

Induction and repression of *DAN1* and the family of anaerobic mannoprotein genes in *Saccharomyces cerevisiae* occurs through a complex array of regulatory sites

Brian D. Cohen¹, Odeniel Sertil, Natalia E. Abramova, Kelvin J. A. Davies² and Charles V. Lowry*

Center for Immunology and Microbial Disease, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208, USA, ¹Laboratory of Reproductive and Metabolic Disorders, Wadsworth Center, PO Box 22002, Albany, NY 12201-2002, USA and ²The Andrus Gerontology Center, University of Southern California, Los Angeles, CA 90089-0191, USA

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ABSTRACT

The *DAN/TIR* mannoprotein genes of *Saccharomyces cerevisiae* (*DAN1*, *DAN2*, *DAN3*, *DAN4*, *TIR1*, *TIR2*, *TIR3* and *TIR4*) are expressed in anaerobic cells while the predominant cell wall proteins Cwp1 and Cwp2 are down-regulated. Elements involved in activation and repression of the *DAN/TIR* genes were defined in this study, using the *DAN1* promoter as a model. Nested deletions in a *DAN1/lacZ* reporter pinpointed regions carrying activation and repression elements. Inspection revealed two consensus sequences subsequently shown to be independent anaerobic response elements (AR1, consensus TCGTTYAG; AR2, consensus AAAAATTGTTGA). AR1 is found in all of the *DAN/TIR* promoters; AR2 is found in *DAN1*, *DAN2* and *DAN3*. A 120 bp segment carrying two copies of AR1 preferentially activated transcription of *lacZ* under anaerobic conditions. A fusion of three synthetic copies of AR1 to *MEL1* was also expressed anaerobically. Mutations in either AR1 site within the 120 bp segment caused a drastic loss of expression, indicating that both are necessary for activation and implying cooperativity between adjacent transcriptional activation complexes. A single AR2 site carried on a 46 bp fragment from the *DAN1* promoter activated *lacZ* transcription under anaerobic conditions, as did a 26 bp synthetic AR2 fragment fused to *MEL1*. Nucleotide substitutions within the AR2 sequence eliminated the activity of the 46 bp segment. Ablation of the AR2 sequences in the full promoter caused a partial reduction of expression. The presence of the ATTGTT core (recognized by HMG proteins) in the AR2 sequence suggests that an HMG protein may activate through AR2. One region was implicated in aerobic repression of *DAN1*. It contains sites for the heme-induced Mot3 and Rox1 repressors.

INTRODUCTION

Saccharomyces cerevisiae cells adapt to anaerobic growth by expressing a number of 'anaerobic' genes (genes which are specifically induced following oxygen depletion) (1). The regulation of some of these genes is still poorly understood, owing to a lack of knowledge about the factors and/or promoter sites which regulate their expression. However, two groups of anaerobic genes have now been identified and assigned to distinct regulons, based on the two different regulatory systems which control them. The best studied are the hypoxic genes (2–8), which are regulated by heme (9) and the Rox1 repressor (3,10,11) through one or more consensus operator sites (YYYATTGTTCTC) (4,12). Regulation occurs via control of repressor activity, with *ROX1* induced by heme during aerobic growth (9,10) and repressed by the Hap1 factor during anaerobiosis (13,14). More recently a second group of heme-repressed anaerobic genes has been identified which encode cell wall mannoproteins (*DAN1*, *DAN2*, *DAN3*, *DAN4*, *TIR1*, *TIR2*, *TIR3* and *TIR4*) (1,15–17; N.Abramova, O.Sertil, S.Mehta and C.Lowry, submitted for publication). We refer to the members of this regulon as the *DAN/TIR* genes. Negative regulation of some of these genes by heme in aerobic cells is quite stringent (17), resulting in undetectable levels of mRNA. The principle elements of the mechanisms which regulate them are different from those controlling the hypoxic genes (17,18).

The newly identified regulatory factors controlling expression of the *DAN/TIR* genes are Mox4/Upc2, a transcriptional activator (18,19), and Mox1, Mox2 and Rox7/Mot3, which are repression factors (18). Like Rox1, expression of Mot3 (20,21) is induced during aerobic growth by heme (O.Sertil, R.Kapoor, B.Cohen and C.Lowry, submitted for publication). The three Mox factors appear to be specific for the *DAN/TIR* regulon, while Mot3 is required for efficient repression of subsets of genes from both the hypoxic and *DAN/TIR* regulons (18) (O.Sertil, R.Kapoor, B.Cohen and C.Lowry, submitted for publication).

Given the number of regulatory factors controlling expression of the *DAN/TIR* genes, we anticipated that the promoter of *DAN1* would be complex. We have identified several sequences involved in the induction of transcription under

*To whom correspondence should be addressed. Tel: +1 518 262 5866; Fax: +1 518 262 5689; Email: cvlowry@aol.com

anaerobic conditions and others involved in repression under aerobic conditions. Surprisingly, we find that two different consensus sequences within the promoter serve as independent anaerobic response elements targeted by different mechanisms. One of the response elements (designated AR1) is present in all of the *DAN/TIR* promoters and has been implicated as the target of the Mox factors (18). The second response element (designated AR2) is targeted by a still unidentified anaerobic activation mechanism. In addition to sites mediating the two activation mechanisms, we have also identified a segment mediating aerobic repression which contains sites for Mot3 and Rox1. In addition, we have identified a second inhibitory segment which appears to be the target of a non-specific repression mechanism which may have an indirect role in maintaining aerobic repression.

MATERIALS AND METHODS

Plasmids

***DAN1/lacZ* reporter plasmids.** A *DAN1/lacZ* reporter fusion was constructed in three steps. First the *DAN1* coding region was inserted into YCplac22 (22) as a *KpnI*–*EcoRI* fragment from pBSDAN1 to create YCpDAN1-1. Then the *DAN1* promoter region (–1260/+17) was positioned 5' to the coding region on the same plasmid. For this purpose it was amplified by PCR from a library containing *HindIII*–*NcoI* inserts carried on plasmid YCplac33 (22) using the M13 reverse primer (AGCGGATAACAATTTTCACACAGGA) and the non-coding strand primer A2 (5'-AGAGGGATCCATACTAATCCTAGACATTACTTGGGG), which contains a *BamHI* site followed by a sequence complementary to the region from –8 to +17. The PCR fragment was digested with *XhoI* (in the region of the polylinker adjacent to the M13 primer site) and *BamHI* and inserted into the *SalI* and *BamHI* sites in the polylinker region of YCpDAN1-1, creating YCpDAN1-2. To join the promoter to *lacZ*, a *BamHI*–*NcoI* fragment carrying the *lacZ* ORF was excised from the *ANB1/lacZ* reporter plasmid YCpAZ-H4 (13) and inserted into the *BamHI* (+17) and *NcoI* (+321) sites of YCpDAN1-2. This insertion placed *lacZ* in-frame with the first five amino acids of *DAN1* and 5' to the 3'-end of the *DAN1* ORF, creating YCpD/Z(22). The *DAN1/lacZ* fusion was then placed into two other vectors. It was excised from YCpD/Z(22) with *EcoRI* and *HindIII*, generating a fragment which includes the promoter region (–879/+17) joined to *lacZ* flanked on the 3'-side by a small piece (+321/+408) of the *DAN1* ORF, and ligated to the *EcoRI* and *HindIII* sites of YIplac128 (8) and YCplac33, creating YIpD/Z(128) and YCpD/Z(33), respectively. To facilitate mutagenesis of the promoter the *BglII* site at –207 of plasmid YCpD/Z(33) was converted to an *XhoI* site by insertion of a *BglII*–*XhoI* linker, generating plasmid D1 (Fig. 1).

***MEL1* reporter plasmids.** Fusions of sequences or subfragments of the *DAN1* promoter to the *MEL1* gene were derived from a *DAN1/MEL1* reporter fusion, which was constructed in two steps. To make use of a *BamHI* site at –37 in the 5'-UTR of *MEL1* a *BamHI* site was introduced into the UTR of the *DAN1* promoter segment. For this purpose the region from –879 to –1 was amplified using the coding strand primer D1 (GACGAATTC AACGATTTCAACAGTC) and non-coding strand primer D0

(GCGGCGGGATCCTACTTGGGGTATATATTTAGTATG). The PCR product was digested with *EcoRI* and *BamHI* and ligated into the same sites of D1 to create D1-X. YCpDAN1/*MEL1* was then constructed by inserting the *MEL1* ORF plus 37 bp of the 5'-UTR into D1-X as a *BamHI*–*HindIII* fragment from plasmid pBSKMEL1 (23) (a gift from M. Ryan and R. Morse) into the same sites of the D1-X backbone, replacing the *lacZ* segment. The *MEL1* ORF, as configured in YCpDAN1/*MEL1*, was then introduced into a number of different constructs derived from D1, replacing the *lacZ* ORF. It was inserted as a *SalI*–*HindIII* fragment, ligated to the *SalI* site just 3' to the TATA site and to the *HindIII* site 3' to the *lacZ* segment. (AR1)₃/*MEL1* and AR2/*MEL1* were derived from (AR1)₃/*lacZ* and AR2/*lacZ*. For construction of the latter two plasmids a promoterless vector for cloning synthetic oligonucleotides was constructed by inserting an *EcoRI*–*BstEII*–*XhoI* linker (coding, AATTCAGCTCA-CCC; non-coding, TCGAGGGTGAGCTG) into plasmid C6, replacing the 46 bp segment with a *BstEII* site, to create D0-BST. To construct (AR1)₃/*lacZ* a phosphorylated double-stranded oligonucleotide, containing the AR1₆ site and three adjacent 3' nucleotides flanked by *BstEII* cohesive ends (coding, GTCACATCGTTTAGCACGTGT; non-coding, GTGACACACGTGCTAAACGAT; repeat interval 21 bp), was ligated to the *BstEII* site, with directional insertion (intrinsic in the *BstEII* site). A plasmid containing three forward-oriented concatenated copies of AR1 (repeat interval 21 bp) was isolated and converted to a *MEL1* version as described above, designated (AR1)₃/*MEL1*. AR2/*MEL1*, which contains a single synthetic copy of the A1 site and 14 flanking nucleotides (coding, GTCACATATAGTTAAAAATTGTTGAGCTCAAT; non-coding, GTGACATTGAGCTCAACAATTTTTAACTATAT) fused to *MEL1*, was constructed in analogous fashion. A control promoterless vector, D0-BST-*MEL1*, was constructed in analogous fashion from D0-BST.

***DAN2/lacZ* reporter plasmid.** YCpDAN2/*lacZ* was constructed by inserting the promoter region of the *DAN2* gene (–1105 to +3) in place of the *DAN1* promoter in plasmid D1-Sal, a derivative of plasmid D1 in which a *SalI*–*BamHI* linker containing a 9 nt segment of the 5'-UTR (GATCCATTTTTGTCGACAAAATG) had been inserted at the *BamHI* site. A *DAN2* promoter fragment was amplified using a coding strand primer (D2A, GAGAGAATTCTACTAACGAAATGATG-GCTG) containing an *EcoRI* site followed by homology from –1105 to –1086 and a non-coding strand primer (D2B, GAGAGTCGACTGAACTTTTG TAGATCTTTTTAGTG) containing a *SalI* site followed by homology from –11 to –35. The amplified promoter fragment was digested with *EcoRI* and *SalI* and ligated to the same sites of D1-Sal, placing the region from –1105 to +3 in-frame with the *lacZ* ORF, with an A→G substitution at –10 and an A→C substitution at –5.

Promoter deletion and subfragment plasmids. A series of plasmids derived from the *DAN1/lacZ* reporter plasmid was constructed containing single or double deletions of various promoter segments. To obtain the desired deletion constructs, truncated promoter fragments were generated by PCR using primers homologous to segments at the points indicated. The PCR template was either the D1 plasmid or, in the case of double deletions, a derivative deletion plasmid. For cloning purposes all of the primers contained appropriate restriction sites 5' to the homologous sequence. Plasmid D16 carries a

deletion of the segment from -207 to -107, adjacent to the TATA site at -101. For the construction a PCR fragment carrying the segment from -107 to +17 was amplified using the A2 non-coding strand primer and a coding strand primer containing an *Xho*I site followed by the sequence from -107 to -85. This was digested with *Xho*I and *Bam*HI and ligated to the same sites in D1. Plasmid D0 carries a deletion of the entire promoter region from -897 to -107. It was constructed by excising that region from D16 with *Eco*RI and *Xho*I, end-filling with Klenow polymerase and religating.

Three sets of nested promoter deletions used in localization of regulatory sites were generated in the D1 plasmid by inserting truncated PCR fragments in place of the full-length promoter: one set of deletions extended 3'-wards from -897, one set extended 5'-wards from -107 and one set extended 5'-wards from -207. For the 3'-ward deletions, fragments of decreasing length were generated with the A2 non-coding strand primer and a set of coding strand primers, each having an *Eco*RI site followed by homologous sequences beginning at -762 (plasmid D2), -633 (plasmid D3), -524 (plasmid D4), -423 (plasmid D5), -354 (plasmid D6), -323 (plasmid D7), -304 (plasmid D8) or -207 (plasmid D9). The PCR fragments were digested with *Eco*RI and *Bam*HI and ligated to the *Eco*RI and *Bam*HI sites of D1 (located at -897 and +17). For deletions extending 5'-wards from -107 a set of fragments was generated with the A3 coding strand primer (GACGAATTCAAACGATTTCAACAGTC, homologous from -897 to -880) and a set of non-coding strand primers, each containing an *Xho*I site followed by homologous sequences beginning at -301 (plasmid D17), -353 (plasmid D18), -424 (plasmid D19), -547 (plasmid D20) or -547 (plasmid D24). The set of PCR fragments was digested with *Eco*RI and *Xho*I and ligated to the *Eco*RI and *Xho*I sites of D16 (located at -897 and -107). For an equivalent set of deletions extending 5'-wards from -207 the same *Eco*RI- and *Xho*I-digested PCR fragments were ligated to the *Eco*RI and *Xho*I sites of D1 (at -897 and -207).

Plasmids containing small segments of the promoter fused to *lacZ* were constructed by deleting the sequences flanking them from D1 or from a derivative. To construct plasmid C6, which contains the segment from -570 to -525 inserted at -107, the flanking segments, -879 to -570 and -525 to -107, were deleted, leaving the segment in the desired position. The insert for this construction was amplified from the segment between -570 and +17 in plasmid D20 (in which the segment from -525 to -107 is deleted); the primers used were a coding strand primer containing an *Eco*RI site followed by the sequence from -570 to -547 and the A2 non-coding strand primer. The 169 bp PCR product was digested with *Eco*RI and *Bam*HI and ligated to the *Eco*RI and *Bam*HI sites of D1. Plasmid C5 carries the -570 to -525 segment inserted at -207 instead of -107 and was derived in an analogous way using the same PCR primers to amplify a 269 bp fragment from plasmid D13 (in which the segment from -525 to -207 was deleted). Plasmids C1 and C2 are similar to C5 and C6, but contain the region from -633 to -525 inserted at -207 and -107, respectively. The insert for the two constructs was amplified from the D13 and D20 templates using a coding strand primer containing an *Eco*RI site followed by the sequence from -633 to -615 and the A2 non-coding strand primer. The two products were digested with *Eco*RI and *Bam*HI and ligated to the same sites in D1. Plasmid C11 contains the segment from -524 to -403 inserted at -107. It

was amplified from the -524 to -403 region using a coding strand primer containing an *Eco*RI site followed by a homologous sequence from -524 to -504 and a non-coding strand primer containing an *Xho*I site followed by homologous sequence from -403 to -425. The product was digested with *Eco*RI and *Xho*I and ligated to the same sites in D16.

Nucleotide substitutions in the DAN1 and DAN2 promoters. Putative regulatory sites were mutated in some of the plasmid constructs described above by substituting PCR-derived segments containing nucleotide substitutions. Plasmids M9 and M10 are mutant derivatives of C11 in which the AR1_b sequence (-413) near the end of the -524 to -403 segment was altered. The mutation in M9 (TCGTTTAG→GTACCTAG) was created by using a substituted non-coding strand primer (GCGCTCGAGTGCTAGGTACCAGATAAAAATAGGAAA-TTCTCCC) plus the same coding strand primer used for C11 to generate an altered fragment, which was then digested with *Eco*RI and *Xho*I and inserted into the same sites of D16. M10 contains a point mutation in AR1_b (TCGTTTAG→TCATT-TAG). This was introduced on a PCR fragment generated as above, but with a non-coding strand primer containing the indicated substitution GCGCTCGAGTGCTAAATGACAGAT-AAAATAGG.

Plasmid M11 contains a triple substitution in the AR1_a site at -495 (TCTTCGTTTGAG→TCTAGATTTGAG) and was generated by insertion of two mutated fragments flanking AR1_a (from -524 to -495 and from -495 to -403) in place of the wild-type -524 to -403 fragment of C11, in a triple ligation reaction. The 5'-insert was a synthetic double-stranded oligonucleotide containing the sequence from -524 to -495 flanked by *Eco*RI and *Xba*I cohesive ends (coding, AATTCCCGTTT-CITCAATCCTCACGTGCTTT; non-coding, CTAGAAAG-CACGTGAGGATTGAAGAAAACGGG). The 3'-insert was a PCR product generated with the same non-coding strand primer used for the C11 construct and a coding strand primer containing an *Xba*I site followed by homology from -492 to -470 (GAGATCTAGATTTGAGTGCAAAAGTTTCATATG). This fragment was digested with *Xba*I and *Xho*I. The two fragments were ligated together into the *Eco*RI- and *Xho*I-digested C11 backbone. In the resulting plasmid nucleotides 495-496 (TCG) have been replaced by AGA, disrupting the *ARE* and generating the 3'-half of the *Xba*I site.

Plasmids M2, M4 and M6 contain substitutions in the A₁ consensus site beginning at -562. These were made using the same scheme as for C6 with substitutions in the coding strand primer. In each case a PCR fragment generated with one of the substituted primers and the A2 anchor primer was digested with *Eco*RI and *Bam*HI and ligated to the D1 backbone as before. The plasmids contain the following substitutions: M2, AAAAATTGTTGA→AAAAATTATTGA, generated with coding strand primer GCGGAATTCATATAGTTAAAAAT-TATTGAGCTCAATTC; M4, AAAAATTGTTGA→AAAA-ACCGTTGA, generated with coding strand primer GCGGA-ATTCATATAGTTAAAAACCGTTGAGCTCAATTC; M6, AAAAATTGTTGA→GTCTCTTGTTGA, generated with coding strand primer GCGGAATTCATATAGTTGTCTCTTG-TTGAGCTCAATTCACG.

A series of plasmids containing multiple substitutions in AR2_a and/or AR2_b (S8, S9 and S10) and a control plasmid (S7) were constructed in the following sequence. In the first step in

the series of constructs plasmids S1 and S2 were generated in parallel. S1 contains the segment -878 to -547 inserted at -207 , leaving the region from -547 to -207 deleted and replaced by an *XhoI* site. The insertion fragment was generated by PCR using the coding strand primer A3 and the non-coding strand primer D21, digested with *EcoRI* and *XhoI* and ligated into the same sites in YCpD1. S2 is analogous to YCpS1, containing the same -878 to -547 segment but with a TTGTTG \rightarrow CAATTG substitution in the AR_{2a} site at -557 . The insertion fragment was generated using primer A3 and non-coding strand primer S2 (CAGCGTGTCTCGAGCTCAATTGTTTT-TAACTATATCAATC).

In the second step of the series, plasmids S3, S4, S5 and S6 were constructed. In each case the segment from -546 to -301 was inserted into the *XhoI* and *BamHI* sites of S1 or S2. For S4 and S6 this fragment contained a mutation in the AR_{2b} site; for S3 and S5 the unmutagenized control fragment was used. These fragments were generated using the coding strand primer S11 (GAGGTTCGACACAGGCTGGATTTCGGCGATC) and either the wild-type non-coding strand primer S3 (GCGGGATCCGAGCTCGAGGATAACATTCATCAATTTTTTC) or the AR₂-substituted non-coding strand primer S4 (GCGGGATCCGAGCTCGAGGATAACATTCATTCTAGATTCCTC) (this primer introduces a AAATTG \rightarrow TCTAGA substitution at -317). The fragments were digested with *SalI* and *BamHI* and inserted into the *XhoI* and *BamHI* sites of S1 or S2 (the inserted fragments contained an *XhoI* site just 5' to the *BamHI* site). In this way mutant and intact AR_{2a} and AR_{2b} segments were joined to generate the four possible combinations of intact and mutant sites. In the resulting plasmids S3 contained intact AR_{2a} and AR_{2b} sites, S4 contained intact AR_{2a} and mutant AR_{2b}, S5 contained mutant AR_{2a} and intact AR_{2b} and S6 contained both mutant sites. In the final step the fragment from -301 to $+17$ was restored by insertion of a PCR product generated with the coding strand primer S7 (GACCTCGAGCGTAGACGCTCCTCTGAAAG) and the non-coding strand primer A2. The product was digested with *XhoI* and *BamHI* and inserted into the same sites in S3, S4, S5 and S6 to generate S7, S8, S9 and S10, respectively.

Plasmid YCpDAN2/*lacZ*-AR_{1x} contains a substitution in the AR₁ site at -847 (TCGTTTCAG \rightarrow ACCGGTAG) and was constructed by a triple ligation of two altered promoter subfragments (-1105 to -848 and -847 to $+3$) in place of the promoter region in YCpDAN2/*lacZ*. The fragment flanking the AR₁ site on the 5'-side was generated using the D2A coding strand primer and a non-coding strand primer containing an *AgeI* site followed by the sequence complementary to the segment from -848 to -866 (GAGAACCGGTGGGCAATATCCAATGTTTTGAC). The 3'-fragment was generated using the D2B non-coding strand primer plus a coding strand primer containing an *AgeI* site followed by a sequence homologous to the segment from -842 to -821 (GAGAACCGGTAGAGGGCACAGAATAAAATTTG). The two PCR fragments were digested with *EcoRI* and *AgeI* and *SalI* and *AgeI*, respectively, and ligated to the *EcoRI*- and *SalI*-digested D1-Sal backbone.

Cell growth and analysis of gene expression

For β -galactosidase assays (24) FY23 cells (25) were grown in SD medium (2% glucose) lacking uracil to maintain selection of the reporter plasmid. Overnight cultures in early stationary phase were diluted 1:25 (to $\sim 5 \times 10^6$ /ml) and grown aerobically

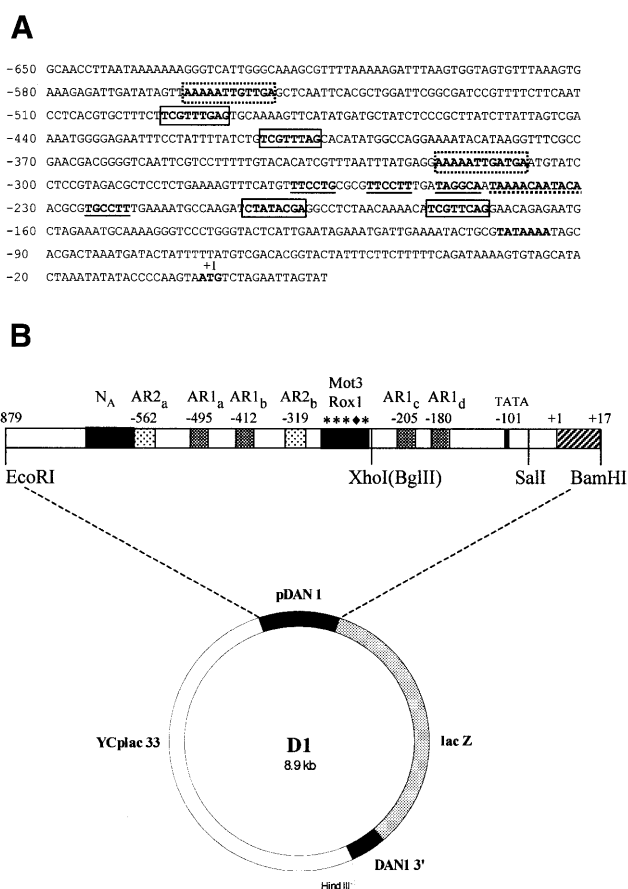


Figure 1. Plasmid D1 and the *DAN1* promoter region. (A) The region of the *DAN1* promoter from -879 to $+17$ included in the *lacZ* fusion is shown. AR₁ sites are boxed with solid lines; AR₂ sites are boxed with dotted lines; Mot3 sites are underlined with solid lines; the Rox1 site is underlined with a dotted line. (B) The D1 plasmid is shown; it contains the *DAN1* promoter region joined in a coding fusion to the *lacZ* gene beginning with the first six amino acids of the *DAN1* ORF. The *DAN1* promoter region is expanded to show the position of regulatory sites. Restriction sites used in manipulation of the plasmid to generate deletions and point mutations in the promoter are indicated.

for 2 h and then anaerobically or aerobically for 7 h. Anaerobic conditions were imposed by bubbling cultures in sealed tubes with high purity nitrogen (99.999% according to the manufacturer, though this was not measured; very similar results were obtained with different lots). At 7 h cells are still growing in mid to late log phase under either condition. There is an ~ 3 h lag in *lacZ* expression, but 7 h anaerobic culture is long enough to give consistent and robust expression from the *DAN1*/*lacZ* fusion. For α -galactosidase assays cells were grown in the same way and assayed as described (23). Data shown are for assays replicated three times.

RESULTS

Anaerobic response sequences in the *DAN1* promoter

Expression of a *DAN1*/*lacZ* reporter (plasmid D1; Fig. 1) reporter is strictly dependent on hypoxic growth conditions,

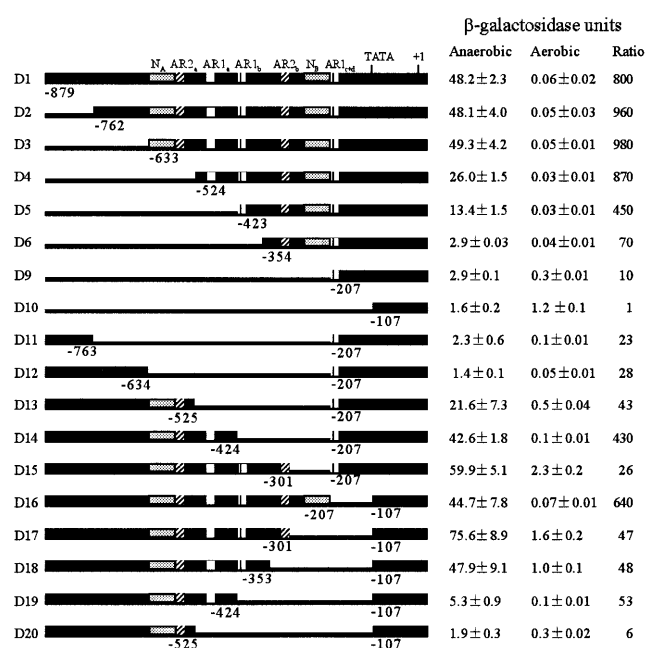


Figure 2. Nested deletions in the *DAN1* promoter. FY23 cells carrying the D1 plasmid or derivatives carrying the indicated deletions (see Materials and Methods) were grown in SD-ura medium under aerobic and anaerobic conditions (8 h) and harvested for β-galactosidase assay, with the results shown in Miller units. The induction ratio, i.e. the ratio of activity in anaerobic cells to activity in aerobic cells, is indicated.

showing a >300-fold difference in expression in anaerobic and aerobic cultures (Fig. 2). Anaerobic conditions imposed by bubbling log phase cells with high purity nitrogen may not be absolute but are sufficiently stringent to observe induction either of the *DAN1lacZ* reporter or of *DAN1* mRNA (18). *DAN1* mRNA, like the mRNAs of most of the other *DAN/TIR* genes, is absent from aerobic cells and is first detected after 30 min of hypoxia and fully induced by 80 min (17). *DAN2* and *DAN3* are expressed only after a prolonged delay, being fully induced at 3 h (N.Abramova, O.Sertil, S.Mehta and C.Lowry, submitted for publication). We earlier demonstrated that heme, which is synthesized only in aerobic cells, is the primary regulatory signal: accumulation of *DAN1* mRNA is completely blocked by addition of heme (17); similarly, expression of the *DAN1lacZ* fusion is >95% inhibited by heme (18), although a role for other environmental changes or for metabolites or nutrients generated or depleted during anaerobiosis has not been ruled out.

To locate regions containing sequences involved in anaerobic activation and/or aerobic repression of *DAN1* we constructed a set of nested deletions in plasmid D1 and tested them for activity under aerobic and anaerobic growth conditions. Several segments appeared to be involved in anaerobic induction, as evidenced by reduced anaerobic expression when they were deleted (Fig. 2). These segments contained two consensus sequences, TCGTTYAG and AAAAATTGTTGA (Table 1), which were designated AR1 and AR2 (for anaerobic response element). There is at least one copy of AR1 in the promoters of each of the *dantir* genes (Table 1) and four in the

DAN1 promoter. The AR2 sequence is found in *DAN1*, *DAN2* and *DAN3*. Sequences within one segment (–301 to –207) also appear to be involved in aerobic repression, judging from the ~20-fold increase in aerobic expression when it was deleted (plasmid D15; see below).

Table 1. Anaerobic induction sites in the *DAN/TIR* regulon

Gene	ORF	Sites		
(A) AR1 sites				
<i>DAN1</i>	YJR150C	AR1 _a	TCGTTAGAG	–495 to –487
		AR1 _b	TCGTTT-AG	–412 to –405
		AR1 _c	TCGTAT-AG (←)	–197 to –204
		AR1 _d	TCGTTC-AG	–180 to –173
<i>DAN2</i>	YLR037C		TCGTTC-AG	–447 to –440
<i>DAN3</i>	YBR301W		TCGTTC-AG (←)	–425 to –432
			TCGTTCAAG	–234 to –226
<i>DAN4</i>	YJR151C		TCGTTC-AG	–195 to –188
		<i>TIR1</i>	YER011W	TCGTAT-AG (←)
			TCGTTTAAG	–350 to –342
<i>TIR2</i>	YOR010C		TCGTAA-AG	–374 to –367
<i>TIR3</i>	YIL011W		TCGTTT-AG (←)	–853 to –860
<i>TIR4</i>	YOR009W		TCGTTC-AG (←)	–734 to –741
			TCGTTT-AG (←)	–215 to –222
Consensus			TCGTTYAG	
(B) AR2 sites				
<i>DAN1</i>	YJR150C	AR2 _a	AAAAATTGTTGA	–561 to –550
		AR2 _b	AAAAATTGATGA	–318 to –307
<i>DAN2</i>	YLR037C		ATAAATTGTTGA (←)	–142 to –153
<i>DAN3</i>	YBR301W		CAAAAATGTTGA (←)	–571 to –581
Consensus			AAAAATTGTTGA	

Properties of the *DAN/TIR* anaerobic response element (AR1)

We tested for anaerobic expression from a reporter plasmid (C11) which carries AR1_a and AR1_b (but no AR2 sites) on a 120 bp segment (–524 to –403) inserted at the TATA site of the *lacZ* fusion. Reporter activity was about four times higher in anaerobic than in aerobic cells, showing that this segment contains a response element (Fig. 3A). To test the importance of the sequence in transcriptional activation we introduced nucleotide substitutions into either AR1_a or AR1_b in C11. Substitutions at either site caused a drastic loss of activity (Fig. 3B). We also observed a strong effect of a single substitution for the 5'-most of the two G residues in AR1₂ (not shown).

The fact that mutation of either AR1 site in the –524 to –403 fragment eliminated nearly all activity showed that two copies of AR1 are required for substantial transcriptional activation by the –524 to –403 segment of *DAN1*. This suggested that there is a cooperative interaction between neighboring activation complexes forming on this segment.

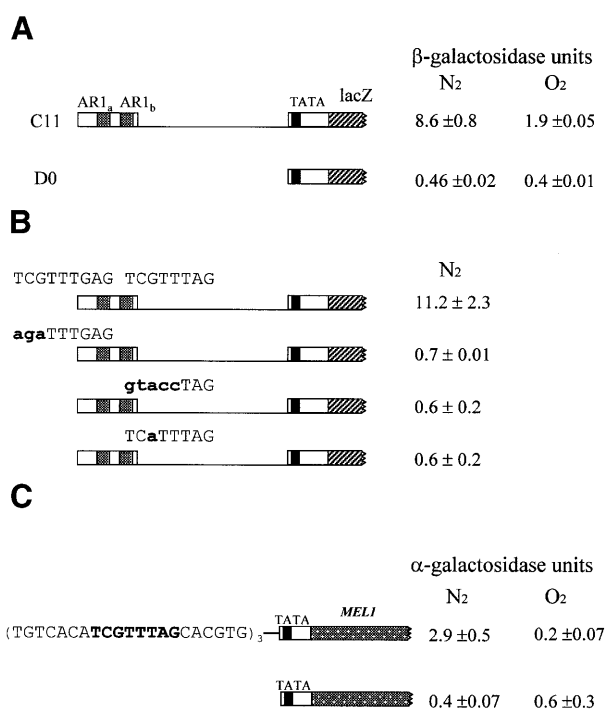


Figure 3. Activation of transcription dependent on AR1 sites. FY23 cells carrying plasmids with fragments fused to the *lacZ* ORF or oligonucleotides fused to *MEL1* (see Materials and Methods) were grown as described in Figure 2. (A) Cells were transformed with plasmid C11, which contains the promoter fragment from -524 to -403 fused at the TATA site to the *lacZ* reporter. Transformants were grown under aerobic and anaerobic conditions. (B) Cells transformed with plasmid C11 or mutant derivatives containing the indicated nucleotide substitutions were grown under anaerobic conditions. (C) Cells were transformed with the (AR1)₃/*MEL1* plasmid, which contains three copies of the AR1 sequence fused at the TATA site to the *MEL1* reporter; control cells were transformed with the promoterless vector D0-BST. Transformants were grown under anaerobic and aerobic conditions as above and harvested for α-galactosidase assay as described in Materials and Methods.

The AR1 site is sufficient for anaerobically induced promoter activity

Given that the AR1 site is necessary for expression from a *DAN1* activation segment, we tested whether it is *sufficient* for activation in a plasmid containing three synthetic concatenated copies fused to the *MEL1* gene. Cells transformed with the (AR1)₃/*MEL1* construct showed significant anaerobically induced activity (Fig. 3C), compared to aerobic cells. Anaerobic expression driven by AR1 implies that it is sufficient to provide a binding site for a transcriptional activator which responds to heme regulation.

The AR1 site of *DAN2* is also involved in anaerobic induction

To determine whether the AR1 sequence plays an equivalent role in the promoters of other genes in the regulon we constructed two plasmids containing a fusion of the *DAN2* promoter to *lacZ*. YCp*DAN2*/*lacZ* contains the wild-type promoter and YCp*DAN2*/*lacZ*-AR1_x contains a mutated version in which the single AR1 site at -447 is disrupted by a triple nucleotide substitution. We observed weak but signifi-

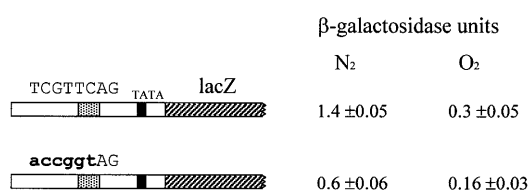


Figure 4. Importance of the AR1 site to expression of *DAN2*. FY23 cells transformed with YCp*DAN2*/*lacZ* or YCp*DAN2*/*lacZ*-AR1_x, a derivative containing a disruption of the single AR1 site in the *DAN2* promoter (-847), were grown under anaerobic and aerobic conditions and harvested for *lacZ* assay.

cant anaerobic induction from the wild-type fusion (Fig. 4). *DAN2* expression is characterized by a prolonged delay (>2 h) in expression; this delay, combined with the 3–4 h lag in β-galactosidase synthesis from *lacZ* fusions driven by anaerobically induced promoters, may account for the relatively low output of the reporter during the 7 h incubation. Substituting 5 nt in the AR1 site caused a 60% decrease in expression (Fig. 4). This shows that the AR1 site plays a role in induction of *DAN2*. However, the effect of eliminating this site is much less pronounced than the effect of eliminating either of the AR1 sites in the -524 to -403 segment of *DAN1* (Fig. 3); the residual activity suggests that a neighboring site(s) in the *DAN2* promoter (e.g. the AR2 site at -142) plays a combinatorial role with the AR1 complex. The fact that this activity is still oxygen regulated implies that the hypothetical site is also an anaerobic response element (such as AR2; see Discussion).

A second activation mechanism works through the AR2 response element

There are two copies of the AR2 consensus (AR2_a and AR2_b) in the *DAN1* promoter and one each in those of *DAN2* and *DAN3* (Table 1B). AR2_a contains an ATTGTT core, which is of interest because it is also the core of the binding sites of a number of HMG proteins, including Rox1. We tested a fusion of a 46 bp fragment (-570 to -525) containing AR2_a to the *lacZ* reporter (plasmid C6) in aerobic and anaerobic cells. Expression was substantial (Fig. 5A) and was 8-fold higher in anaerobic cells than in aerobic, indicating that the segment contained an anaerobic response element. Aerobic repression of expression from C6 was not as stringent as repression of the full promoter. Nucleotide substitutions across the AR2 sequence in the C6 construct caused full or partial loss of expression (Fig. 5A), confirming its role as an activation site. These substitutions included two within the ATTGTT core, indicating the importance of this segment. Where mutations resulted in partial activity, it appeared still to be regulated, as would be expected for a mutant activation site with reduced affinity for a heme-inhibited activator, i.e. one controlled through its activation function rather than its affinity. To narrow down the segment sufficient for expression we tested a 27 bp synthetic fragment (-570 to -544) including the AR2_a site and 15 flanking nucleotides in a fusion to the *MEL1* gene in plasmid AR2/*MEL1* (Fig. 5B). Again we observed elevated expression in response to hypoxia (Fig. 5B).

To assess the contribution of the two AR2 sites to anaerobic induction in the full promoter we constructed derivatives of

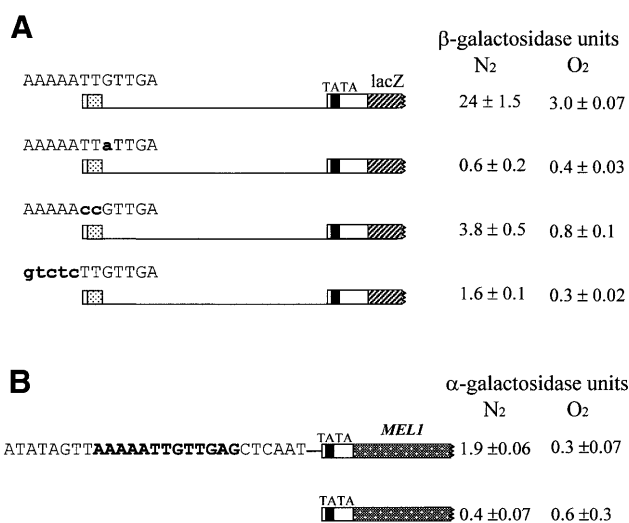


Figure 5. Function of the AR2 site in anaerobic induction. (A) FY23 cells were transformed with the C6 plasmid, which contains the AR2 site on a 46 bp fragment, or, in descending order, by plasmid M2, M4 or M6. The latter three plasmids are derivatives of C6 generated by introduction of the indicated nucleotide substitutions within the AR2 site (M2, AAAAATTaTTGA; M4, AAAAAccGTTGA; M6, gtctcTTGTTGA). Transformed cells were grown under anaerobic and aerobic conditions and analyzed for β-galactosidase as described in Figure 2. (B) FY23 cells were transformed with the plasmid AR2/MEL1 or the MEL1 vector. Transformants were grown under anaerobic and aerobic conditions and analyzed for α-galactosidase as described in Materials and Methods.

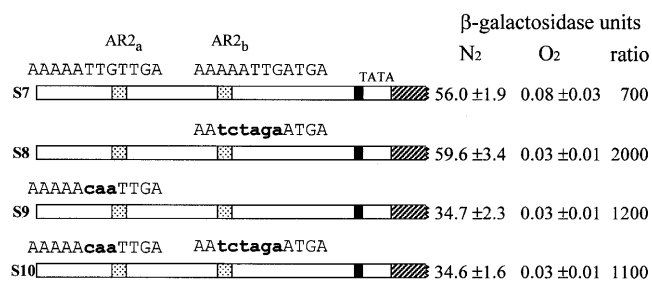


Figure 6. Role of AR2 sites in the full promoter. FY23 cells carrying derivatives of plasmid D1 containing nucleotide substitutions in either or both AR2 sites (AR2_a and AR2_b as defined in Fig. 1) and a control plasmid (S7) with normal AR2 sites were grown and analyzed as in Figure 1. All four plasmids contain identical substitutions adjacent to the AR2 sites introduced to facilitate plasmid construction.

plasmid D1 containing nucleotide substitutions in either the AR2_a or AR2_b site, or both. Loss of AR2_a caused a 40% reduction in activity, as did loss of both AR2_a and AR2_b; loss of AR2_b alone had no effect (Fig. 6). This showed that the activation mechanism operating through the AR2_a site does contribute to the activity of the full promoter. However the AR2_b site appears to play no role; significantly, it differs from AR2_a in the six base core (ATTGAT instead of ATTGTT). One simple explanation for anaerobic induction through the AR2 site is that it is alternately occupied by an activator during anaerobic growth and by Rox1 in aerobic cells; however, elim-

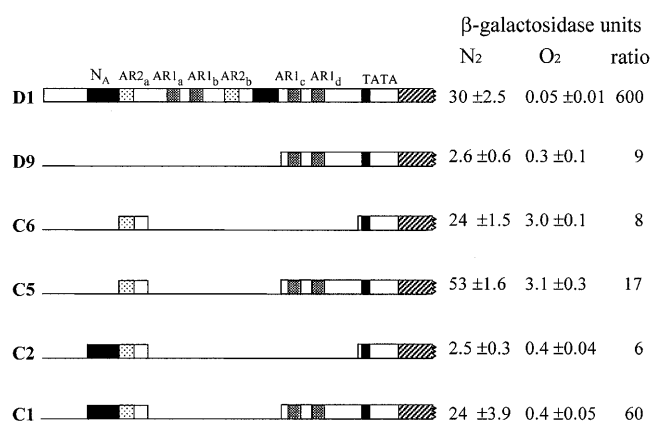


Figure 7. Expression by plasmids containing combinations of regulatory segments. FY23 cells were transformed with various plasmids containing segments of the *DAN1* promoter fused to *lacZ* and grown under anaerobic or aerobic conditions and analyzed as described in Materials and Methods. Cells carried D1 or plasmids containing the fragments indicated fused at -107, 5 bp upstream from the TATA site: D9, (-207 to -107); C6, (-570 to -525); C2, (-633 to -525); C5, (-570 to -525 plus -207 to -107); C1, (-633 to -525 plus -207 to -107).

ination of the two AR2 sites did not cause any increase in aerobic expression, arguing against a role for these sites in mediating repression.

The role of Mot3 and Rox1 sites in aerobic repression

Extensive deletions in the *DAN1* promoter failed to reveal any element essential for blocking aerobic expression, i.e. none of the deletions eliminated the difference between aerobic and anaerobic expression levels. However, we would not expect any repression sites which are present to be solely responsible for blocking aerobic expression, because the Mox4 activator is itself the target of a heme-dependent repression mechanism, i.e. it is less active in aerobic cells (18). Even so, the segment between -301 and -207 does appear to play a contributory role in aerobic repression; deletion of this region from the full promoter of the D1 plasmid, generating plasmid D15, resulted in a significant increase in aerobic expression (Fig. 2) and, consequently, a decrease in the anaerobic/aerobic induction ratio from >500 to 26. This suggested that a heme-responsive repressor binds in this region. The segment contains a cluster of four moderate to high affinity sites for the heme-induced repression factor Mot3 (see Fig. 1 for sites at -268, -258, -249 and -225) (9,19,22) and a potential Rox1 site (-242), providing a likely explanation for the importance of this segment to aerobic repression. We earlier observed that deletion of *MOT3* caused a substantial loss of repression of *DAN1* and other *DAN/TIR* genes (O.Sertil, R.Kapoor, B.Cohen and C.Lowry, submitted for publication), as did deletion of *ROX1*, but to a lesser extent.

A non-specific inhibitory site

A comparison of anaerobic and aerobic expression from two of our *lacZ* constructs revealed an inhibitory segment in the region between -630 and -570: the C6 construct, which contains only the 46 bp AR2_a segment (-570 to -525) fused to the TATA box, showed strong activity (Fig. 7), while the D20

construct, which contains the same segment plus the adjoining 5'-region (-878 to -571), was only weakly active. To further localize the inferred inhibitory segment we tested a plasmid (C2) which contains the AR2_a segment plus the adjacent 63 bp upstream segment. Inclusion of this segment, designated N_A, upstream from the AR2_a element caused a strong reduction in expression of both anaerobic and aerobic activity (compare C2 and C6) (Fig. 7), indicating that a sequence within N_A is targeted by a non-specific repression mechanism.

Combinatorial activation and repression in minimal constructs

The *DAN1* promoter contains a large number of sites which mediate the combined action of several regulators. Clearly both the AR1 and AR2 mechanisms can function independently, as demonstrated by the activity of the C6 and C11 constructs (Figs 3 and 5). To explore further how these mechanisms combine we constructed a plasmid (C5) containing the weakly active 100 bp AR1_c-AR1_d segment and the 46 bp AR2_a segment in tandem, fused to the *lacZ* reporter at the TATA site (Fig. 7). The activity of C5 was twice the sum of the activities of the C6 and D9 plasmids, which contain the AR2_a and AR1_c-AR1_d segments, respectively, indicating a modest synergy in their contributions to activation of expression. There was also a net increase in the induction ratio resulting from the combination. Another, more striking, kind of synergy between the AR2 and AR1_c-AR1_d segments was evident as decreased sensitivity to repression by the N_A mechanism. This segment inhibited the AR1-AR2 combination far less than it did the AR2 site alone (compare C1 and C2), hence, with the inhibitory segment present the activity of the combined segments was now about eight times higher than the sum of the activities of the equivalent individual segments (compare C1 with D9 plus C2).

Comparison of the expression of these constructs under anaerobic and aerobic conditions suggested that the N_A segment might contribute to heme repression in aerobic cells. As described above, inclusion of the N_A segment had an equally strong effect in aerobic and anaerobic cells on activation by the AR2_a segment (compare C6 and C2). However, it had a differential effect on aerobic and anaerobic expression driven by the combined AR1-AR2 segments; thus inhibition resulting from inclusion of the segment was greater in aerobic cells (~8-fold) than in anaerobic cells (~2-fold) (compare C5 and C1). Hence, in this artificial context it appeared that the 'non-specific' repressor functioned to improve aerobic repression and, consequently, the anaerobic/aerobic induction ratio. Non-specific repressors have been shown to enhance induction ratios in other settings (26).

DISCUSSION

The *DAN1* promoter is a mosaic of positive and negative regulatory elements, targeted in combinatorial fashion by various complexes which activate transcription during anaerobiosis and block expression during aerobic growth. Although we have not directly demonstrated the role of heme in regulating through the separate elements of the *DAN1* promoter, it is clear that heme is a primary signal co-effector for the promoter as a whole, since addition of heme to anaerobic cultures causes complete loss of expression (17); the fact that heme is

metabolically inert under anaerobic conditions implies that it functions directly in regulation rather than through an effect on metabolism. However, it is possible that metabolic or environmental changes associated with anaerobic growth (e.g. reduced pH, glucose depletion, alcohol production, unsaturated fatty acid, sterol depletion, etc.) are also factors in gene induction through one or more of the sites we have identified in this study.

Our observations revealed an unusual situation in which two different activation mechanisms were controlled by the same signal function on the same promoter. The first of the contributing activation mechanisms operates through the AR1 site, which appears at least once in each of the *dan/tir* promoters. Thus it is a common response element defining the regulon. We found that AR1 is necessary and sufficient for anaerobically induced transcriptional activation in two different experimental settings: first, AR1 site mutations in promoter segments of *DAN1* and *DAN2* caused loss of activity; second, there was substantial anaerobically induced expression from a fusion of concatenated AR1 sites to a *MEL1* reporter [(AR1)₃/*MEL1*] and also from *lacZ* reporter constructs (C11, D9, etc.) driven by promoter subsegments carrying AR1 sites.

We have reported elsewhere (18) evidence that Mox4, Mox1 and Mox2 function through the AR1 site. Mox4 is a binucleate zinc cluster protein and it might be expected to function in a dimer or a heterodimer complex. Since there was no dyad symmetry or repeated motif apparent to us in the AR1 site or its context, dimers might be expected to incorporate neighboring AR1 sites. One observation supporting this notion was that the individual complexes (presumably including Mox4) which bind to AR1 sites are not able to function independently, as shown by a nearly total loss of promoter activity when either AR1 site was eliminated from C11, indicating that the activation factor which binds to these sites functions cooperatively.

The second anaerobic induction mechanism operates independently through the AR2 site in the promoter of *DAN1*, and possibly in *DAN2* and *DAN3*. This was clear first because a synthetic AR2_a site stimulated anaerobic expression of reporters and second because nucleotide substitutions in the AR2_a site reduced the activity of the full *DAN1* promoter and eliminated the activity of a fragment carrying AR2_a. Significantly, the fact that the putative AR2 activation complex activates transcription efficiently from a 46 bp fragment carrying only one AR2 site (plasmid C6) indicates that cooperative interactions are not required for it to function, in contrast to the AR1 site mechanism.

The factor(s) which activates expression through AR2 has not been identified. We speculate that it may be an HMG protein, because AR2 contains an HMG core binding sequence (ATTGTT). Mutations affecting this factor would not have shown up in our earlier search for activators of *DAN1* (18), since elimination of both AR2 sites caused only a 40% reduction in expression. The AR2 site does not appear to be a target for repression by Rox1: we observed that ablation of the two AR2 sites reduced anaerobic expression as expected, but did not cause detectable loss of aerobic repression of *DAN1/lacZ* expression. This argues against the possibility that the AR2 activator is replaced by the repressor during aerobic growth.

There are single AR1 sites in the regulatory regions of four of the *DAN/TIR* genes (Table 1) and we found that the one in the *DAN2* promoter is important for induction. This was

surprising, since we had also found that *both* the AR1_a and AR1_b sites are strongly required for expression of a reporter construct containing the -524 to -403 segment of *DAN1*, implying that a single complex is ineffective and that some form of cooperativity is needed. Hence, it is not clear how single AR1 sites (in *DAN2*, *DAN4*, *TIR2* and *TIR3*) can contribute significantly to anaerobic induction and, conversely, how loss of the AR1 site in *DAN2* can have any impact (Fig. 3). One way in which a single AR1 site could participate in activation would be through an interaction with another factor bound at another site, e.g. one which binds to the AR2 site at -142. This would also explain why the residual activity is still oxygen regulated, since AR2 is also an anaerobic response site. Fortunately, two of the *DAN1* deletion constructs were analogous to the *DAN2/lacZ* and *DAN2/lacZAR1_x* constructs and behaved in a similar manner: plasmid D19, which contains the AR2_a site and a single AR1 site (AR1_a) was moderately active, but plasmid D20, in which the AR1_a site was deleted, was ~60% less active (Fig. 2). Another factor which may join with Mox4 in activation of *DAN2* is the homologous Ecm22 protein, which is needed for full expression of *DAN2* and *DAN3* (N.Abramova and C.Lowry, unpublished results) and is known to play a role in cell wall synthesis (27). Clearly the full set of regulators and regulatory sites controlling expression of the *DAN/TIR* regulon has yet to be accounted for. Some of these are likely to be involved in mediating signals other than heme, such as temperature (the *TIR* genes) and temporal ordering of cellular events (*DAN2* and *DAN3*).

In several contexts the AR1 and AR2 complexes appear to act in concert, either in synthetic constructs or in the full promoter. The most striking instance of combinatorial action was provided by a construct (C1) in which a 108 bp fragment containing AR2_a and the N_A segment was joined to a 100 bp segment containing AR1_c and AR1_d; the combined segments showed strong well-regulated activity about 10 times higher than that of either segment alone (D9 and C2). The synergizing effect of the inhibitory mechanism suggests that the AR1 and AR2 mechanisms overwhelm a repressing effect when their combined strength passes a certain threshold. We observed a similar effect when we combined two AR2_a sites with the N_A site: in this case the activity was increased ~20-fold by addition of the second AR2 site (data not shown). It is difficult to extrapolate from the properties of promoter subfragments to those of the full promoter, but these experiments emphasize the need to understand the balance of repression and activation in accounting for the net output of the combinatorial ensemble.

Like activation, repression of the *DAN/TIR* genes is also combinatorial: we earlier found that at least four heme-responsive repressors and Tup1 and Ssn6 are required to fully repress *DAN1* and other members of the regulon during anaerobiosis (18). Two of these repression factors, Mox1 and Mox2, appear to function through the AR1 site, possibly through an interaction with Mox4 or by competing with it for binding to AR1. In addition, Mot3, and to a lesser extent Rox1, play a role in aerobic repression. The one region identified in our deletion study which clearly contributes to aerobic repression (-301 to -207) does contain several Mot3 sites (at least two of high affinity; 21), as well as a Rox1 site.

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