Identification of an Upstream Repressor Site Controlling the Expression of an Anaerobic Gene (ANBl) in Saccharomyces cerevisiae

Kamal D. Mehta and Michael Smith
From the Department of Biochemistry, Faculty of Medicine, and the Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia V6T 1W5, Canada

The Saccharomyces cerevisiae anaerobic gene (ANBl) is negatively regulated both by oxygen and heme. A 299-base pair-long fragment from the 5′-flanking region of the ANBl gene was found to confer oxygen-mediated negative regulation to an heterologous CYCl-LacZ hybrid gene. Studies with deletions of predefined length in this fragment demonstrated the presence of separate elements that comprise an upstream promoter that is active in the absence or presence of oxygen, and an upstream repressor site (URS) which confers strong repression upon the promoter element when oxygen is present. The promoter element is located 5′ to the URS in the ANBl gene. Mixed oligonucleotide-directed mutagenesis was used to obtain nucleotide substitutions in the URS which partially or completely inactivated this sequence without affecting the promoter activity. The URS region has three short direct repeats which seem to be important for function, as nucleotide substitutions within the repeats and not outside them, inactivated URS function. A model to explain the negative regulation of the ANBl gene by oxygen and heme is proposed.

In Saccharomyces cerevisiae, like other higher eukaryotes, regulation at the transcriptional level is often mediated by upstream regulatory elements located in the 5′-flanking region of a given gene (reviewed in Refs. 1–4). In inducible genes, upstream activation sites (UAS)1 stimulate transcription by providing sites for the binding of specific regulatory factors which are activated by an inducing agent. On the other hand, in genes that are negatively regulated, it has been shown that the UAS are located close to a regulatory site that in turn controls the action of UAS (5 and references therein). In S. cerevisiae, the iso-1-cytchrome c (CYCl) gene and the anaerobic gene (ANBl)2 are two neighboring genes that are transcribed in opposite directions and that are regulated in an opposite manner by oxygen and heme (6, 7). Transcription from the CYCl gene is induced in the presence of oxygen or heme, but the adjacent ANBl gene is transcribed only in their absence (6). Earlier studies have shown that the intergenic region between CYCl and ANBl contains DNA sequence elements that impart transcriptional regulation (8). In the case of CYCl, the UAS and the regulatory factors which bind to this sequence have been characterized (9–12). Similar detailed studies have not been carried out on the ANBl gene; however, it is known that a 299-bp-long Smal-Xhol fragment located about 200 nucleotides upstream from the transcription initiation sites may contain regulatory elements that respond to oxygen and heme (8). The molecular mechanisms involved in the negative regulation of the ANBl gene by oxygen or heme are at present not understood. It is of general interest to know the regulatory elements involved in its regulation, as it provides the only system to study the negative regulation of gene expression by factors such as oxygen or heme, which are important regulators of respiratory metabolism.

The objective of the present study was to identify the cis-acting elements involved in the regulation of the ANBl gene. In the present paper, by using a tridrib gene in which the 299-bp-long Smal-Xhol fragment from the 5′-flanking region of the ANBl gene is placed in front of a CYCl-LacZ hybrid gene, and, by obtaining deletions of defined lengths in this fragment, we provide evidence for the presence of a promoter element and an URS in this fragment. Complete or partial deletions in the URS led to constitutive expression of the otherwise normally inducible hybrid gene. Furthermore, by combining mixed-oligonucleotide-directed mutagenesis with a selection for inactivation of the URS function, point mutations in the URS region have been identified which partially or completely inactivate it without affecting the promoter activity. These studies demonstrate that the ANBl URS confers oxygen-mediated repression upon the promoter under aerobic conditions.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—Strains of Escherichia coli JM101 (13) and RR1 (14) were used for propagation of plasmids based on pEMBl (15) and pLG699-Z (16), respectively. The S. cerevisiae strain GMID-1 (a, cyc-1, ura3-52) used for analysis was obtained by a cross of ura3-52 (a, ura3-52; obtained from S. Roeder, Yale University) (17) with the GM3C-2 (a, leu2-3, leu2-112, top1-1, his3-519, cyc-1, cyc3-1) (18) and selecting for CYCl1 and URA3+ by its inability to grow on lactate plates as well as on synthetic media lacking uracil (SC-Ura) plates. The S. cerevisiaae strain, GM 5D-1, which lacks URA3, CYCl, and ANBl loci, was grown in YPD medium, and GM 5D-1 transformed with the indicated plasmid was grown in SC-Ura medium to maintain the plasmid.

Enzymes and Plasmid Construction—Standard procedures were used for restriction digestion and plasmid construction (19). Restric-
tion enzymes and T4 DNA ligase (Bethesda Research Laboratories, Inc., Gaithersburg, MD) were used according to the instruction of the supplier. The plasmid pLG669-Z was obtained from L. Guarente (Massachusetts Institute of Technology) (16), and the plasmid mpLG2 was constructed from pLG669-Z as described in the text. DNA fragments were purified from agarose gels as described by Maniatis et al. (19).

Oligonucleotide-directed Mutagenesis—For the construction of mutants, a 1.3-kb pair-long SphI-PstI fragment, containing the 299-bp-long Smal-XhoI fragment (see above), was isolated by digestion of mpLG2 with the restriction enzymes SphI and PstI. This fragment was cloned into pEMBL18 to create the plasmid JPK2. The single-strand complement of the above plasmid used for mutagenesis and sequencing was prepared as described earlier (15). The DNA was mutagenized according to the method of Zoller and Smith (20), as modified by Kunkel (21). Desired mutants were identified by direct sequencing using the dideoxy-chain termination method (22). Mutagenic oligonucleotides were synthesized on an Applied Biosystems 380 A DNA synthesizer. The 299-bp-long Smal-Xhol fragment in plasmid mpLG2 was replaced with Smal-Xhol fragment containing the mutation obtained from the above mutagenesis.

Strategy for the Isolation of Point Mutants in the URS Function—For the isolation of point mutants affecting the URS function, another modification of the method of Zoller and Smith was employed (23). Instead of using a single mutagenic oligonucleotide, a mixed pool of mutagenic oligonucleotides was used. These mixed oligonucleotides were synthesized by using a mutagenase that is active at low doses of three other nucleotides, and the levels of contaminating nucleotides used in the mixed synthesis cycles were calculated as described in McNeil and Smith (24). Here, a mixed 42-mer (5'-GGGGCGAAAAACAGGCCACGAGCCACATGGAAAAACGAAA-3') corresponding to positions -227 to -186 was synthesized such that at least two point mutations were introduced in the 42-bp-long target region. The single-stranded DNA that was synthesized with 35.1% of the wild type phosphoramide was mixed with 1.63% of each of the other three phosphoramides. The single-stranded template of plasmids JPK2 and JPK3 was annealed and then ligated to create the plasmid mpLG2, has the intact transcript initiation sites and the proper orientation. The XhoI site in plasmid mpLG2 is at position 413.

RESULTS

Measurement of β-Galactosidase Activity—Yeast cells were grown overnight at 30 °C either aerobically with vigorous shaking or anaerobically in flasks packed into sealed jars containing a Gas-Pak anaerobic system (BBL Microbiology Systems). For anaerobic growth, freshly autoclaved medium was degassed for 1 h and supplemented with 20 μg of ergosterol per ml and 0.2% Tween 80. Cells were harvested at a midlog density of growth, and β-galactosidase activity was determined by using the method described earlier (27). Variation in the assay when freshly transformed yeast cells were employed was always less than 15% for the same sample prepared from different cultures on different days.

Construction of the Vector—In order to define the cis-acting sequences that regulate the expression of the ANB1 gene and to ensure that they reside within the 299-bp-long Smal-Xhol fragment located at the 5'-end of the ANB1 gene, an analysis vector mpLG2 was constructed from the yeast-E. coli shuttle vector pLG669-Z (16). The nucleotide sequence of the 299-bp-long fragment is shown in Fig. 1. The vector pLG669-Z is a multicopy plasmid and contains a 2-μm origin.

It bears a CYC1-LacZ hybrid gene, whose expression is driven solely by the UAS of CYC1. For making mpLG2, pLG669-Z was digested with the restriction enzymes Smal and Xhol, and the 299-bp-long Smal-Xhol fragment was ligated with the backbone plasmid. The resultant plasmid mpLG2 contains all three functional TATA boxes located at positions -106, -52, and -22 and all six transcription initiation sites of CYC1 (the most upstream of which is +1) without the UAS, but instead having the Smal-Xhol fragment containing the regulatory elements of ANB1 gene in front of the CYC1-LacZ hybrid gene in the right orientation. The XhoI site in plasmid mpLG2 is at position -178, and the numbering continues over to the ligated Smal-Xhol fragment. The thick bar in the smaller Smal-Xhol fragment of plasmid PGL 699-Z denotes UAS of CYC1.

Construction of Deletions in the 5'-Regulatory Region of

FIG. 1. Nucleotide sequence of the 299-bp-long Smal-Xhol fragment from the 5'-flanking region of the ANB1 gene. Nucleotide sequence was determined on both strands by using the dideoxy-chain termination method (22). The thick line represents the URS identified in the study described here.

FIG. 2. Construction of the parent plasmid mpLG2 from the plasmid pLG669-Z which bears a 2-μm origin and a CYC1-LacZ hybrid gene whose expression is driven solely by the UAS of CYC1. For making mpLG2, pLG669-Z was digested with the restriction enzymes Smal and Xhol, and the 299-bp-long Smal-Xhol fragment was ligated with the backbone plasmid. The resultant plasmid mpLG2 contains all three functional TATA boxes located at positions -106, -52, and -22 and all six transcription initiation sites of CYC1 (the most upstream of which is +1) without the UAS, but instead having the Smal-Xhol fragment containing the regulatory elements of ANB1 gene in front of the CYC1-LacZ hybrid gene in the right orientation. The XhoI site in plasmid mpLG2 is at position -178, and the numbering continues over to the ligated Smal-Xhol fragment. The thick bar in the smaller Smal-Xhol fragment of plasmid PGL 699-Z denotes UAS of CYC1.

It bears a CYC1-LacZ hybrid gene, whose expression is driven solely by the UAS of CYC1 located in the smaller 143-bp-long Smal-Xhol fragment (10). This plasmid was modified by removing the CYC1 promoter fragment and replacing it with the 299-bp-long Smal-Xhol fragment containing the 5'-flanking DNA of the ANB1 gene (Fig. 2). The resulting plasmid, mpLG2, has the intact transcript initiation sites and the TATA boxes of the CYC1 gene coupled to the regulatory sequences of the ANB1 gene.

The recipient strain GM5D-1 which lack URA3, ANB1, and CYC1 loci was transformed with the starting plasmid pLG669-Z and the newly constructed plasmid mpLG2, and the β-galactosidase activity was measured in transformants grown aerobically or anaerobically. The results from a typical experiment are presented in Table I. As expected, the plasmid pLG669-Z showed more than 100-fold induction of β-galactosidase activity under aerobic conditions. Conversely, transformants carrying the plasmid mpLG2 showed activity only under anaerobic conditions (Table I), indicating that the Smal-Xhol fragment from the ANB1 gene must contain the regulatory elements involved in anaerobic expression.

Construction of Deletions in the 5'-Regulatory Region of

FIG. 2. Construction of the parent plasmid mpLG2 from the plasmid pLG669-Z which bears a 2-μm origin and a CYC1-LacZ hybrid gene whose expression is driven solely by the UAS of CYC1. For making mpLG2, pLG669-Z was digested with the restriction enzymes Smal and Xhol, and the 299-bp-long Smal-Xhol fragment was ligated with the backbone plasmid. The resultant plasmid mpLG2 contains all three functional TATA boxes located at positions -106, -52, and -22 and all six transcription initiation sites of CYC1 (the most upstream of which is +1) without the UAS, but instead having the Smal-Xhol fragment containing the regulatory elements of ANB1 gene in front of the CYC1-LacZ hybrid gene in the right orientation. The XhoI site in plasmid mpLG2 is at position -178, and the numbering continues over to the ligated Smal-Xhol fragment. The thick bar in the smaller Smal-Xhol fragment of plasmid PGL 699-Z denotes UAS of CYC1.
The above deletions can be divided into four classes. In the oligonucleotide-directed mutagenesis, Fig. 3 shows the coordinates of the deletions constructed and their effect on \( \beta \)-galactosidase activity under aerobic and anaerobic conditions. The \( \beta \)-galactosidase activity is expressed as described by Guarente (27).

**TABLE I**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Controlling promoter</th>
<th>( \beta )-Galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aerobic</td>
</tr>
<tr>
<td>pLG669-Z</td>
<td>CYC1</td>
<td>180.00</td>
</tr>
<tr>
<td>mpLG2</td>
<td>ANB1</td>
<td>00.00</td>
</tr>
</tbody>
</table>

**ANB1 Gene**—To further map the apparent boundaries of the DNA sequences that are involved in the regulation of the ANB1 gene, we initially constructed a series of deletion mutations that start at the 3' XhoI site by treating the linear plasmid JPK2 with Bal31 nuclease followed by the addition of XhoI linkers. DNA fragments containing these deletions were then used to replace the wild type promoter fragment in mpLG2. The \( \beta \)-galactosidase activity was measured in the transformants under both aerobic and anaerobic conditions of growth. We observed that the regulation of the hybrid gene is not affected by deleting the 5' non-coding region up to position -195 (results not shown). However, deletions beyond this position led to a constitutive expression of the hybrid gene, suggesting that these deletions removed a negative regulatory element.

To localize this putative negative regulatory element more precisely, a number of internal deletions of defined lengths were generated in the 299-bp-long fragment by using the oligonucleotide-directed mutagenesis. Fig. 3 shows the coordinates of the deletions constructed and their effect on \( \beta \)-galactosidase activity under aerobic and anaerobic conditions. In all experiments, the \( \beta \)-galactosidase activity levels in yeast cells transformed with the starting plasmid mpLG2, which contains the native ANB1 sequence, were used as a control. Based on the effects of the internal deletions on the \( \beta \)-galactosidase activity under aerobic and anaerobic conditions, the above deletions can be divided into four classes. In the first class of deletions, internal deletions within the 299-bp-long fragment have no effect on the gene regulation as \( \beta \)-galactosidase activity remains unchanged. Examples are mutants A and F. A second class of deletions led to a decrease or complete loss of \( \beta \)-galactosidase activity under anaerobic conditions without affecting the pattern of expression of the hybrid gene. Examples are mutants B and C. The third class represents a special and perhaps the most interesting deletion; removal of 47 bp (positions -237 to -189) in mutant E abolished suppression by oxygen of the hybrid gene expression and led to its constitutive expression. Thus, this deletion suggests the presence of an URS in this fragment, and the essential part of the URS lies within the deleted sequences. In the fourth class, deletion of sequences within the 299-bp-long fragment led to a constitutive expression of the hybrid gene as well as a decrease in its expression under aerobic or anaerobic conditions. Mutant D is an example of this class.

**Point Mutants in the URS Function**—A novel strategy was employed as to precisely define nucleotides in the URS that contribute to regulatory function (Fig. 4). In this strategy, we combined the ability to induce random multiple substitutions in a target region with phenotypic selection for inactivation of the desired function. It is important to note that such a protocol only selects for point mutations that cause loss of function. A series of mutations were first constructed by mixed-oligonucleotide-directed mutagenesis throughout the entire URS segment (positions -227 to -186). As described under "Experimental Procedures," the mixed oligonucleotide was synthesized such that it can introduce at least two or three point mutations throughout the target region. We reasoned that multiple mutations would be required to drastically affect URS function.

The efficiency of the mixed-oligonucleotide mutagenesis was tested by sequencing plasmid DNA from 72 bacterial transformants obtained after the mutagenesis reactions. The results of this experiment suggested that multiple point mutations were generated randomly throughout the target region except in the two nucleotides located at either end of the mixed oligonucleotide. The 299-bp-long Smal-XhoI fragment containing the different point mutations was isolated from a pool of transformants and used to replace the wild-type Smal-

<table>
<thead>
<tr>
<th>Mutant</th>
<th>SmaI Promoter XhoI SmaI-LacZ</th>
<th>Base Pairs Deleted</th>
<th>( \beta )-Galactosidase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Aerobic</td>
</tr>
<tr>
<td>A</td>
<td>-450-380</td>
<td></td>
<td>69.00</td>
</tr>
<tr>
<td>B</td>
<td>-243-257</td>
<td></td>
<td>145.00</td>
</tr>
<tr>
<td>C</td>
<td>-380-248</td>
<td></td>
<td>149.00</td>
</tr>
<tr>
<td>D</td>
<td>-237-217</td>
<td></td>
<td>74.00</td>
</tr>
<tr>
<td>E</td>
<td>-237-199</td>
<td></td>
<td>47.00</td>
</tr>
<tr>
<td>F</td>
<td>-237-199</td>
<td></td>
<td>19.00</td>
</tr>
</tbody>
</table>
Effect of Single-point Mutations Within and Outside URS—To explore further whether the URS is represented by the direct repeats, point mutations were generated within the repeats (mutants O, P, and Q) as well as outside (mutants R and S), and the effect of each on hybrid gene expression was determined under aerobic and anaerobic conditions. Fig. 5 also shows the results obtained with these mutants. Interestingly, all three changes, at two positions −211 (A→C, mutant O; A→T, mutant P) and one at position −199 (C→T, mutant Q) within the direct repeats, led to constitutive expression of the hybrid gene. Most remarkable is the finding that a single point change from A to C at position −211 (mutant O, Fig. 5) caused an induction of β-galactosidase similar to that measured when the entire URS was deleted (Fig. 3). On the other hand, two separate point changes at position −197 (C→A, mutant R) or −196 (A→C, mutant S), which are located outside the direct repeats had no major effect on the suppression by URS.

**DISCUSSION**

The current studies were designed to identify the cis-acting regulatory elements involved in the negative regulation of the ANBl gene by oxygen. We have used a fusion gene in which the 299-bp-long Smal-Xhol fragment from the 5′-flanking region of the ANBl gene is placed in front of the CYCl1-LacZ hybrid gene, and the effects of internal deletions of predefined lengths as well as point mutations in the specific region of the above fragment were determined. Results presented here indicate that the 299-bp-long fragment contains two functionally distinct regulatory sites, an upstream promoter element that acts in the absence or presence of oxygen and an URS which confers strong repression upon the promoter when oxygen is present.

The presence of the promoter in the 299-bp-long fragment from the 5′-flanking region of the ANBl gene is supported by the following results. First, mutant B, which involves a deletion of 145 bp from positions −403 to −257 led to at least a 7-fold decrease in β-galactosidase levels under anaerobic conditions without affecting the regulation; this mutant suggests that this deletion is affecting the promoter which is required for the expression of the hybrid gene. Furthermore, deletion of 149 bp from position −380 to −248 and position −237 to −217 in mutant C showed no detectable β-galactosidase activity under anaerobic conditions, indicating that the promoter lies within position −380 to −217. In addition, mutant F has a deletion of 19 bp from position −237 to −217 and has no effect on the regulation or promoter activity, which suggests that nucleotides from positions −380 to −237 are absolutely required for promoter activity. From the coordinates of the above deletions, it is not possible to further narrow down the promoter to a more specific region. The DNA sequence between positions −380 and −237 contains long stretches of poly(dA:dT) sequences (Fig. 1). Long dA:dT tracts have earlier been shown to be present in the 5′-flanking region of many yeast genes (4). For example, in the case of ADR2 gene, mutants that contain longer stretches of upstream poly(dA:dT) sequence have been shown to exhibit higher levels of constitutive expression of the gene (28). Also, naturally occurring poly(dA:dT) sequences have been shown to act in a bidirectional manner as a component of the upstream promoter elements for the constitutive basal level transcription of the HIS3 and PET56 genes (29). In view of these results, it is tempting to speculate that one or more of direct repeats (represented by arrows in Fig. 5), and it is possible that the direct repeats may represent URS and play a part in its function.

![Diagram](https://example.com/diagram.png)
pressive activity of the URS. Thirdly, single nucleotide sub-
transcription. The observation that URS mediates repression
without affecting the constitutive level of expression of the
hybrid gene under anaerobic conditions. In the other four
above strategy, a complete loss of URS activity was observed
mutants, nucleotide substitutions led to partial loss of sup-
and anaerobic conditions binds to the promoter and facilitates
positions -237 and -189. Furthermore, deletion of 19 bp in
region of 42 bp as tested by sequencing, only mutations
indicated that the essential part of the URS lies between
constitutive promoter elements located between positions
structural promoter elements located between positions
-380 and -237. The coordinates of deletion in mutant E
the negative element is located 3' to the
region corresponding to the URS are shown with arrows above the sequence. The base substitution mutations are
in the noncoding strand (bottom strand), and the dashes indicate nucleotides that are identical in the normal and
sequences. The \( \beta \)-galactosidase values at the right side show the mean values obtained for each mutant.
The mixed oligonucleotides used for inducing random mutations in the region -227 to -166 is shown by a solid
Mutants G to N were isolated by using the scheme outlined in Fig. 3, and mutants O to S were obtained by using
the oligonucleotides containing the desired mutation. The effect of nucleotide substitutions on the hybrid
gene expression was determined by replacing the Smal-Xhol fragment in the plasmid mpLG2 with the mutant
\( \beta \)-galactosidase activity of the transformants under aerobic and anaerobic conditions. * indicates the nucleotide which does not fit in the direct repeats structure.

Evidence for the presence of an URS in the 299-bp-long
fragment from the 5'-flanking region of \( ANB1 \) gene is pro-
vided by the following results. First, deletion of 47 bp from position -237 to -189 in mutant E led to constitutive expres-
sion of the hybrid gene without affecting \( \beta \)-galactosidase levels under anaerobic conditions. This suggests that sequences
within this region are required to suppress hybrid gene expression
under aerobic conditions. This region is distinct from the
constitutive promoter elements located between positions
-380 and -237. The coordinates of deletion in mutant E
indicate that the essential part of the URS lies between
positions -237 and -189. Furthermore, deletion of 19 bp in
mutant F from position -237 to -217 does not affect regula-
tion of the hybrid gene, and this further narrows down the
functional URS to a 28-bp region (positions -217 to -189).

Secondly, mixed-oligonucleotide-directed mutagenesis was
used to introduce random multiple nucleotide substitutions
between positions -227 and -186. A phenotypic loss of func-
tion selection was carried out to isolate mutants containing
an inactive URS. All the transformants isolated in this
manner with constitutive expression of the hybrid gene
carried plasmids with mutations clustered between positions
-215 and -201. It is important to note that although the
nucleotide substitutions were induced randomly in the entire
target region of 42 bp as tested by sequencing, only mutations
mapping to positions -214 to -202 affected URS function.
Furthermore, in four out of the eight mutants isolated by the
above strategy, a complete loss of URS activity was observed
without affecting the constitutive level of expression of the
hybrid gene under anaerobic conditions. In the other four
mutants, nucleotide substitutions led to partial loss of sup-
pressive activity of the URS. Thirdly, single nucleotide sub-
stitutions were made at positions -211 and -199 (mutants O, P and Q) as well as at -197 and -196 (mutants R and S).

Nucleotide substitutions in mutants O, P, and Q led to con-
stitutive expression of the hybrid gene, whereas mutants R
and S had no effect on regulation. Interestingly, in mutants
O and P, a single nucleotide substitution led to complete loss
of URS activity, and the resulting level of \( \beta \)-galactosidase was
similar to that observed when the entire URS region has been
deleted. In mutants O, P, and Q, the mutation is located
within the direct repeat structures shown in Fig. 5. Many
protein-binding sites for transcription factors are composed
direct repeats (30). In view of these observations, it is likely
that the URS is mainly composed of three direct repeats.

The fusion of heterologous promoter elements to \( \beta \)-galac-
tosidase has been applied successfully to determine the regu-
lar elements of a number of yeast genes (5, 31, 32). The results
obtained here suggest that a constitutive promoter and an
URS is involved in the negative regulation of the \( ANB1 \)
gene by oxygen. In the \( ANB1 \) gene, the URS is located 294
bp upstream from the translation initiation codon and is 3'
to the promoter element. The relative positions of the positive
and negative regulatory elements in other yeast genes is
variable. In the case of \( CYC7 \), the negative element is located 5'
to the UAS (33), whereas, in other yeast genes, the negative
control sequence is located 3' to the UAS. The 3' location of
regulatory sequences in the \( ANB1 \) gene is similar to the
arrangements observed for promoters of the \( TR1, STE6 \), and
\( HO \) genes (34–36).

The mechanism by which the URS silences the activity of
the upstream promoter in the presence of oxygen or heme is
at present not known. However, a simple model can be pro-
posed to account for the negative regulation of the \( ANB1 \)
gen by oxygen and perhaps by heme. According to this model,
a transcription factor(s) that is functional under both aerobic
and anaerobic conditions binds to the promoter and facilitates
transcription. The observation that URS mediates repression
of the promoter under aerobic conditions raises the possibility
that a trans-acting factor binds to this site in response to

\[
\text{FIG. 5. Mutations in the URS region of the } ANB1 \text{ gene. The nucleotide sequence of the 5'-flanking region of the } ANB1 \text{ gene from position } -231 \text{ to } -182 \text{ is shown in this figure. The short direct repeats observed in the region corresponding to the URS are shown with arrows above the sequence. The base substitution mutations are in the noncoding strand (bottom strand), and the dashes indicate nucleotides that are identical in the normal and mutant sequences. The } \beta \text{-galactosidase values at the right side show the mean values obtained for each mutant. The mixed oligonucleotides used for inducing random mutations in the region } -227 \text{ to } -166 \text{ is shown by a solid line. Mutants G to N were isolated by using the scheme outlined in Fig. 3, and mutants O to S were obtained by using the oligonucleotides containing the desired mutation. The effect of nucleotide substitutions on the hybrid gene expression was determined by replacing the Smal-Xhol fragment in the plasmid mpLG2 with the mutant Smal-Xhol fragment and determining the } \beta \text{-galactosidase activity of the transformants under aerobic and anaerobic conditions. * indicates the nucleotide which does not fit in the direct repeats structure.}
\]

\[
\begin{array}{c|cc|cc}
\text{Mutant} & \text{G} & \text{H} & \text{I} & \text{K} & \text{L} & \text{M} & \text{N} & \text{O} & \text{P} & \text{Q} & \text{R} & \text{S} \\
\hline
\text{mpLG2 (WT)} & \text{G} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} \\
\text{G} & \text{G} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} \\
\text{H} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} \\
\text{I} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} \\
\text{J} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} \\
\text{K} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} \\
\text{L} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} \\
\text{M} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} \\
\text{N} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} \\
\text{O} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} \\
\text{P} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} \\
\text{Q} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} \\
\text{R} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} \\
\text{S} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} & \text{T} \\
\end{array}
\]
Negative Regulation of the ANB1 Gene

oxygen or heme. Under physiological aerobic conditions, heme levels are high in the cell, and it is possible that heme is an essential co-factor required to activate the URS-binding factor or heme regulates the URS-binding factor. The binding of this factor to the URS is postulated to prevent the necessary interactions between the transcription factor and the promoter. Under anaerobic conditions, heme biosynthesis is decreased leading to low intracellular heme levels. The absence of heme may prevent the URS-binding protein from interacting with the ANB1 repressor site or decreasing the expression of the repressor protein, leading to derepression. This model is consistent with the following observations. First, heme has been implicated as the mediator of the presence of oxygen for the activation of the CYCI gene (7, 11). Secondly, genetic studies on the regulation of the ANB1 gene suggest that heme along with a trans-acting factor is required for the aerobic suppression of the ANB1 gene (7). Studies are now in progress to identify the URS-binding factor as assumed above.

Acknowledgments—We thank Susan Porter and Johny Ngsee for helpful suggestions and T. Atkinson for oligonucleotide synthesis. We thank David Russell for critical review of the manuscript.

REFERENCES