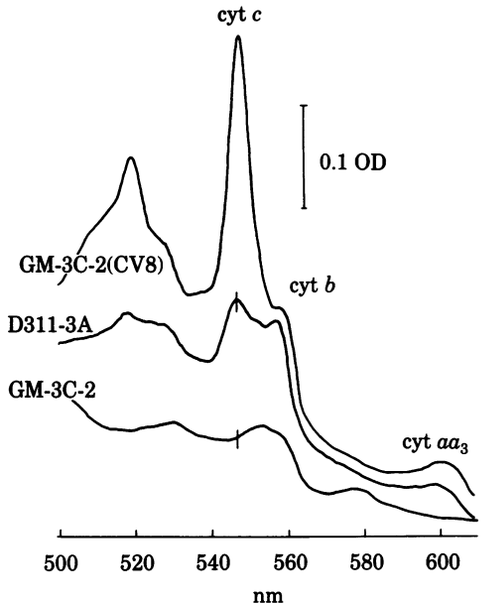

FIG. 2. Low temperature absorption section of yeast cells GM-3C2 grown in yeast extract/bactopeptone/1% glucose and D311-3A and GM-3C-2(CV8) grown in yeast extract/bactopeptone/2.5% glycerol; GM-3C-2(CV8) is the strain transformed with plasmid YEp13*CYC*1(2.5). cyt, Cytochrome.

coding region plus 243 bp of the 5′ flanking DNA). After nuclease S1 digestion to degrade unhybridized DNA sequence and subsequent denaturation, the size of the protected DNA strand was measured by polyacrylamide gel electrophoresis (Fig. 3) Protected fragments of seven different sizes are seen, indicating 5′-leader extensions of various lengths for different *CYC*1 RNA molecules. An alternative explanation, that the mixture of RNA ends reflects an intracellular mixture of mature *CYC*1 mRNA molecules and unprocessed precursors with intervening sequences, has been ruled out by an experiment with exonuclease VII. The possibility remains open that some of the RNA 5′ ends observed may reflect terminal processing rather than transcription starting points. The same seven bands were observed regardless of the carbon source on which the yeast culture was grown. The positions of these 5′ ends in relation to the corresponding DNA sequence are shown in Fig. 1*B*.

When polyA$^+$RNA from YEp13*CYC*1(2.5)-transformed yeast was used to carry out a similar nuclease S1 protection experiment with the 5′ end-labeled *Xho* I–*Eco*RI probe, only bands I, II, and III were visible (Fig. 3, leftmost lane). However, by varying 15-fold the amount of input polyA$^+$RNA (results not shown) bands IV, V, and VI were seen. The level of total *CYC*1 transcripts appeared to be about 15 times higher in cells containing YEp13*CYC*1(2.5) than in strain D311-3A and, without an adjustment of the ratio of total RNA:DNA probe during hybridization, this higher content caused a skewing of the apparent 5′ end distribution toward longer RNA molecules. The complete correspondence between the pattern of transcripts from the chromosomal gene and that from a plasmid-localized *CYC*1 gene can be seen by comparing the second and third lanes in Fig. 3. Additional detailed evidence for the transcriptional equivalence of chromosomal and extrachromosomal *CYC*1 genes was obtained by *in vitro* reverse transcription experiments with purified *CYC*1 mRNA from yeast strain D311-3A and with total polyA$^+$RNA from GM3C2 cells bearing the YEp13*CYC*1(2.5) plasmid. The complex pattern of 5′ ends observed was the same in both cases and agreed with that in Fig. 1 (unpublished results).

5′-End Mapping of *in Vivo* Transcripts from Deleted *CYC*1 Genes. For 17 of the 90 mutant *CYC*1 genes made by the *in vitro*

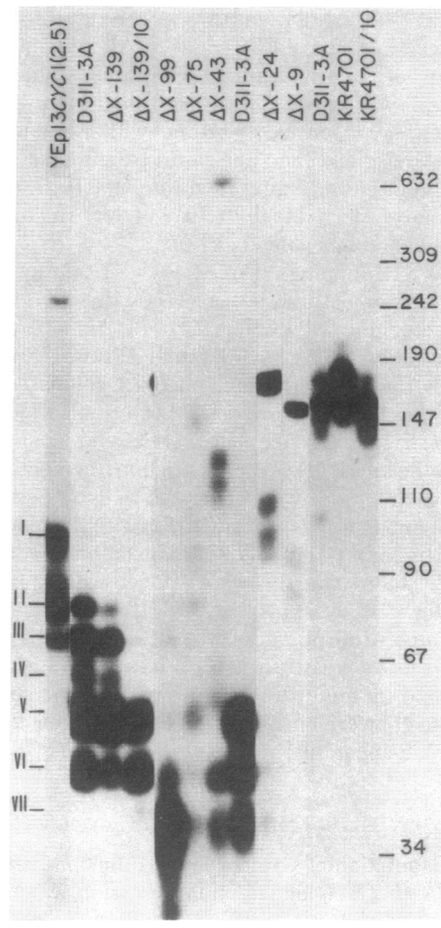

FIG. 3. Nuclease S1 mapping of 5′ ends of iso-1-cytochrome *c* mRNA from D311-3A and GM-3C-2 transformed with different plasmids. The protected DNA strands were run on 6% (wt/vol) polyacrylamide denaturing gels. Except as indicated, the quantity of polyA$^+$RNA used during hybridization was 30 μg/25 μl. For each hybridization with RNA from plasmid-transformed GM-3C-2 cells, the DNA probe (strand-separated) was derived from a restriction fragment of the homologous plasmid. For RNA from cells transformed by YEp13*CYC*1(2.5) and for the adjoining D311-3A lane, the *Xho* I-*Eco*RI fragment was used as probe. For the deletion plasmids, the probes were *Sma* I–*Eco*RI for ΔX-139, ΔX-24, and ΔX-9 and *Sal* I–*Eco*RI for ΔX-99, ΔX-75, and ΔX-43. For the experiment in lane 8, a *Sal* I–*Eco*RI probe from the ΔX-43 deletion was hybridized to RNA from yeast strain D311-3A. For hybridization with RNA from GM-3C-2 cells transformed by the internal deletion plasmid KR4701 and for the adjoining D311-3A lane, the probe was the *Taq* I–*Taq* I fragment of KR471 DNA, with ends at −247 and +351. Note that in hybrids between D311-3A and this probe, the DNA was poorly cut by nuclease S1 opposite the nonhomologous RNA loop.

deletion procedure, the exact deletion end point was determined by DNA sequence determination. The extent of deletion for each mutant used in this study is shown in Fig. 4. To characterize the degree of remaining *CYC*1 gene activity for each deletion mutant, we used RNA hybridization analysis in preference to growth rate studies on glycerol or lactate medium. For each of the six mutant plasmids studied, polyA$^+$RNA was isolated from transformed yeast cells grown in leucine-free medium with 2% glucose and hybridized to a 5′ end-labeled *Eco*RI–*Sal* I or *Eco*RI–*Sma* I probe derived from the homologous plasmid. After nuclease S1 digestion, the protected DNA strands were separated and sized on a denaturing polyacrylamide gel (Fig. 3).

The pattern of protected fragments obtained with DNA and RNA from deletion ΔX-139 was identical to that observed when an equivalent quantity of RNA from strain D311-3A (chromo-

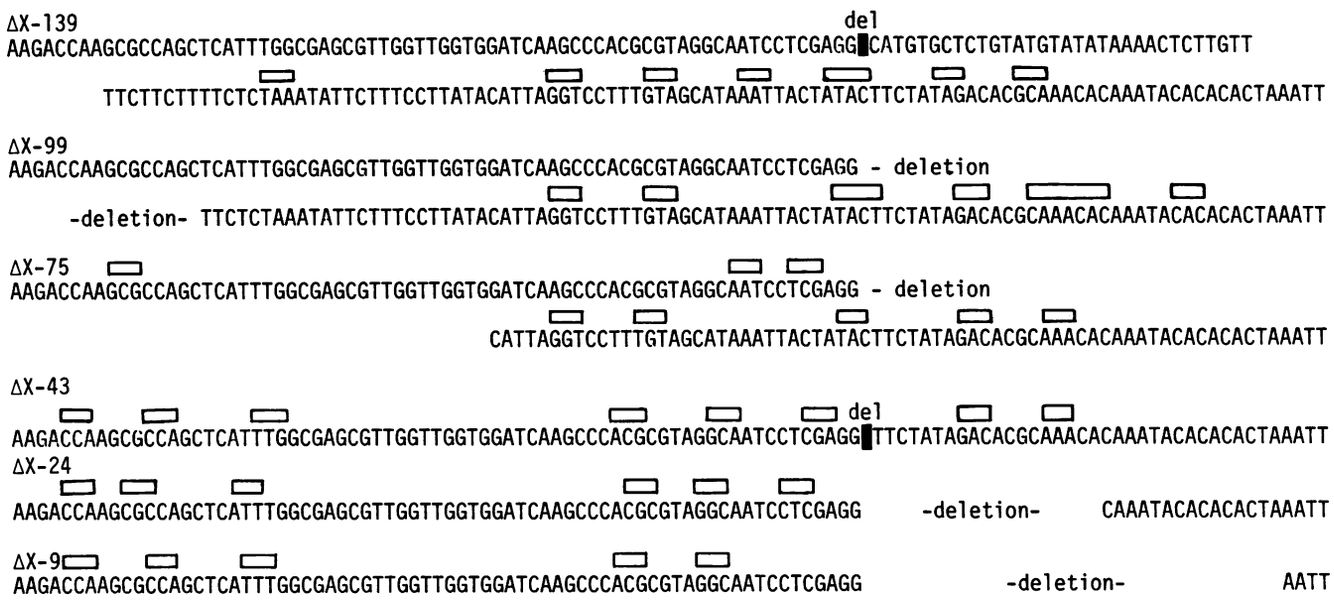

FIG. 4. Extent of the six deletions generated between the 5′ end of iso-1-cytochrome *c* coding sequence (ATG) and the *Xho* I site, 245 bp upstream. The approximate positions of the 5′ ends of their respective transcripts are indicated by boxes.

somal *CYC1*, no plasmid) was hybridized to an undeleted probe (Fig. 3). We have seen that RNA from glucose-grown yeast cells containing YEp13*CYC1*(2.5) formed a darker pattern of larger DNA bands (Fig. 3) when this same RNA concentration was used. Only by diluting the RNA from the undeleted *CYC1*$^+$ plasmid by a factor of 15 prior to hybridization could the pattern be made to resemble that for ΔX-139 plasmid-transformed cells and for D311-3A. Therefore, deletion of nucleotides −242 to −138 produced a reduction approximately by a factor of 15 in the level of cytochrome *c* mRNA as compared to wild-type but did not alter the distribution of 5′ ends observed (Fig. 4).

For deletion ΔX-99, the quantity of *CYC1* transcript remained about equal to that for ΔX-139; however, the pattern of transcripts was altered. Bands I and IV disappeared, band VII (−29 to −23) became the major RNA, and a new band appeared corresponding to a 5′ end at −16 (Figs. 3 and 4).

The overall levels of *CYC1* transcripts were similar for deletions ΔX-75, ΔX-43, ΔX-24, and ΔX-9; in all cases, these levels were 1/10th to 1/20th the transcript levels observed for ΔX-139 and ΔX-99. Whereas RNAs II, III, and V–VII were transcribed from ΔX-75 and VI and VII were transcribed from ΔX-43, for these deletions new transcription starts were observed in the region immediately to the left of the deleted sequence. Beginning with protected fragments of lengths 90–95 and 140–145 for deletion ΔX-75, these bands occured in a pattern which moved diagnonally downward (left to right) as the deletion length increased (Fig. 3), indicating the presence of a family of *CYC1* transcription starts at fixed locations on the DNA sequence (Fig. 4).

For the three longest deletions, a hybridization band was observed corresponding to a fully nuclease S1-protected DNA probe. The results in Fig. 3 show that RNA from deletion mutant ΔX-43 protects the entire *Sal* I–*Eco*RI probe and that deletion mutants ΔX-24 and ΔX-9 yield high levels of an RNA that protects the entire *Sma* I–*Eco*RI fragment (Fig. 1). RNA transcribed from the latter two mutant genes was also able to protect the entire *Sal* I–*Eco*RI probe (results not shown); in that experiment, no 5′ ends of *CYC1* RNA were found within the *Sal* I–*Sma* I interval. Therefore, the 5′ ends of all of these longer transcripts produced by deletions ΔX-43, ΔX-24, and ΔX-9 must lie to the left of the *Sal* I site.

Transcription of an Internally Deleted *CYC1* Gene. To determine whether sequences within the main body of the *CYC1* gene are required for its transcription, the deletion mutation Δ +10 to +247 (KR4701) was created by removal of the DNA sequence between the *Eco*RI and *Kpn* I sites (11) and subsequent ligation together of the 5′ and 3′ extremities. This internally deleted *CYC1* gene was transferred to vector YEp13, and the resulting hybrid plasmid was introduced into the *cyc*1-1 *cyp*3-1 *leu*2 recipient yeast strain by selection for *LEU*$^+$. For the analysis of transcripts from this yeast strain, we constructed a strand-separated *Taq* I–*Taq* I probe spanning the deletion (ends: −247 and ^{32}P +351; Fig. 1). This probe was hybridized on the one hand to RNA from strain D311-3A and on the other hand to an equal quantity of RNA from cells transformed by the *Eco*RI–*Kpn* I-deleted plasmid or to 1/10th this amount of RNA (Fig. 3). With diluted RNA made from the *Eco*RI–*Kpn* I mutant plasmid, the same pattern of protected bands was observed as with D311-3A RNA; however, the intensity is 1.4 times greater for the deletion RNA. From this result, it is evident that the rate of RNA synthesis by the internally deleted *CYC1* gene is at least 14 times that observed for yeast cells carrying a single chromosomal *CYC1* gene. Because this extent of difference is very similar to that observed between RNA from YEp13CYC1(2.5)-transformed cells and D311-3A, we conclude that the sequences within the region deleted are not required for *CYC1* transcription. The distribution of 5′ ends of *CYC1* RNA molecules is also the same for molecules transcribed from the *Eco*RI–*Kpn* I deletion and those from the *CYC1*$^+$ gene.

DISCUSSION

The objective of this study was to define the role of DNA sequences at the *CYC1* locus required for iso-1-cytochrome *c* mRNA production by using deletion mutants constructed *in vitro* and a homologous *in vivo* test system. Preliminary studies showed that all of the *CYC1* genes with deletions in the 5′-flanking sequence produced sufficient cytochrome *c* to support the growth of yeast cells on glycerol medium. Presumably this is a consequence of the gene-dosage effect of a multiple-copy plasmid coupled with a low requirement for iso-1-cytochrome *c* for respiratory growth. Hence, a change of one or two orders of magnitude in the production of mRNA together with an accompanying decrease in cytochrome *c* apoprotein does not produce a large change in growth phenotype. For this reason, we have characterized the deletions by their effects upon the intracel-

lular level of hydribidizable *CYC1* mRNA molecules and upon the positions of 5′ ends of these RNA molecules.

Deletion mutants that extended toward the *CYC1* coding region from a fixed point 242 bp upstream produced a variety of effects upon the level and the 5′ termini of iso-1-cytochrome *c* RNA molecules. These were of four types.

***(i)*. Reduction in Overall *CYC1* Gene Transcription.** This effect was produced by the deletion of each of two blocks of sequences. Deletion ΔX-139 had a *CYC1* RNA level 1/15th that of wild type; deletion ΔX-75 had a level of *CYC1* transcripts 1/20th that of deletion ΔX-99. These results suggest that sequences controlling the level of *CYC1* transcription are located in the regions −242 to −139 and −99 to −75.

***(ii)* Large Changes in the Relative Levels of One or More of the Seven *CYC1*.** This was most pronounced for deletion ΔX-99, which in comparison to ΔX-139 and to $CYC1^+$, contains increased levels of transcript VII and greatly decreased levels of transcripts II–V (Figs. 3 and 4). The large differences between the 5′ termini of ΔX-139 and ΔX-99 RNAs suggest that a sequence in this region, most likely −121 T-A-T-A-A-A-A, has a role in directing transcription initiation to starting sites II–V (25). Upon deletion of this sequence, the majority of starts are shifted to the region from −23 to −29 (Fig. 4), approximately 30 nucleotides downstream from −57 C-A-T-A-A-A. The activation of transcription initiation in this region by deletion of an upstream sequence required for starts II–V suggests that events required for initiation by RNA polymerase normally occur in a polarized manner. According to this model, sequences upstream of −121 T-A-T-A-A-A-A would serve as the initial binding site for RNA polymerase molecules that transcribe *CYC1*. Some form of polarized linear transport along the DNA would follow, culminating in a phasing event by the first T-A-T-A-A-A-like sequence to be encountered. For the undeleted *CYC1* gene, phasing by −121 T-A-T-A-A-A-A effectively captures nearly all approaching RNA polymerase II molecules, directing them to start points I–V. Starting point VII is relatively minor for the $CYC1^+$ allele because very few RNA polymerase molecules reach the corresponding T-A-T-A-A-A-like sequence (−57 C-A-T-A-A-A). This situation is relieved in deletion ΔX-99, with the result that those RNA polymerase molecules that approach the *CYC1* gene from upstream do reach −57 C-A-T-A-A-A without having been phased by a previous T-A-T-A-A-A-like sequence.

***(iii)* The Appearance of Novel *CYC1* Transcripts with 5′ Ends Mapping 0–80 bp Upstream of *Xho* I.** A family of transcripts with discrete 5′ termini mapping at the same positions in the interval −245 to −325 begins to be observed in deletion ΔX-75 and is found for all larger deletions as well. It appears that the signals that define these 5′ ends must be located in the DNA to the left of the *Xho* I site. This family of transcripts is not detectable in the ΔX-99, ΔX-139, or $CYC1^+$ genotype. This suggests that a sequence between −99 and −75 normally acts to prevent the synthesis of RNAs extending from the *Xho* I site rightward into the *CYC1* coding region.

***(iv)* Appearance of *CYC1* Transcripts with Long 5′ Extensions.** From the ΔX-43, ΔX-24, and ΔX-9 mutant *CYC1* genes, a very long RNA is transcribed which initiates to the left of *Sal* I, either in the *CYC1*-linked *Bam* I–*Sal* I segment or in YEp13 vector sequences that lie beyond *Bam I*. Because these very long transcripts are not seen in deletion ΔX-75, sequences that prevent their formation must be present between −75 and −43 and perhaps also between −43 and −24. Although our results implicate sequences between −75 and −24 in preventing such long transcripts from reaching the *CYC1* gene, they offer no clue as to the mechanism of prevention.

Is There a Control Region in the Middle of *CYC1*? The persistence of functional *CYC1* expression and the presence of a low but appreciable amount of *CYC1* RNA even in deletion ΔX-9 suggested a possible role for sequences within the *CYC1* coding region in specifying RNA transcribed by eukaryotic RNA polymerase III (19, 26–28). However, it was possible to rule out a similar role of the *CYC1* coding sequence in the production of RNA molecules with normal 5′-end positions. A *cyc*1 gene deleted for the sequences between +10 and +247 continues to be transcribed, yielding the same array of 5′ ends and the same overall level of *CYC1* transcripts as the intact gene. Although it is still possible that the sequence from −8 to +10 or the *CYC1* gene sequence beyond +247, or both, may have a role in mRNA initiation, this result eliminates models of transcription initiation in which a central control region (19) specifies transcription initiation a fixed distance upstream.

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