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Yeast Intragenic Transcriptional Control: Activation and Repression Sites within the Coding Region of the *Saccharomyces cerevisiae LPD1* Gene

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Though widely recognized in higher eukaryotes, the regulation of *Saccharomyces cerevisiae* genes transcribed by RNA polymerase II by proteins that bind within the coding sequence remains largely speculative. We have shown for the *LPD1* gene, encoding lipoamide dehydrogenase, that the coding sequence between +13 and +469 activated gene expression of an *LPD1::lacZ* fusion by up to sixfold in the presence of the upstream promoter. This downstream region, inserted upstream of a promoterless *CYC1::lacZ* fusion, activated gene expression in a carbon source-dependent manner by a factor of 15 to 111, independent of orientation. Deletion and mutational analysis identified two downstream activation sites (DAS1 and DAS2) and two downstream repressor sites (DRS1 and DRS2) that influence the rate of *LPD1* transcription rather than mRNA degradation or translation. Activation from the DAS1 region (positions +137 to +191), encompassing a CDEI-like element, is twofold under derepressive conditions. Activation from DAS2 (+291 to +296), a CRE-like motif, is 12-fold for both repressed and derepressed states. DRS1, a pair of adjacent and opposing ABF1 sites (+288 to +313), is responsible for a 1.3- to 2-fold repression of transcription, depending on the carbon source. DRS1 requires the concerted action of DRS2 (a RAP1 motif at position +406) for repression of transcription only when the gene is induced. Gel mobility shift analysis and in vitro footprinting have shown that proteins bind in vitro to these downstream elements.

Transcriptional control of the expression of most protein-coding (type II) genes requires *cis*-acting sequences which lie upstream of the TATA box. The mechanisms by which these sequences and the cognate transcription factors influence the rate of transcription are beginning to be realized (reviewed in reference 66). Less understood are the regulatory sequences of a number of bacterial, viral, and mammalian type II genes that are located within transcribed sequences, within either the 5′ untranslated leader sequence, introns, or translated sequences.

The location of regulatory sequences within transcribed (downstream) regions places them under a variety of constraints not encountered by upstream elements. The intragenic sequence must perform two roles, specifying a motif for a DNA-binding protein while coding for mRNA and the resultant protein. Furthermore, the passage of the bulky transcriptional machinery after every initiation event results in a variety of disturbances to the coding sequence. These include the overwinding and then underwinding of the DNA (36), disruption of nucleosomes (30), and the potential for interference with the DNA-bound regulatory protein. Regulatory proteins that bind to transcribed sequences must therefore have mechanisms that either allow rapid reestablishment of the DNA-protein complex or modify the transcriptional state of the gene which is maintained after the protein is released. To date, RNA polymerase III-transcribed genes have provided a model for downstream regulation of transcription; however, their limited numbers and diverse structures do not allow generalizations to be drawn (62).

Though well documented in higher eukaryotes, intragenic transcriptional regulation of *Saccharomyces cerevisiae* type II genes remains largely speculative. Attempts to construct functional intragenic elements in yeast spp. by moving upstream activation sites into coding regions have been unsuccessful (reviewed in reference 59). The yeast retrotransposons Ty1 and Ty2 have been shown to contain multiple downstream elements that either activate (DASs) or repress (DRSs) transcription (17, 21). The regulation of yeast retrotransposons may, however, differ from that of normal yeast genes since retrotransposons are under considerable selection pressure for compact genomes and, as a consequence, economical regulatory mechanisms (4).

In several cases, the expression of heterologous sequences by using upstream promoters from yeast genes has been shown to be well below the level of the wild-type gene that retains the coding sequence (12, 29, 38, 45, 65). In the case of the *LPD1* gene, deletion of a large section of the coding region resulted in a carbon source-dependent decrease in the rate of transcription of between 6- and 21-fold (65). This phenomenon has been attributed, in part, to the loss of an intragenic activator, though other hypotheses have been proposed (12). In this study, we extend the work on the *LPD1* coding region to localize and identify the DNA-protein interactions responsible for downstream transcriptional regulation in *S. cerevisiae*.

The *LPD1* gene of *S. cerevisiae* encodes lipoamide dehydrogenase, which forms an essential component of at least three separate multienzyme complexes: pyruvate dehydrogenase; 2-oxoglutarate dehydrogenase (47); and branched-chain 2-oxoacid dehydrogenase (15). Each complex consists of multiple subunits of a dehydrogenase (E1), a transferase

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(E2), and lipoamide dehydrogenase (E3). Pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase supply and maintain the metabolic turnover of the citric acid cycle and are therefore essential for oxidative metabolism, while the branched-chain 2-oxoacid dehydrogenase catalyzes the second step in the degradation of branched-chain amino acids. To accommodate the requirement for E3 subunits, the rate of *LPD1* transcription increases dramatically when cells are transferred from glucose-containing media to media containing nonfermentable carbon substrates such as glycerol or lactate (6).

The upstream regulatory region of *LPD1* is one of the most complex described to date, with potential binding sites for the transcription factors ABF1, HAP2/3, GCN4, CPF1, and yAP1 and heat shock elements. Considerable progress has been made in determining the functions of many of these sites, using a combination of techniques including deletion, site-directed mutagenesis, and analyses of both DNA-protein interactions and chromatin structure. Some of the ABF1 binding sites are required for maintaining transcription during both repressive and derepressive conditions, whereas the three HAP2/3 sites act in a cooperative manner to increase transcription of the gene during shifts to aerobic metabolism. Mutagenesis of the upstream CPF1 site does not influence the level of gene expression under fermentative, respiratory, or phosphate-limited growth conditions, and as yet their role, if any, remains unclear.

In this study, we report the localization of yeast intragenic regulatory elements in a housekeeping gene and show that they interact with DNA-binding proteins. Site-directed mutagenesis and transplacement of the motifs indicate that the regulation of *LPD1* transcription requires a complex interplay of both positively and negatively acting proteins that bind to the coding region to modulate the rate of transcription at levels comparable to those directed by the upstream promoter.

MATERIALS AND METHODS

Yeast strains and media. Yeast cells were grown with shaking at 30°C in rich medium (2% Bacto Peptone, 1% yeast extract) containing either 2% glucose (YEPD), 2% glycerol (YEPG), or 2% lactate (YEPLact) as the carbon source. Synthetic minimal medium (2% glucose, 0.17% yeast nitrogen base, 0.5% ammonium sulfate, supplemented with appropriate auxotrophic requirements) was used to select and maintain strains transformed with plasmids containing the *URA3* gene.

Strains. General plasmid preparation was routinely performed from transfected *Escherichia coli* JM101 (40). For in vitro site-directed mutagenesis, *E. coli* BHM71-18 *mutS* (31) was used. Unmethylated DNA for mobility shift experiments was prepared from *E. coli* GM2163 (46).

S. cerevisiae BWG1-7A (*MAT***a** *ade1-100 his4-519 leu2-3 leu2-112 ura3-52*) was used for transformation with recombinant plasmids and determination of β-galactosidase and mRNA levels. DNA-binding proteins used in mobility shift and footprinting experiments were isolated from strain DC5 (*MAT***a** *leu2-3 leu2-13 his3 can1*). The stability of the *LPD1*::*lacZ* fusion mRNA was determined in strain Y262 (*MAT***a** *ura3-52 his4-539 rpb1-1*).

Plasmids and deletion constructs. The reporter plasmid pDS1 was constructed by inserting the *Bam*HI 1.46-kb fragment consisting of the *LPD1* upstream promoter and the first 673 bp of coding sequence into the *Bam*HI site of the integrative *lacZ* reporter plasmid YIp357 (41). Unidirectional deletions (3′ to 5′) were generated by linearizing pDS1 with *Xba*I and *Sph*I at the *LPD1*::*lacZ* fusion boundary. Nucleotides were removed at a rate of 75 bp min^{-1} at 22°C with 15 U of exonuclease III per μg of DNA. Deleted plasmids were treated with both S1 nuclease and the Klenow fragment of DNA polymerase to create blunt ends, which were ligated at 15°C for 8 h, and the resulting plasmids were transformed into *E. coli*. The extent of deletion was determined by sequencing across the fusion boundary, and out-of-frame *LPD1*::*lacZ* fusions were discarded.

To generate plasmids pCTDAS+ (forward orientation of downstream promoter) and pCTDAS− (reverse orientation), the 425-bp *Sau*3A fragment from *LPD1* (+13 to +438) was excised and ligated in both orientations into the *Bam*HI site of M13mp18. This insert was then removed with *Eco*RI and *Sal*I and inserted upstream of the basal promoter of the *CYC1*::*lacZ* fusion in pCT (9). The integrative plasmid pDS-UAS was constructed by ligating the *LPD1* *Bam*HI-to-*Hin*dIII fragment from the pJW11.3 deletion to YIp358 cut with the same enzymes. An integrative minimal *LPD1* promoter was constructed by partially digesting *LPD1* from pDS1 with *Sau*3A and ligating the fragment (−793 to +13) to YIp357 cut with *Bam*HI. The upstream promoter (−793 to −185) was then removed by first digesting the fragment with *Sma*I and *Sac*II. The *Sac*II 5′ overhang was digested with S1 nuclease and then treated with Klenow fragment in the presence of excess deoxynucleoside triphosphates. The resulting blunt ends were ligated to produce pDSmin.

Yeast transformations and β-galactosidase assays. Each construct was linearized within the *URA3* sequence by digestion with *Stu*I and integrated in the genome of BWG1-7A at the *ura3* locus by high-efficiency lithium acetate transformation (22). Each transformant was checked by Southern analysis to ensure the presence of a single integrated copy of the plasmid.

For assay of β-galactosidase, yeast cells were grown at 30°C to an A_{600} of 0.3 and resuspended in 3 ml of 50 mM potassium phosphate buffer. Cells were broken by agitation with glass beads in a Braun MSK homogenizer, and 20 μl of a saturated solution of phenylmethylsulfonyl fluoride in ethanol was added. β-Galactosidase assays were performed by the method of Guarente (23). Protein concentration was determined by the Bradford method (7).

Site-directed mutagenesis. The oligonucleotides used for site-directed mutagenesis are listed in Table 1. Base changes were chosen to abolish motifs for DNA-binding proteins without altering the protein code, where possible. In cases where direct substitution of a particular amino acid codon was impossible, it was replaced with a similar amino acid or the order of two consecutive amino acids was changed. In all cases, codons of similar usage in *S. cerevisiae* were used. Site-directed mutagenesis was performed as previously described (6) by annealing a kinase-treated mutagenic oligonucleotide to single-stranded DNA prepared from an M13mp9 derivative containing *LPD1* sequence (−793 to +673) inserted at the *Bam*HI site. After annealing, the oligonucleotide was extended to completion with T7 polymerase, ligated, and transfected into *E. coli* BHM71-18. Mutations were identified by plaque hybridization using the ^{32}P-labelled mutagenic oligonucleotide as a probe and by detecting altered restriction sites that were included in the design of some oligonucleotides. The presence of the desired mutation was confirmed by sequencing, and the 1.46-kb *Eco*RI-to-*Hin*dIII fragment of the mutant *LPD1* gene was excised and ligated in frame with *lacZ* of plasmid YIp358 cut with the

TABLE 1. Oligonucleotide sequences used in this study[a]

Oligonucleotide	Sequence	Use
ABF1-GH mut	CGTCAACGGTatTgaCAAAATTAACGTAGC	Mutation of ABF1 sites
CDEI-D1 mut	CATTAACAAGagcCATGATGT	Mutation of CDE-D1 site
CDEI-D2 mut	TAACACTGCtTGcGTAGAAAAAG	Mutation of CDE-D2 site
RAP1 mut	CCTATTATAAgGGcAAcGGTagtTTCGAAGACG	Mutation of RAP1 site
yATF1 mut	GAGGTATTGcCGTgcACGGTGATATCA	Mutation of yATF1 site
ABF1 cmp-a	TATAATATCATTTTTAACGAAGCT	Competition (strand a)
ABF1 cmp-b	TCGTTAAAAATGATAT	Competition (strand b)
RAP1 cmp	CCATACACCGGTGTATGGGTTTAAAC	Competition
yATF1a cmp	CCGGGAGACGTCAACTCGAGTTGACGTCTC	Competition
yATF1b cmp	CCCGGGATGACGTCAT	Competition

[a] Bases which alter the sequence from the wild type are shown in lowercase. The wild-type sequences are shown in Fig. 1.

same enzymes. Resultant plasmids were transformed into yeast strain BWG1-7A as described above.

Northern (RNA) analysis. For steady-state analyses, cell cultures were grown to an A_{600} of 0.5 and RNA was extracted by the method of Schmitt et al. (50). Total RNA (20 μg in 5 to 10 μl) was denatured at 60°C for 15 min with 4.5 μl of deionized formamide and 1 μl of formaldehyde and separated by electrophoresis in a 0.8% formaldehyde gel. Nitrocellulose filters were probed separately for *lacZ* and actin transcripts with the appropriate *Sac*I fragment of YIp357 and a *Bam*HI-to-*Eco*RI fragment of pYA301 (3), respectively.

For analyses of transcript stability, cultures in 100 ml of YEPD were grown at 25°C to an A_{600} of 1. Cells were harvested, resuspended in YEPLact medium, and shaken vigorously at 25°C for another 4 to 6 h to induce *LPD1* expression. Cultures were added to 100 ml of preheated YEPLact (50°C) and incubated with aeration at 37°C. Samples (20 ml) were taken at 10-min intervals, and RNA was extracted, separated, and probed as described above. Quantitative estimation of probed transcripts was performed with ImageQuant software on a Molecular Dynamics PhosphorImager. Quantification of 28S rRNA was performed on a scanned image of an ethidium bromide-stained gel, using the Apple Macintosh program Image from the National Center for Supercomputing Applications, University of Illinois, Champaign-Urbana.

Preparation of DNA-binding protein extracts. Nuclear extracts of *S. cerevisiae* DC5 were prepared by the method of Stanway et al. (55). Heparin-Sepharose-purified extracts of *S. cerevisiae* DC5 were prepared from a 10-liter culture of cells grown to mid-log phase (A_{600} of approximately 2) in YEPD or a 15-liter culture grown in YEPG. Cells were harvested by centrifugation at 2,000 × *g* and washed twice in 0.1 volume of buffer C containing 0.3 M $(NH_4)_2SO_4$ [200 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10% (wt/vol) glycerol, 300 mM $(NH_4)_2SO_4$]. The cells were broken in a Braun MSK homogenizer in 45 ml of buffer C containing 0.3 M $(NH_4)_2SO_4$ and centrifuged at 40,000 × *g* at 4°C for 30 min, and the aqueous phase was diluted with an equal volume of buffer C and applied to a 25-ml column of heparin-Sepharose as described by Ruet et al. (49). Protein fractions (10 ml) were eluted with a salt gradient of column buffer containing 0.1 to 0.75 M $(NH_4)_2SO_4$. The fractions were assayed for protein, dialyzed against 20 mM Tris-HCl (pH 8.0)–50 mM KCl–1 mM dithiothreitol–0.2 mM EDTA–10% (vol/vol) glycerol, and stored at −20°C in 1-ml aliquots.

DNA binding assays. Protein extracts (0.7 μg of total protein per reaction) were incubated with 4 fmol of ^{32}P-end-labelled DNA fragment in 12 μl of footprint buffer (25) at 25°C for 15 min. After incubation, 10 μl was loaded onto a preelectrophoresed 5% polyacrylamide gel (20 by 20 cm; 98.8:1.2 acrylamide/bisacrylamide) containing 5% (vol/vol) glycerol and 1× TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA [pH 8.3]). The complexes were electrophoresed at 5°C in 1× TBE buffer containing 5 mM 2-mercaptoethanol. Electrophoresis was performed at 280 V (40 mA per gel) for 3 to 4 h with buffer recirculation and was monitored by the addition of bromophenol blue and xylene cyanol to the free DNA lane. Electrophoresis was stopped when the bromophenol blue marker dye was 1 cm from the bottom of the gel. The gels were dried under vacuum and autoradiographed overnight at −70°C with two DuPont Lightning-Plus autoradiography screens and Fuji RX film.

In competition experiments with concatenated oligonucleotides, the unlabelled competing DNA was added to the protein extract and preincubated for 10 min before the addition of end-labelled DNA. In assays using anti-ABF1 antibody (16), protein extracts were incubated with DNA in a final volume of 12 μl, and 1 μl of a 1:10 dilution of polyclonal antibody was added. Electrophoresis was carried out in the absence of 2-mercaptoethanol.

Footprinting analysis. Cu^{2+}-phenanthroline footprinting assays were performed as previously described (32) after binding activities were determined by mobility shift assays. Forty femtomoles of ^{32}P-end-labelled DNA was incubated with enough protein to completely complex the DNA in a total of 300 μl of footprint buffer. After incubation for 15 min at room temperature, the mixture was treated with 48 μl of 1 mM phenanthroline–0.23 mM $CuSO_4$ and 48 μl of 58 mM 3-mercaptopropionic acid. The reaction mixture was incubated at 37°C for 9 min and then quenched with 48 μl of 28 mM 2,9-dimethylphenanthroline, chilled on ice, and ethanol precipitated. The DNA was resuspended in 10 μl of formamide dye mixture (95% formamide, 1% bromophenol blue, 1% xylene cyanol FF), heated to 95°C for 5 min, and electrophoresed on an 8% sequencing gel. A G+A sequencing ladder was prepared from 40 fmol of the same fragment (37).

RESULTS

The *LPD1* coding sequence can activate gene expression in both downstream and upstream locations. The downstream region from +13 to +673 contains consensus motifs for regulatory proteins that bind to elements normally located in yeast upstream promoters (Fig. 1). CDEI motifs that bind the protein CPF1 are located at positions +77 and +158. Further distal is a potential regulatory cluster consisting of

FIG. 1. Comparison of sequences within the coding region with sequences of known transcription factor binding motifs. The consensus motifs for ABF1 (14), CPF1 (19), HAP2/HAP3 (20), yATF1 (28), SKO1 (42) and ACR1(64), RAP1 (61), and GCR1 (26) are shown below the corresponding downstream *LPD1* sequence(s). The position of each motif relative to the ATG start codon is shown. Potential motifs are written relative to the consensus sequences.

overlapping and divergent motifs for the yeast activating transcription factor (yATF) family. A SKO1/ACR1 motif overlaps this yATF1 dyad, which itself overlaps one of two tandem and opposing ABF1 sites. A potential RAP1 motif and a number of GCR1 motifs are clustered further downstream. Beyond this region of coding sequence (+674 to +1500), there are no other recognizable motifs.

To test whether the *LPD1* coding sequence regulates transcription, the majority of the coding region (between +13 and +673) was deleted from the wild-type *LPD1*::*lacZ* fusion, pDS1. The resulting plasmid, pDS1+13, expressed about 10-fold less β-galactosidase than did pDS1. This observation supports the proposal of Zaman et al. (65) that *LPD1* contains a transcriptional activator between +13 and +697.

The nature of the *LPD1* DAS was analyzed initially by constructing a series of gross deletions and downstream fragment insertions (Fig. 2). Removal of either the upstream sequence (pDS-UAS) or both upstream and downstream sequences (pDSmin) led to a 5-fold and a 25-fold decrease in gene expression, respectively. To test the promoter activity of the *LPD1* downstream region in an upstream context, the downstream sequence between +13 and +438 was fused upstream of a minimal *CYC1*::*lacZ* promoter to create pCTDAS+ and pCTDAS−. On glucose medium, the fragment increased gene expression by about 15-fold above the basal level; on lactate medium, expression was increased up to 111-fold. Furthermore, the expression was regulated in a carbon source-dependent manner and was independent of orientation.

Identification of both positive and negative downstream elements. To more accurately determine the location of the elements responsible for downstream activation, a series of 19 nested deletions of the *LPD1* coding sequence was constructed. These deletions were generated from the boundary of the *LPD1*::*lacZ* fusion (+697) and extended 5′ toward the upstream region of *LPD1* (to −210 relative to the start of the coding sequence). Approximately one in three deletion constructs maintained the correct reading frame, as detected by both β-galactosidase production in *E. coli* and sequencing across the fusion boundary. Each deletion construct was integrated at *ura3*, and all transformants were rigorously screened by Southern analysis for the presence of a single copy of the construct.

The level of β-galactosidase expressed by each of the *LPD1*::*lacZ* deletion constructs was then determined in response to growth of the cells on various carbon sources (Fig. 3). The full-length fusion construct (pDS1) was repressed in cells grown on glucose medium, whereas growth on either glycerol or lactate medium resulted in partial (18-fold) or full (22-fold) relief of carbon catabolite repression, respectively. Deletions from the region containing three consensus GCR1 motifs and the potential RAP1 motif, between +697 to +343, did not result in any major changes to β-galactosidase expression in cells grown on any of the media tested. However, deletion to +312 (ΔE) removed one of the pair of adjacent ABF1 binding sites and resulted in a 1.3- to 2-fold increase in expression of β-galactosidase. This implied that a sequence within the region, perhaps an ABF1 motif, binds a repressor of gene expression. Larger deletions that removed both ABF1 binding sites (ΔF and ΔG) had similarly elevated levels of expression.

A subsequent twofold reduction in β-galactosidase specific activity resulted after deletion of the sequence beyond +291 (ΔH) which removed the yATF1 dyad motifs at +285 and +287. This decrease was similar in cells grown on all of the carbon sources tested. A further twofold decrease in enzyme levels resulted upon removal of the CDE-D2 element (ΔI). This effect was most marked in cells grown on nonfermentable substrates promoting high levels of gene expression.

The rate of transcription is regulated by downstream elements. The different β-galactosidase activities observed as a result of deleting the coding sequence from pDS1 may not have been a direct consequence of changes to the rate of transcription but may have resulted from differences in the stability or efficacy of the resultant hybrid transcripts or polypeptides. In addition, the deletions may have led to inefficient 3′-end formation of the transcript as reported for *lacZ* gene fusions and the *URA3* gene (1).

To investigate whether the downstream effect was due to changes in transcript level rather than translational or post-translational events, the steady-state levels of mRNA from representative deletion constructs were determined by probing Northern blots for *LPD1*::*lacZ* transcripts (Fig. 4). In each transformant, the *lacZ*::*LPD1* transcripts were the appropriate length, indicating that the fusion transcript was terminating efficiently. Furthermore, the relative levels of mRNA closely paralleled the levels of β-galactosidase de-

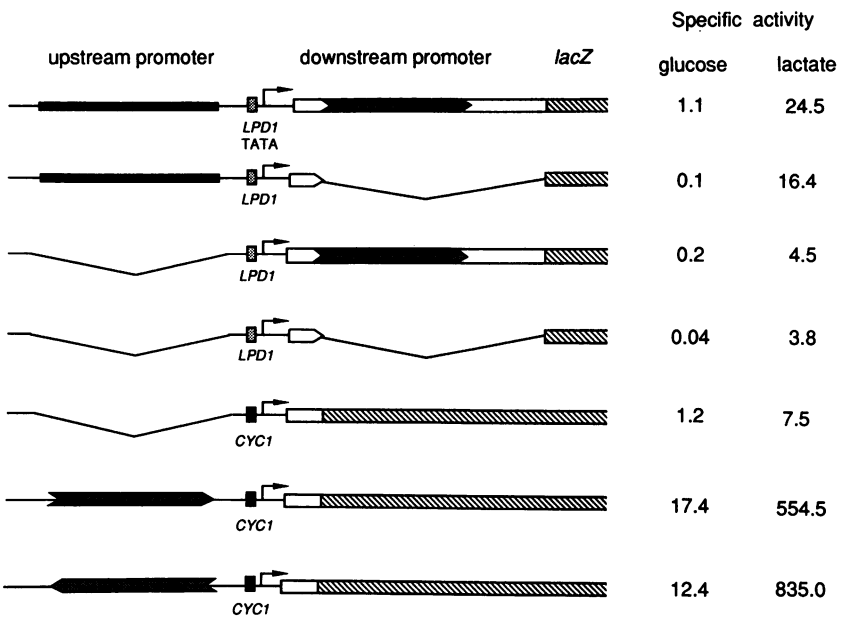

FIG. 2. Effects of transplacing or deleting the downstream regions of *LPD1* on the expression of *LPD1*::*lacZ* or *CYC1*::*lacZ* constructs. The constructs are depicted diagrammatically (descending order): wild-type, pDS1+13, pDS-UAS, pDSmin (minimal *LPD1* promoter), pCT (minimal *CYC1* promoter), pCTDAS+ (*LPD1* coding region upstream of pCT, forward orientation), pCTDFAS− (*LPD1* coding region upstream of pCT, reverse orientation). The *LPD1*-based constructs were integrated as single copies, and the *CYC1*-based constructs are centromeric vectors. Transformants were grown in glucose (YEPD)- or lactate (YEPLact)-based medium, and β-galactosidase assays were performed as described in Materials and Methods. Specific activities are expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside cleaved per minute per milligram of protein; standard deviation is <6.8.

tected in each of the strains. Thus, deletions that removed *LPD1* coding sequence between the *lacZ* boundary and +469 (ΔA and ΔC) produced levels of mRNA similar to those from the full-length construct. When grown on glucose, transformants containing the construct with one ABF1 binding site deleted (+288, ΔF) contained approximately twice the level of mRNA seen in the full-length construct (data not shown). This transformant had 140% of the level of β-galactosidase produced from the full-length construct when grown under identical conditions. Deletions that removed most of the coding sequence (ΔL, ΔN, and pDS+13) led to an eightfold decrease in transcript relative to the full-length construct, which is in agreement with the level of β-galactosidase detected in enzyme assays. Thus, the Northern analysis confirmed that regions of coding sequence of *LPD1* influence steady-state transcript levels.

Since the level of an mRNA species is determined by the relative rate of its synthesis and degradation, the effects described above may have been due to differences in the stability of the transcripts rather than control exerted over the rate of transcription. To resolve this question, the in vivo rates of degradation of the transcripts produced from the wild-type *LPD1*::*lacZ* fusion, ΔF, and pDS+13 were determined by using the temperature-sensitive RNA polymerase II mutant strain Y262. By shifting the cells to 37°C, thereby preventing further mRNA synthesis (43), the transcripts were shown not to differ significantly in their rates of decay (Fig. 5). This result extends the qualitative observations that the full-length (+700) and minimal (+13) *LPD1*::*lacZ* fusions had similar stability (65). Thus, the steady-state levels of mRNA detected from the deletion constructs represent the rates of *LPD1*::*lacZ* transcription, and the results from deletion analysis, taken together with those from insertion of the coding sequence upstream of a minimal *CYC1* promoter, indicate that regions within the coding sequence act to regulate the rate of *LPD1* transcription.

The downstream region forms complexes with nuclear proteins. The coding region of the gene was found to contain motifs for known yeast upstream binding factors. In addition, downstream transcriptional regulation may be controlled, at least in part, by novel proteins that function only within the coding sequence. To characterize the binding motifs and associated proteins, end-labelled restriction fragments encompassing the downstream region were used in mobility shift assays with heparin-Sepharose-purified extracts and nuclear extracts from YEPD- and YEPG-grown cells (Fig. 6). These assays indicated that at least six different proteins of moderate to high abundance bound in vitro specifically to the downstream region.

The complex of shifts (Fig. 6A, lanes 1 to 9) associated with a 145-bp downstream fragment was confirmed as being due to interaction with ABF1 by using polyclonal antibodies (16) directed against the protein, as indicated by the supershifts in Fig. 7B, lanes 10 to 14. Binding to the correct ABF1 motif was shown by competition with an oligonucleotide containing the ABF1 binding site (Fig. 7B, lanes 1 to 9) and in vitro Cu^{2+}-phenanthroline footprinting (Fig. 8). DNA-ABF1 complexes containing proteins derived from glucose-grown cells showed fewer higher-order gel mobility shifts, consistent with the observation that ABF1 is less phosphorylated during growth under fermentative conditions (53).

A second DNA-protein interaction in this region was localized by using a nested series of end-labelled restriction fragments in a mobility shift assay and by competition of the interaction with two different oligonucleotides containing the yATF1 binding site (Fig. 7C), one of which was identical to that originally used to isolate yATF1 protein (35). Two bands

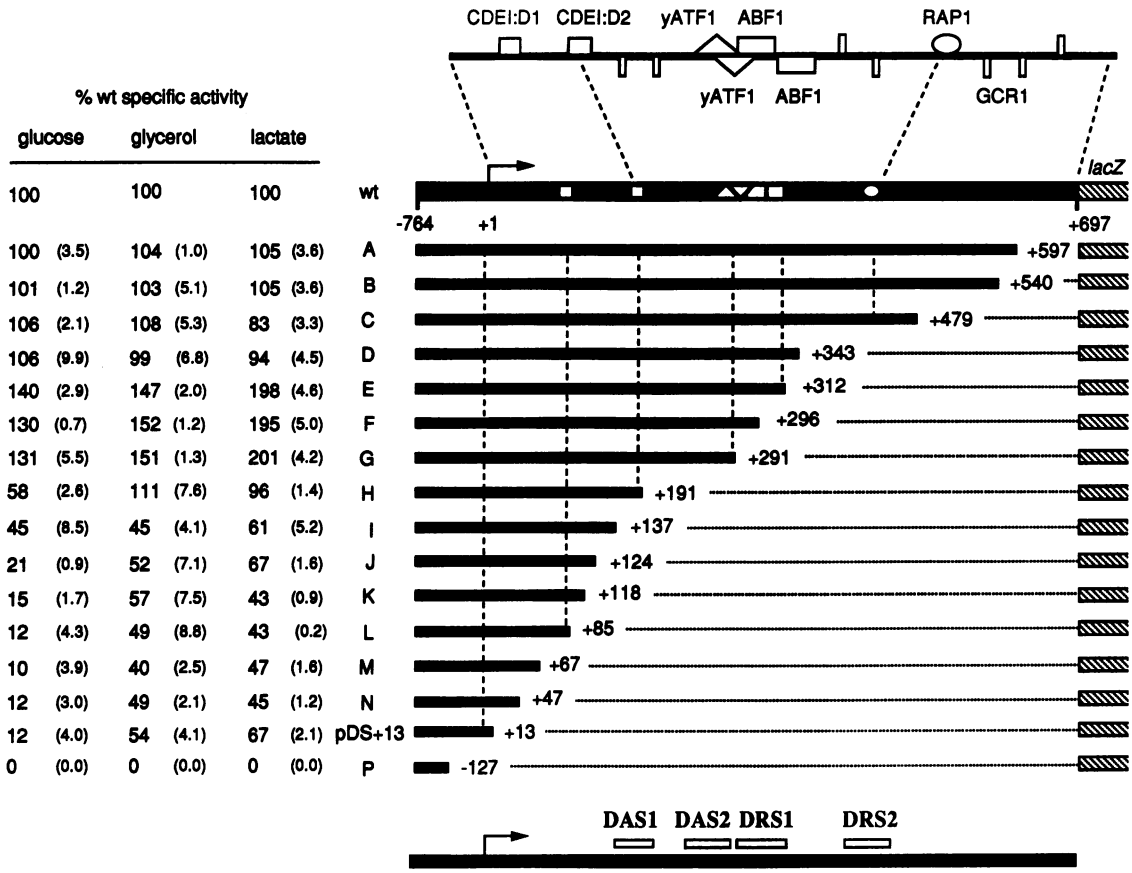

FIG. 3. Relative levels of β-galactosidase produced by *LPD1::lacZ* deletion constructs. The translation start point is designated by a horizontal arrow. Single-copy integrants were grown on glucose (YEPD), glycerol (YEPG), and lactate (YEPLact) as carbon sources. Assays were performed as described in Materials and Methods; values are given as a percentage of wild-type (wt) activity (± standard deviation). Specific activities of the wild-type integrant were 1.1 (±0.2) × 10^{-2}, 20.2 (±0.4) × 10^{-2}, and 24.5 (±0.9) × 10^{-2} nmol of product min^{-1} mg of $protein^{-1}$ for YEPD, YEPG, and YEPLact, respectively. At the bottom is a diagrammatic representation of the relative positions of downstream elements identified by deletion analysis and site-directed mutagenesis.

were seen (Fig. 7C, lane 3), both of which were competed with an equal rate by either of the oligonucleotides. An oligonucleotide which contains only an ABF1 site did not compete with either of the complexes. Although the arrangement of the two yATF1 sites forms a potential SKO1/ACR1 motif (42, 52, 64), the formation of the dimeric complex and the use of the yATF1a competition oligonucleotide (which contains a single base mismatch to the SKO1 consensus motif) indicated that the complex was most probably due to yATF1 protein and not SKO1 or ACR1.

A protein interacting downstream of the ABF1 sites was localized to a 30-bp fragment by using a series of nested DNA fragments (data not shown). This fragment encompasses a potential RAP1 motif which shows a 10/13 match to the consensus sequence described by Buchman et al. (9) or an 11/13 match as judged from a comparison of yeast glycolytic genes (Fig. 1).

A protein binds to the CDEI element at +77, as shown by using gel mobility shifts (Fig. 6C) and in vitro footprinting (data not shown). Site-directed mutation of this site abolished the gel mobility shift (Fig. 7A). Furthermore, this interaction was efficiently competed with by an oligonucleotide containing the CDEI element.

The other major shifts (Fig. 6A, e; Fig. 6B, a; and Fig. 6C, b) have yet to be localized to specific regions.

Localization of DASs by site-directed mutagenesis. Several of the elements shown to bind protein fall within regions defined by deletion analysis as being responsible for either the activation or repression of the transcription of the gene. To localize these downstream regulatory sequences and to eliminate the possibility that any effects observed were due to the shortening of the gene, the potential transcription factor binding sites were altered without affecting the size of the fusion and with minimal, if any, change to the amino acid sequence encoded. The effects of these mutations on expression of the *LPD1::lacZ* fusion are summarized in Fig. 9.

The first recognizable motif downstream of the start codon is the CDEI element at +77. Deletions through this region did not show any alteration in β-galactosidase activity in either fermenting or respiring conditions. A mutation of this site also did not result in any significant change in activity under these conditions. Thus, the role of the *LPD1* CDEI:D1 site remains uncertain, as does its function in the upstream region of other genes (11, 39).

Mutations of the remaining four sites (CDE-D2, yATF1/SKO1/ACR1, ABF1:ABF1, and RAP1) all showed changes in β-galactosidase activity, some consistent with the results of the deletion studies. Mutation of the CDE-D2 motif at +163 resulted in an 18% decrease in β-galactosidase activity when cells were fermenting but led to 20% increase in

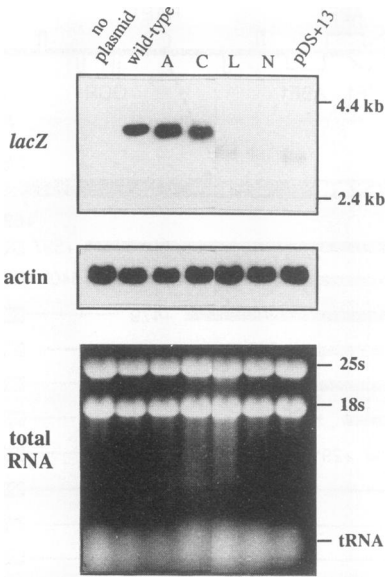

FIG. 4. Steady-state levels of *LPD1*::*lacZ* mRNA produced from representative deletion constructs depicted in Fig. 3. Total RNA was extracted from transformants grown to exponential phase in YEPD medium. Twenty micrograms of RNA per lane was probed for *lacZ* in a formamide-based buffer at 40°C. Relative amounts of RNA loading were assessed by probing the same filter for actin transcripts.

actively respiring cells. Since the activity of the site-specific mutant differed from that of deletions through the CDE-D2 site (ΔH to ΔI), it appears that this element does not operate independently and regions downstream of the CDEI motif may also be required. This site has been called DAS1.

The most dramatic effect on β-galactosidase activity resulted from mutation of the yATF1/SKO1 motif, which led to a 12-fold decrease in gene expression on all media tested. This is in agreement with the decrease detected when the yATF1 motif was deleted (ΔH). In conjunction with the analysis of mRNA and DNA-protein interactions, this finding indicates that the motif (termed DAS2) binds a transcription-activating protein, most likely yATF1.

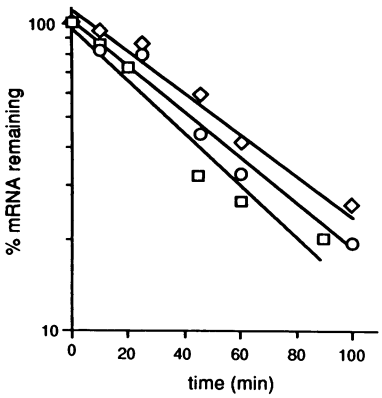

FIG. 5. Comparison of the stability of mRNA produced from wild-type and deletion constructs. Kinetics of mRNA depletion in a temperature-sensitive RNA polymerase mutant (Y262) is shown. After a temperature shift, RNA was extracted from the wild type (□), ΔE (◇), and pDS1+13 (○), subjected to electrophoresis, stained with ethidium bromide, and transferred to nitrocellulose. The blot was probed with the *lacZ* sequence, quantified with a Molecular Dynamics PhosphorImager, and standardized against the 25S rRNA band.

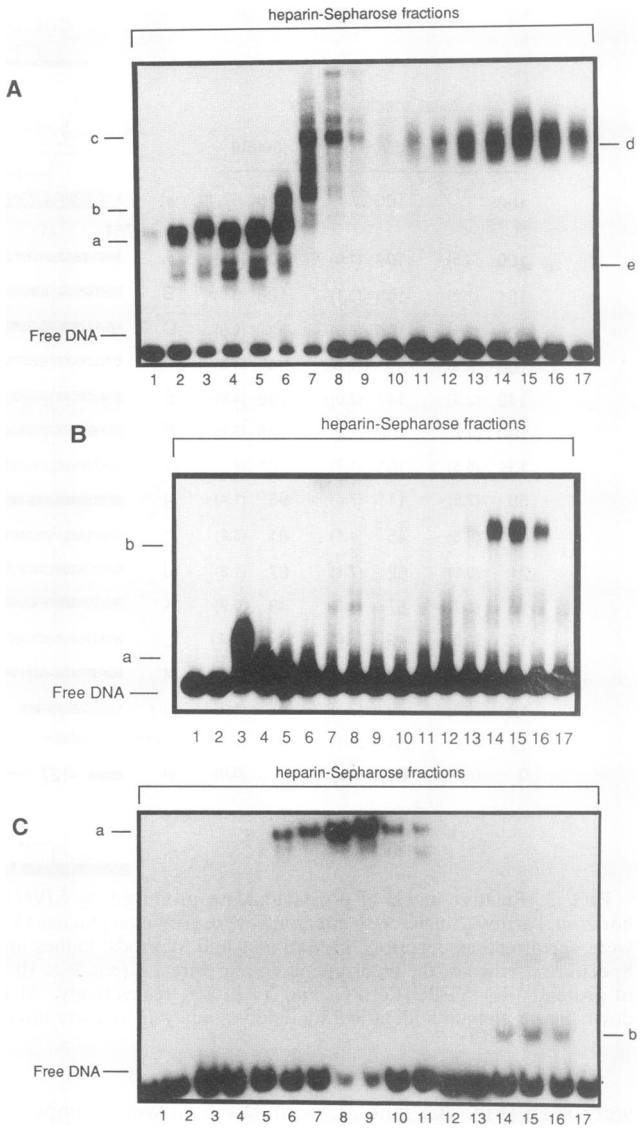

FIG. 6. Interaction of proteins from partially purified extracts of YEPG-grown cells with the downstream control region of the *LPD1* gene. (A) Mobility shift analysis of heparin-Sepharose-purified protein fractions with the 145-bp *Kpn*I-to-*Dde*I DNA fragment which encompasses the yATF1 and ABF1 elements. Four femtomoles of DNA was incubated with 0.7 μg of total protein from each fraction of the 0.1 (lane 1) to 0.75 (lane 17) M $(NH_4)_2SO_4$ gradient. ABF1 protein bound to one (a) or both (b) of the downstream ABF1 sites. The interaction shown by shift c and the higher-order shifts are due to phosphorylated forms of ABF1. The mobility shift (d) is the result of interaction of the DNA with yATF1 or a similar CRE motif-binding protein. (B) Interaction of the 345-bp *Dde*I-to-*Xba*I fragment with the same fractions. This fragment contains the majority of the GCR1 elements and the putative RAP1 motif. (C) Complexes formed between the fractions and a 236-bp region encompassing the two CDEI elements. Band a is the result of a protein binding to the CDEI-D1 motif at +77. At higher DNA/protein ratios, a second, higher band is visible in these lanes.

Downstream elements show functional redundancy and

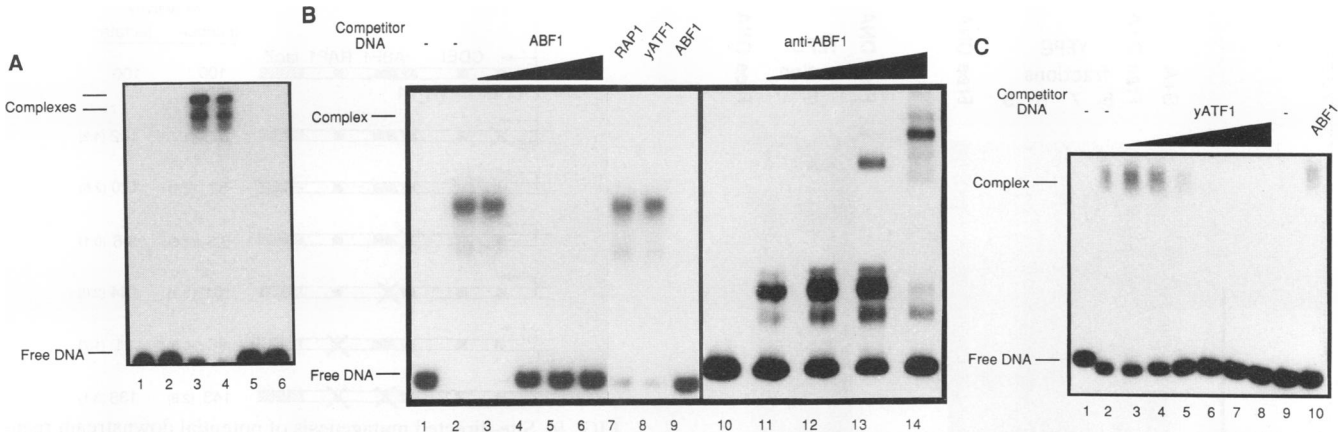

FIG. 7. Characterization of transcription factor binding motifs in the coding region of the *LPD1* gene. (A) Complexes formed with the CDE-D1 motif. Four femtomoles of end-labelled DNA from the wild-type *LPD1* gene (lane 1) was incubated with 0.7 μg of heparin-Sepharose-purified extract (lanes 3 and 4). The same region of DNA containing a mutated CDEI motif (lane 2) was not able to bind protein in the extract (lanes 5 and 6). (B) Confirmation of ABF1 protein binding to the ABF1 motifs. DNA binding mixes contained 4 fmol of DNA and enough protein extract (2 μg) to completely shift the free DNA band representing complete occupancy of one of the two sites. Lane 1 contains DNA alone. Lanes 2 to 9 contain DNA and protein either without competitor (lane 2) or with 2.5 fmol (lane 3), 25 fmol (lane 4), 250 fmol (lane 5), or 2.5 pmol (lanes 6 and 9) of ABF1 competition oligonucleotide. Lane 7 contains 2.5 pmol of the RAP1 competition oligonucleotide, and lane 8 contains an equal amount of the yATF1 competitor DNA. Lanes 10 to 14 show the supershifting of the DNA-ABF1 complex with polyclonal antibody raised against ABF1 (16). Lane 10 contains DNA alone. Lanes 11 to 14 contain 2 μg of protein extract and either no antibody or a 1:1,000 (lane 12), 1:100 (lane 13), or 1:10 (lane 14) dilution of antibody. Preimmune serum does not result in a supershift at any concentration tested. (C) Competition of the yATF1 binding sites with a double-stranded yATF1 oligonucleotide. DNA binding mixes contained 4 fmol of DNA and 2 μg of protein extract except lanes 1 and 9, which contained DNA alone. Lanes 2 to 8 contain increasing amounts of competitor DNA: lane 2, no competitor; lane 3, 0.025 fmol; lane 4, 0.25 fmol; lane 5, 2.5 fmol; lane 6, 25 fmol; lane 7, 250 fmol; lane 8, 2.5 pmol. Lane 10 contains 2.5 pmol of ABF1 competition DNA.

cooperativity. The deletion analysis for lactate-grown cells differed from the results of the site-directed mutagenesis (Fig. 3 and 9). This difference can be traced largely to the yATF1/ABF1 region of overlapping motifs since the yATF1 mutation had a strong effect in both carbon sources, but the deletion analysis (which removed both yATF1 and ABF1) does not show such a large change with lactate-grown cells. Since the phosphorylation state and activity of ABF1 is carbon source dependent (53), the results obtained could be explained in terms of the ABF1 repressor modulating activation by yATF1 by competition for binding sites in glucose-grown cells. Other explanations based on interactions between yATF1 and ABF1 in its various phosphorylated forms are possible.

Another possible interaction was indicated by comparing the deletions and site-directed mutations of the ABF1 and RAP1 motifs. The deletion (ΔE) which removed the ABF1 motifs gave an increase in expression of 1.5- and 2-fold in cells grown on glucose and lactate, respectively. However, site-directed mutation of the ABF1 motifs alone led to a 1.5-fold increase in cells grown on lactate but no change in those grown on glucose. The double mutation of the RAP1 and ABF1 motifs led to an increase in expression in both lactate- and glucose-grown cells. Thus, the ABF1 sites appear to be required for repression during fermentation, but both the ABF1 and RAP1 elements are needed for repression of transcription during growth on lactate. Although the trends are the same, the magnitude of the changes between targeted mutations and the corresponding deletion constructs implies that other sequences (possibly the GCR1 motifs) that lie between these two sites may also contribute to the regulatory activity. This hypothesis is supported by reports of ABF1-RAP1 and GCR1-RAP1 cooperativity in upstream activation sequences (8, 51). We have termed the ABF1 dyad at +288 DRS1 and the RAP1 motif at +406 DRS2.

DISCUSSION

There have been numerous reports of heterologous constructs utilizing upstream yeast promoters that are expressed at levels well below that of the wild-type yeast gene. It has been suggested that this reduction in expression is due to the removal a transcriptional activator that resides in the wild-type coding region. In addition, there have been reports of potential motifs for transcription factors within the coding region of yeast genes, but the majority of these have been either neglected or removed when constructing promoter fusions (33). The finding that a deletion of the *LPD1* coding sequence between positions +13 and +673 results in a 6- to 21-fold decrease in the transcription rate of *LPD1*::*lacZ* fusions (65) prompted a detailed search for an *LPD1* DAS.

Initial deletions in this study defined a DAS to within positions +13 and +473. When transformants were grown on a repressing carbon source, a deletion of this region resulted in a greater reduction in gene expression (10-fold) than that resulting from a deletion of the upstream promoter (5.5-fold). Deletion of the downstream region from a construct already lacking the *LPD1* upstream promoter resulted in a further fivefold decrease in expression. In all, this indicates that under repressing conditions, the promoter activity of the downstream region is at least as important as that of the upstream promoter. However, under derepressing conditions, the downstream region appears to play a minor role, similar to the downstream elements of the human gastrin (60) and murine Thy-1 genes (54) which modulate the rate of transcript initiation primarily directed by the upstream promoter.

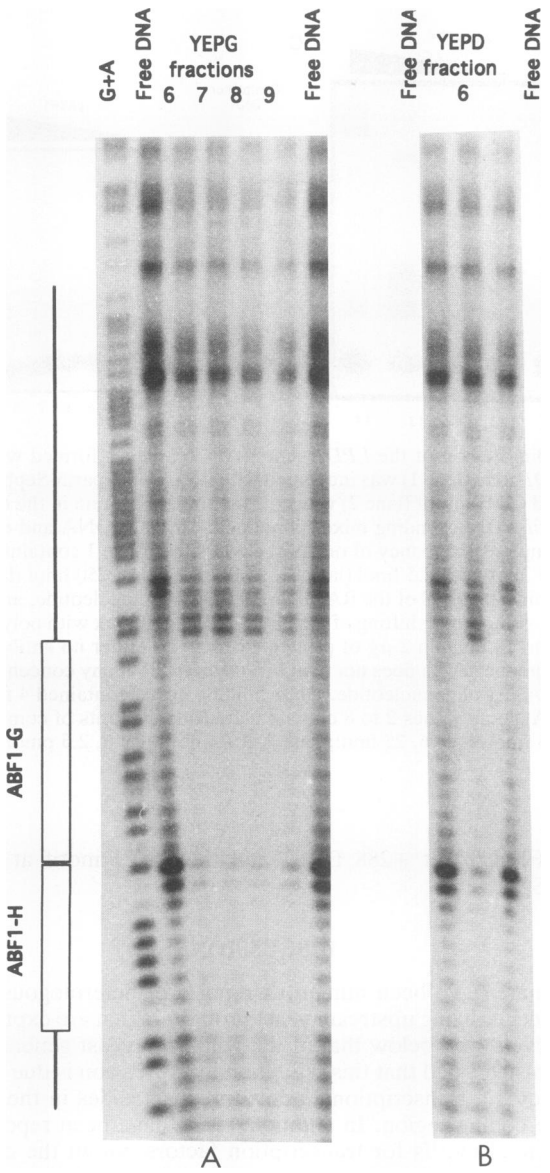

FIG. 8. Binding of ABF1 protein to the downstream region. (A) Cu^{2+}-phenanthroline footprinting analysis of the interaction of heparin-Sepharose-purified extracts with the ABF1 dyad located in the *LPD1* coding sequence. Binding and footprinting reactions were performed as described in Materials and Methods; fraction numbers correspond to the lanes in Fig. 6. The bar represents the location of the two ABF1 binding sites. (B) Footprint resulting from protein prepared from glucose-grown cells.

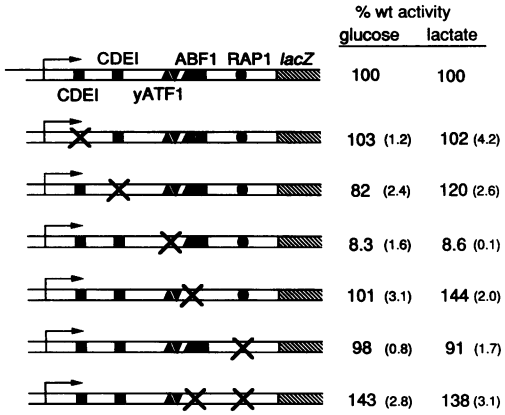

FIG. 9. Site-directed mutagenesis of potential downstream regulatory sites. Crosses represent mutated motifs for CDEI:D1, CDEI:D2, yATF1, ABF1 dyad, and RAP1. Motifs were mutated without alteration of the amino acid sequence where possible or by substituting the amino acid with one of a similar charge. The β-galactosidase activities for cells grown on YEPD (glucose) and YEPLact (lactate) are given as percentages of wild-type activity (± standard deviation).

When the 460-bp downstream region was placed upstream of a *CYC1* basal promoter in either orientation, it activated gene expression between 10- and 111-fold, depending on the carbon source. The region from +13 to +473 thus has the qualities of an enhancer by functioning both outside and inside the coding sequence, in either orientation, while maintaining its carbon source dependence. Similar properties have been described for three DRSs of Ty2-917 which autonomously repress transcription between two- and fourfold when placed upstream of the TATA box (18). These findings imply that downstream regulatory proteins may not be specific for downstream regulation; rather, their action may be determined by specific contexts or accessory factors. This may explain why upstream activation sites have failed to function when blindly inserted into the coding region (58).

The increased activity of the downstream region in an upstream context may have been due to either an increase in the efficiency of an activator element within this region or, conversely, a decrease in the efficiency of a repressor element. Both of these hypotheses are consistent with the finding that the downstream activators of Ty2 are more efficient in the upstream location, whereas repressors act less efficiently (18).

By mutation and deletion analyses, we have shown that the *LPD1* gene contains multiple intragenic regulatory elements and that these elements affect the rate of transcription, thus ruling out mechanisms related to RNA stability or processing. Deletion and site-directed mutation of motifs showed that the yATF1/ABF1 cluster is responsible for carbon source-dependent transcriptional regulation. The ABF1 elements (DRS1) repress transcription by approximately 2-fold, and the overlapping yATF1 element (DAS2) activates transcription rates by 12-fold. Mutation of the RAP1 site (DRS2) demonstrated that the repressor is redundant during basal levels of transcription; however, under elevated levels of expression, the concerted action of both the RAP1 and ABF1 sites is required. Despite a well-characterized association between GCR1 and RAP1 in the upstream region of glycolytic genes (51), gross deletion of the *LPD1* GCR1 sites did not affect transcription rates. However, it is possible that the deletions removed accessory elements or that the GCR1 elements are functionally redundant, as shown for the multiple downstream elements of the adenovirus major late promoter (34).

By using specific antibody, DRS1 has been shown to bind the ABF1 protein in vitro; however, the identities of the proteins which have been shown to bind the other downstream motifs (CDEI, yATF1, and RAP1) are still mostly speculative. Vincent and Struhl (64) have recently proposed that like mammalian ATF/CREB proteins, multiple ATF proteins with different transcriptional activation properties may be expressed by *S. cerevisiae*. Judging by its sequence, the DAS2 element most likely binds yATF1, but another

protein such as SKO1 or ACR1 may also bind and thus compete for binding. Similarly, the DAS1 element, comprising an imperfect CDEI site, may bind a protein other than CPF1 since there is a family of bZIP helix-loop-helix proteins (including CPF1 and PHO4) that bind similar sequences.

The low activity of the yATF1 (DAS2) mutant relative to the corresponding nested deletion construct may be explained by competition for binding to the overlapping DAS2 and DRS1 elements. In the wild-type gene, when yATF1 binds to DAS2, ABF1 is excluded from DRS1. In the case of the DAS2 mutant, ABF1 is free to bind to DRS1, resulting in the substantial reduction in activity of this construct. However, in the corresponding DAS2 deletion construct (ΔH; Fig. 3), both DAS2 and DRS1 elements were deleted. With DRS1 inoperable, a relatively minor twofold reduction in activity due to the deletion of DAS2 was observed. A similarly complex interplay between activator and repressor proteins has been described for the regulation of the bovine papillomavirus P1 gene. In most instances, the bovine papillomavirus E2 product is a potent transactivator; however, in the context of the P1 promoter, the E2 transactivator represses transcription by competing with another transcriptional activator for DNA binding (56).

Although lipoamide dehydrogenase is highly conserved between species, the ABF1 dyad and the yATF1 motifs code for a region of the protein that lies between the αII and αIII helices and which plays little role in protein structure or catalytic activity (24, 27). This region shows low homology across species from bacteria to mammals (2, 10, 13, 44, 48, 57, 63). Thus, the evolution of these sites appears to be directed toward sequences that may be altered without reducing the activity of the resultant protein.

There are a number of mechanisms whereby downstream elements may regulate transcription, by influencing either the rate of transcript initiation or the rate of transcript elongation. A model commonly proposed for DAS regulation of transcript initiation involves the physical contact between the transactivator and the transcriptional apparatus, with looping out of intervening DNA. This would explain the different activities of the *LPD1* DAS between downstream and upstream locations, since its ability to make contact with components of the transcriptional complex would be dictated by its position with respect to the transcriptional machinery. Alternatively, downstream elements may interact with an upstream element to influence the rate of initiation. For example, the *LPD1* DRS sites may interact with an upstream repressor to render the TATA box inaccessible to the transcription apparatus.

Another possible mechanism for transcriptional repression involves inhibition of transcript elongation. In this model, the repressor proteins bind to the DRS and either block the passage of the polymerase or induce negative supercoils in the DNA strand to slow its passage. As would be expected from this model, we have preliminary evidence for the production of a shorter *LPD1* transcript of approximately 300 bp that may be produced by the premature termination of transcription at the DRS (data not shown).

Alternatively, the intragenic elements may influence chromatin structure and thus alter either the binding of proteins to the DNA or the ability of the RNA polymerase to pass along the gene. Recent evidence shows that the majority of the *LPD1* gene maintains regular nucleosome spacing during both basal and derepressed states of activity; however, three regions of the gene remain free of nucleosomes (5). These regions span (i) an upstream cluster of binding sites for ABF1, GCN4, and HAP2, (ii) the DAS2/DRS1 cluster, and (iii) the downstream RAP1 element. This finding suggests that chromatin structure is influenced by the DAS and DRS elements and the RAP1 element; however, the implications for transcriptional activation remain unclear.

The finding of multiple regulatory sites in the coding region of *LPD1* prompted a search of the coding regions of other yeast genes. Of nine yeast tricarboxylic acid cycle genes searched, seven contained potential intragenic regulatory elements of the type found in *LPD1*, though none had the number or complexity of the *LPD1* gene. We therefore predict that many yeast genes possess DAS or DRS elements, many of which have been overlooked by the common practice of removing the majority of coding region when constructing heterologous gene fusions that utilize yeast promoters.

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