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Transcriptional Regulation of the *Schizosaccharomyces pombe* Malic Enzyme Gene, *mae2**

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The NAD-dependent malic enzyme from *Schizosaccharomyces pombe* catalyzes the oxidative decarboxylation of L-malate to pyruvate and CO_2. Transcription of the *S. pombe* malic enzyme gene, *mae2*, was studied to elucidate the regulatory mechanisms involved in the expression of the gene. No evidence for substrate-induced expression of *mae2* was observed in the presence of 0.2% L-malate. However, transcription of *mae2* was induced when cells were grown in high concentrations of glucose or under anaerobic conditions. The increased levels of malic enzyme may provide additional pyruvate or assist in maintaining the redox potential under fermentative conditions. Deletion and mutation analyses of the 5′-flanking region of the *mae2* gene revealed the presence of three novel negative *cis*-acting elements, URS1, URS2, and URS3, that seem to function cooperatively to repress transcription of the *mae2* gene. URS1 and URS2 are also present in the promoter region of the *S. pombe* malate transporter gene, suggesting co-regulation of their expression. Furthermore, two positive *cis*-acting elements in the *mae2* promoter, UAS1 and UAS2, show homology with the DNA recognition sites of the cAMP-dependent transcription factors ADR1, AP-2, and ATF (activating transcription factor)/CREB (cAMP response element binding).

The fission yeast *Schizosaccharomyces pombe* efficiently degrades L-malate to CO_2 under aerobic conditions and to ethanol and CO_2 under anaerobic conditions (1). Cells of *S. pombe* are not able to utilize malate as the sole energy source or incorporate the malate into biomass (2) and therefore require glucose or other carbon sources for the energy-dependent transport and efficient degradation of malic acid (3). Three enzymes are involved in malate degradation in *S. pombe*, namely the malate transporter, malic enzyme, and malate dehydrogenase (4). The transporter, encoded by the *mae1* gene (5), uses an H^+-symport system for the active transport of L-malate, and the NAD-dependent malic enzyme (EC 1.1.1.38) catalyzes the oxidative decarboxylation of L-malate to pyruvate and CO_2. The mitochondrial malate dehydrogenase oxidizes L-malate to oxaloacetate in the tricarboxylic acid cycle and is responsible for 10% of the degradation of malate under aerobic conditions.

Molecular analysis of the *S. pombe* malic *mae2* gene showed a high degree of homology with malic enzymes from various organisms (6). Eight highly conserved regions were identified in malic enzymes, including the binding sites for L-malate and the dinucleotide co-factors $NAD(P)^+$ (7, 8). Although the secondary structure of malic enzymes is highly conserved, the coenzyme specificity (NAD^+ or $NADP^+$) and cellular localization (cytosolic or mitochondrial) are strongly linked to their regulation and metabolic function (9). Cytosolic NADP-dependent malic enzymes play an important role in lipid metabolism in higher eukaryotes (10), whereas NAD-dependent malic enzymes provide mitochondrial NADH for electron transport or cytosolic NADH for reductive power for other metabolic pathways (9).

The degradation of malate in yeasts such as *Candida utilis* and *Hansenula anomala* that can utilize intermediates of the tricarboxylic acid cycle as the only source of carbon was reported to be subject to glucose repression and induction by L-malate (11). However, no substrate induction or glucose repression was observed in species such as *S. pombe*, *Saccharomyces cerevisiae,* and *Zygosaccharomyces bailii* that cannot utilize malate as the only source of carbon. A mitochondrial NADP-dependent malic enzyme was recently cloned from *S. cerevisiae* (12), and a role in the provision of intramitochondrial NADPH or pyruvate under anaerobic conditions was ascribed to the enzyme. DNA sequence analysis of the *S. pombe* malic enzyme gene (6) did not indicate the presence of a mitochondrial targeting signal, suggesting that it functions in the cytosol where it catalyzes the decarboxylation of malate to pyruvate. This could serve to regulate the levels of intracellular malate for its function in shuttle systems with oxaloacetate or aspartate or to provide NADH or pyruvate for other metabolic pathways.

Because of the inability of *S. pombe* cells to utilize L-malate as the only source of carbon and energy, the effective transport and intracellular degradation of L-malate by the yeast is intriguing. Transcription of the malic enzyme gene was therefore studied to elucidate the transcriptional regulation and physiological importance of malate degradation in *S. pombe*. Our results showed increased levels of *mae2* transcription when cells were grown in high concentrations of glucose (8%) or under anaerobic conditions. A detailed analysis of the *mae2* promoter enabled us to identify three novel negative *cis*-acting elements, URS1, URS2, and URS3, as well as two positive *cis*-acting elements that have sequence similarity to the binding sites of cAMP-dependent transcription factors.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Media—All strains and plasmids used in this study are listed in Table I. Yeast strains were cultured in SC^{-leu} medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories, Detroit, MI), 0.5% $(NH_4)_2SO_4$, amino acid supplements, and 2% glucose, unless stated otherwise). Media were supplemented with 0.2% L-malic acid (Sigma) when required.

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TABLE I
Strains and plasmids used in this study
pMV refers to the native *mae2* promoter and gene, and pMZ refers to fusions of the *mae2* promoter and *lacZ* gene.

Strain/plasmid	Description	Reference
Strains		
S. pombe wild type	*S. pombe* 972 h$^-$	
S. pombe leu$^-$	*S. pombe* 972 h$^-$ *leu*1–32	18
S. pombe mae2$^-$	*S. pombe* 972 h$^-$ *leu*1–32 *mae2*$^-$ LH67	18
S. cerevisiae	*S. cerevisiae* N114 MATα *his*3Δ200 *leu*2–3,112 *ura*3–52	
E. coli DH5α	*E. coli* DH5α F′/*endA1 hsdR17*($r_K^- m_K^+$) *supE44 thi1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)$_{U169}$ (*m80lacZ*Δ*M15*)	
Plasmids		
pRS315	*LEU*2, ARS/CEN-based cloning vector with pBluescript cloning sites	39
pMV652	4.6-kilobase *mae2*-ORF and flanking regions cloned in pRS315	6
YEp356	*URA3*, 2 μm-based expression plasmid with cloning sites for *lacZ* fusions	40
pMV46	pMV652 with destroyed *Sal*I-*Xho*I sites in cloning site of pRS315	
pMV/pMZ1	$\Delta >$ nt −1928 (exonuclease deletion upstream of nt −1928)	
pMV/pMZ2	$\Delta >$ nt −1678	
pMV/pMZ3	$\Delta >$ nt −1341	
pMV/pMZ4	$\Delta >$ nt −1265	
pMV/pMZ5	$\Delta >$ nt −1107	
pMV/pMZ6	$\Delta >$ nt −898	
pMV/pMZ7	$\Delta >$ nt −820	
pMV/pMZ8	$\Delta >$ nt −746	
pMV/pMZ9	$\Delta >$ nt −542	
pMV/pMZ10	$\Delta >$ nt −529	
pMV/pMZ11	$\Delta >$ nt −467	
pMV/pMZ12	$\Delta >$ nt −362	
pMV/pMZ13	$\Delta >$ nt −284	
pMV/pMZ14	$\Delta >$ nt −245	
pMV/pMZ15	$\Delta >$ nt −218	
pMV/pMZ16	$\Delta >$ nt −182	
pMV/pMZ17	$\Delta >$ nt −73	
pMV/pMZ18	$\Delta >$ nt −47	
pMV/pMZ47	pMV46 with *Xho*I-*Sal*I deletion	
pMV/pMZ48	pMV46 with *Sph*I-*Sal*I deletion	
pMV/pMZ49	pMV46 with *Sph*I-*Xho*I deletion	
pMV/pMZ64	pMV46 with *Sph*I-*Cla*I deletion	
pMV/pMZ69	pMV46 with *Eco*RII-*Xho*I deletion	
pMV/pMZ71	pMV46 with *Cla*I-*Eco*RII deletion	
pMV/MZ77	pMV46 with *Xho*I-*Ava*II deletion	
pMV/MZ79	pMV46 with *Sal*I-*Ava*II deletion	
pMV/pMZ53	pMV46 with mutated GTTGATTGG at nt −298	
pMV/pMZ54	pMV46 with mutated TCATTCATTT at nt −244 (URS1)	
pMV/pMZ55	pMV46 with mutated AAATTGCGAG at nt −202 (URS2)	
pMV/pMZ59	pMV46 with mutated TTATTTAAAA at nt −88 (TATA)	
pMV/pMZ61	pMV46 with mutated TCATTCATTT and AAATTGCGAG (URS1 + URS2)	
pMZ86	pMZ21 with mutated TGGGCTAAT at nt −186 (URS3)	
pMZ89	pMZ21 with mutated URS1, URS2, and URS3	
pMV/pMZ88	pMV46 with mutated TCCCCTGGCA at nt −359 (UAS1)	
pMZ291	pMZ21 with mutated TGACGT at nt −175 (UAS2)	
pMV/MZ292	pMV46 with mutated AGGGGGA at nt −251	
pMV/MZ293	pMV46 with mutated TGACGA at nt −102	

Escherichia coli cells were grown in Luria broth with 200 mg/liter ampicillin for plasmid selection.

DNA Isolation, Transformation, and Analysis—Standard procedures were used for plasmid isolation and transformation of competent cells of *E. coli* (13), *S. pombe leu*$^-$ (14), or *S. cerevisiae* (15). Standard procedures were used for DNA sequencing (13), and computerized analysis (16) was done to identify putative regulatory elements. For primer extention analysis, primers MV2 and MV3 (Fig. 1 and Table II) were annealed to 50 μg of total RNA and treated with Superscript Moloney murine leukemia virus reverse transcriptase in the presence of [α-^{32}P]dATP (17). The products were loaded on a 6% acrylamide urea gel with DNA sequence ladders obtained with the respective primers.

Malic Enzyme Activity Assays—Wild type and transformed strains of *S. pombe* and *S. cerevisiae* were cultured overnight in 10 ml of SC^{-leu} medium with or without 0.2% L-malate. Crude cell extracts and enzyme assays were done as described by Osothsilp and Subden (18). Enzyme activities are given as μmol of NADH produced/min/mg of protein as determined by Bradford assays (Bio-Rad).

RNA Isolation and Northern Analysis—Total RNA was isolated from wild type *S. pombe* cells grown overnight in SC^{-leu} medium with 1% glucose to A_{600} of 0.8. Cells were harvested and resuspended in fresh medium with 2% glucose or 2% glycerol, ethanol, with one set of glucose cultures sealed with mineral oil and incubated stationary so as to mimic anaerobic conditions. Incubation was continued, and total RNA was isolated (19) after 30, 60, and 90 min, respectively. Samples of 15 μg of total RNA were subjected to electrophoresis on a 0.8% formaldehyde agarose gel (13) and transferred to a Nylon membrane (MSI, Westboro, MA). The 510-bp[1] *Bgl*II-*Eco*RV fragment of the *mae2* gene and 1.2-kilobase *Cla*I fragment of the *S. cerevisiae ACT1* gene served as probes.

Cloning of Unidirectional Deletion Fragments and lacZ Fusions—Exonuclease III deletion fragments of the *mae2* promoter (6), plasmids pMV1 to pMV18 (Table I), were selected to construct *mae2* promoter-*lac*Z fusions. These plasmids were digested with *Pst*I and *Apa*I (Fig. 2) to replace the *mae2* open reading frame (ORF) and 3′-flanking region with a 3.7-kilobase *Pst*I-*Apa*I fragment containing the *lacZ* ORF from YEp356, resulting in *mae2*-promoter *lac*Z fusions (pMZ1 to pMZ18).

β-Galactosidase Assays—Yeast cells transformed with the respective pMZ plasmids were inoculated into 10 ml of SC^{-leu} with 0.2% glucose, 2% glucose, 8% glucose, or 2% raffinose as the carbon source. L-Malate was added at 0.2% when required, and anaerobic conditions were obtained with mineral oil and and incubation without shaking. Cultures were grown to A_{600} of 0.8 to 0.9, and β-galactosidase assays were performed with permeabilized cells (13). Assays were done in duplicate with samples representing 8 μl/ml of original cell culture on at least three transformants from different transformation sets.

[1] The abbreviations used are: bp, base pair(s); ORF, open reading frame; nt, nucleotide; ATF/CREB, activating transcription factor/cAMP response element binding.

TABLE II
Primers used for mutation of putative cis-acting sites in the mae2 promoter
The respective annealing positions of the primers are given in Fig. 1.

Primer	DNA sequence	Application
MV2	5′-TTGGTTCCTGCAGGCAT-3′	Primer extention
MV3	5′-ACTCCTTTTAAAGGACA-3′	Primer extention
MV24	5′-GAGTTAGCATGCAAGGTG-3′	5′ primer with native *Sph*I site
MV25	5′-GTTATTAGGTCACCTTACTCGAGT-3′	Mutated GTTGATTGG in pMV53
MV26	5′-ACTCGAGTAAGGTGACCTAATAAC-3′	Mutated GTTGATTGG in pMV53
MV27	5′-AGCCCAGTCGACCTCGCA-3′	3′ primer with native *Sal*I site
MV28	5′-AGGGGGATGGTCACCTTAAAG-3′	Mutated TCATTCATTT in pMV54
MV29	5′-CTTTAAGGTGACCATCCCCCT-3′	Mutated TCATTCATTT in pMV54
MV30	5′-AGCCCAGTCGACGACCCTAATCGTACGTTC-3′	Mutated AAAATTGCGA in pMV55
MV31	5′-AGCCCTACTTTTGGTGACCTATTT-3′	Mutated TATA in pMV59
MV32	5′-AAATAGGTCACCAAAAGTAGGGCT-3′	Mutated TATA in pMV59
MV40	5′-GCGAGGTCGACTACGTACGTCGTG-3′	Mutated TGGGCTAAT in pMV86
MV41	5′-CCATGGTTACCGGCAACACCC-3′	Mutated TCCCCTGGCA in pMV88
MV42	5′-GGGTGTTGCCGGTAACCATGG-3′	Mutated TCCCCTGGCA in pMV88
MV43	5′-CGAGGTCGACTGGGCTAATCGATCGGTTGGTGGT-3′	Mutated TGACGT in pMV291
MV44	5′-GTCACTTTAAATGAATGATCGATCTTCTC-3′	Mutated AGGGGGA in pMV292
MV45	5′-ACGTGGACCGTCTTACCGGAACAACAGATGAAA-3′	Mutated TGACGA in pMV293

Site-directed Deletions and Mutations—Various deletions within the 480 bp upstream of the *mae2* ORF were constructed with restriction enzyme digestions of pMV46 as indicated in Table I. Restriction enzyme overhangs were filled with dNTPs and Klenow enzyme prior to religation, and standard procedures were used for subsequent cloning (13). Nine putative *cis*-acting elements identified in the *mae2* promoter region (Fig. 1) were mutated using polymerase chain reaction primers to alter the native sequence to a *Bst*EII restriction site (GGTCACC). For example, mutation of the GTTGATTGG sequence at nt −298 was introduced into plasmid pMV53 (Fig. 2) with two sets of primers, primers MV24 and MV26 (yielding fragment A) and primers MV25 and MV27 (fragment B). Both fragments were digested with *Bst*EII and ligated, and the product was amplified with primers MV24 and MV27. The product was digested with *Sph*I and *Sal*I and cloned into the native *Sph*I, *Sal*I sites of pMV46.

In pMV54, the TCATTCATTT sequence at nt −244 was mutated in a similar way with primers MV24 and MV29, and MV28 and MV27 (Fig. 1), and mutation of AAAATTGCGAG at nt −202 in pMV55 was obtained with primers MV24 and MV30. Plasmid pMV61 carries mutations in both the TCATTCATTT and AAAATTGCGAG elements and was constructed using primers MV24 and MV30 with pMV54 as template. The polymerase chain reaction products were digested with *Sph*I and *Sal*I and cloned into the native *Sph*I, *Sal*I sites of pMV46 (Fig. 2). The mutated TGGGCTAAT sequence at nt −186 (pMZ86) was introduced with primers MV40 and MV2, with direct cloning into the *Sal*I, *Pst*I sites of pMZ1. Plasmid pMZ89 contains mutations in the TCATTCATTT, AAAATTGCGAG, and TGGGCTAAT elements and was constructed by subcloning the *Sal*I, *Pst*I fragment from pMZ86 (contains mutated TGGGCTAAT element) into the *Sal*I, *Pst*I sites of pMZ61 (mutated TCATTCATTT and AAAATTGCGAG elements).

In pMV88, the TCCCCTGGCA sequence at nt −297 was mutated with primers MV24 and MV42, and MV41 and MV2, and the putative TATA element at nt −88 in pMV59 with primers MV24 and MV31, and MV32 and MV2. Both were subcloned in the *Sph*I, *Pst*I sites of pMV46. Primers MV24 and MV44 were used for mutation of AGGGGGA at nt −251 in pMV292 (subcloned in the *Sph*I, *Dra*I sites of pMV46); primers MV43 and MV2 for mutation of TGACGT at nt −175 in pMZ291 (subcloned in the *Sal*I, *Pst*I sites of pMZ1); and primers MV45 and MV2 for mutation of TGACG at nt −102 in pMV293 (subcloned in the *Ava*II, *Pst*I sites of pMV46). For all the pMV constructs, the *mae2* ORF was replaced with the *lac*Z ORF as described above. All deletions and mutations were verified with restriction enzyme digestions and sequence analysis (13).

RESULTS

Malic Enzyme Activity and Levels of mae2 Transcription—The *mae2* gene carried on the episomal plasmid pMV652 complemented the *S. pombe mae2*$^-$ mutant in its ability to reduce NAD^+ to NADH via the malic enzyme reaction (Table III). There was no difference in the growth rate of *S. pombe* wild type, *mae2*$^-$ or transformed strains (data not shown) that would indicate that the malic enzyme is essential for growth. When cells of *S. cerevisiae* were transformed with pMV652, no increase in malic enzyme activity was observed relative to the untransformed strain. β-Galactosidase assays on *S. cerevisiae* cells transformed with pMZ1 showed only 4% activity relative to *S. pombe* (data not shown), suggesting that the *mae2* promoter is not recognized by *S. cerevisiae*.

No evidence for an increase in either the malic enzyme activity or *mae2* transcription was observed for *S. pombe* cells grown in the presence of 0.2% L-malate (Fig. 3). However, β-galactosidase assays done with pMZ1 (Table IV) showed transcription levels of 122 and 187% for cells grown in 2% glucose and 8% glucose, respectively, relative to the 100% for cells grown in 0.2% glucose and 105% in 2% raffinose. Furthermore, the levels of transcription were increased more than 5-fold when grown under anaerobic conditions in 2% glucose. This was confirmed with Northern analysis (Fig. 4) on the wild type strain; a small increase was observed when cells were shifted to 2% glucose, with a stronger response under anaerobic conditions.

Sequence Analysis of the mae2 Promoter—The DNA sequence of the region upstream of the *mae2* ORF is available in the GenBank™ and EMBL data bases (accession number SPCC794). Computerized analysis (16) of the *mae2* promoter indicated a putative TATA element (TTATTTAAAA) at nt −88 in the *mae2* promoter (Fig. 1). A predicted CAP signal (AC<u>A</u>GTAAT) corresponding to the putative TATA element was identified at nt −51, and primer extension reactions (data not shown) confirmed that it serves as the transcription initiation site. Comparative analysis of the 5′-flanking regions of the *S. pombe mae2* and malate transporter (*mae1*) genes,[2] revealed three conserved elements: GTTGATT at nt −298 in *mae2* (GTTTGATT at nt −292 in *mae1*), a direct repeat TCATTCATTT at nt −244 (TCATTCATTT at nt −227 in *mae1*), and AAATTGCGAG at nt −202 (AAATTGCTAG at nt −152 in *mae1*).

Putative binding sites for several eukaryotic activators were identified in the *mae2* promoter (Fig. 1). The conserved CCAAT sequence that serves as binding site for the CCAAT-binding protein (20) is present in the reverse orientation at nt −397 and −294. Recognition sites for the TGGCA-binding protein (21), a member of the ubiquitous eukaryotic family of NF1-like nuclear proteins that are involved in transcriptional regulation (22), are present at nt −395 and nt −354. Furthermore, the TGGGCTAAT sequence at nt −186 shows strong homology with the conserved (T/G)(G/A)GGCG(T/G)(A/G)(A/G)(T/C) se-

[2] J. Grobler and H. J. J. van Vuuren, unpublished observation.

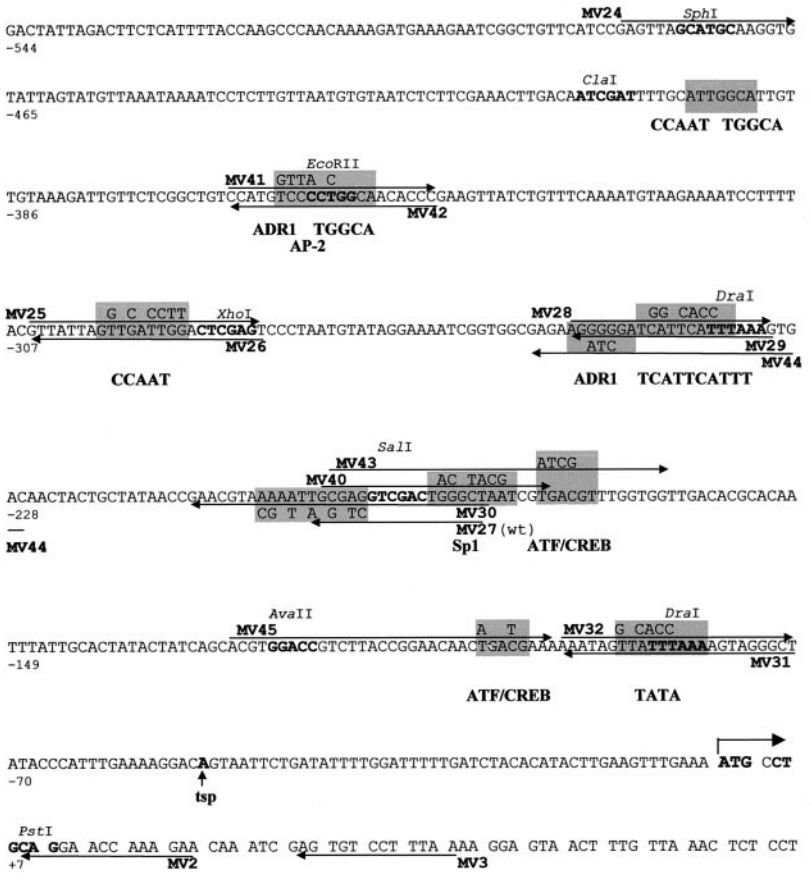

FIG. 1. **DNA sequence of the 544 bp upstream of the *mae2* gene.** Putative regulatory elements indicated by *shaded blocks* include the recognition sites for the TGGCA-binding protein at nt −394 and −354, CCAAT-binding protein at nt −397 and −294, ADR1 at nt −358 and −250, AP-2 at nt −358, Sp1 at nt −186 ATF/CREB at nt −175 and −102, and TATA element at nt −88. The annealing positions of primers used for mutagenesis of putative elements are indicated with *arrows*, and the altered sequences are shown above or underneath the nucleotide sequence. Restriction enzyme sites are indicated in *bold letters* as are transcription initiation (*tsp*) and translation initiation (ATG) sites.

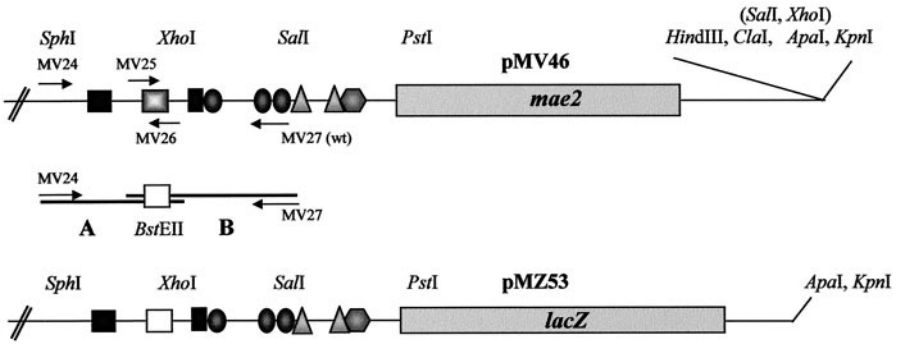

FIG. 2. **Introduction of the mutated CCAAT-like element in pMZ53.** Plasmid pMV46 contains the *mae2* gene cloned in the *Sac*I, *Hin*dIII sites of pRS315 (*Sal*I-*Xho*I sites destroyed with restriction digest). Primers MV24 and MV26 were used to yield fragment A (nt −483 to −282) and primers MV25 and MV27 for fragment B (nt −305 to −180). Both fragments were digested with *Bst*EII and ligated, and the product was amplified with primers MV24 and MV27. The product was digested with *Sph*I and *Sal*I and cloned in the native *Sph*I, *Sal*I sites of pMV46. The *mae2* ORF was replaced by the *lac*Z ORF from YEp356 and flanking regions as a *Pst*I-*Apa*I fragment.

TABLE III

Malic enzyme activity in wild type and transformed yeast strains

Yeast cells were cultured overnight in SC^{-leu} medium with or without 0.2% L-malate, and crude cell extracts were used for malic enzyme assays (18). Activities are given as μmol of NADH produced/min/mg of protein. ND, not determined.

Strain/plasmid	−malate	+malate
S. pombe wild type	3.54	2.96
S. pombe mae2$^-$ mutant	0.40	ND
S. pombe mae2$^-$ mutant + pMV652 (*mae2*)	6.32	6.16
S. cerevisiae N114	0.14	ND
S. cerevisiae N114 + pMV652 (*mae2*)	0.21	ND

quence reported for the binding of the mammalian transcription activator Sp1 (23).

The putative *cis*-acting elements that were identified based on homology with the recognition sites of cAMP-dependent transcription factors include the conserved GG(A/G)G core sequence for the yeast transcription factor ADR1 (24) at nt −251 and inverted at nt −359. The ADR1 binding site was first identified in the upstream-activating sequence UAS1 of the *S. cerevisiae ADH2* gene, where it functions as a *cis*-acting element involved in glucose repression (24). The ADR1 protein (adr1p) is inactivated through phosphorylation by a cAMP-dependent kinase (25), therefore rendering the activity of adr1p sensitive to the levels of glucose. More recently, adr1p was also reported to be involved in derepression of the *S. cerevisiae* acetyl-CoA synthetase gene in response to sugar limitation (26).

The putative ADR1 binding site at nt −359 overlaps with the CCCMNSSS recognition sequence reported for the mammalian transcription factor AP-2 that is involved in cAMP-induced expression of the human metallothionein IIA protein (27). Fur-

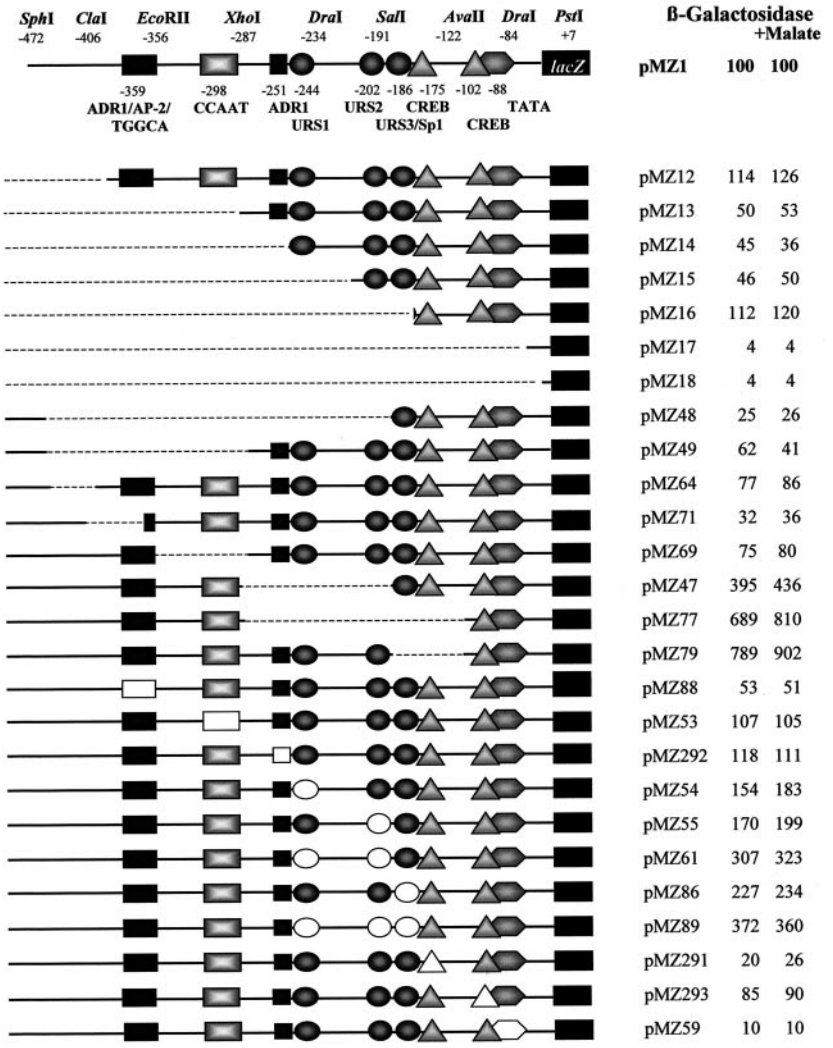

FIG. 3. **Deletion and mutational analysis of the *S. pombe mae2* promoter region.** Yeast transformants were cultured in SC^{-leu} medium with or without 0.2% malate. Assays were done in duplicate on permeabilized cells, and results are given in percentage relative to that obtained for pMZ1.

TABLE IV
β-Galactosidase values for S. pombe 603 transformed with pMZ1 and cultured in various carbon sources

Yeast transformants were cultured in SC^{-leu} medium with the carbon sources as indicated. Anaerobic conditions were obtained with mineral oil. Assays were done in duplicate on permeabilized cells (13), and results are given in percentage relative to that obtained for growth in 0.2% glucose.

Glucose				Raffinose
0.2%	2%	8%	2% Anaerobic	2%
100	122	187	554	105

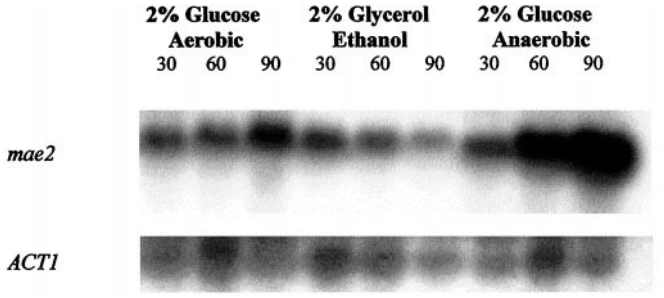

FIG. 4. **Northern analysis of *mae2* expression.** Cells were grown overnight in 1% glucose and shifted to fresh 2% glucose, either aerobic or anaerobic, or 2% glycerol, ethanol medium. Total RNA was probed with the 510-bp *Bgl*II-*Eco*RV fragment of the *mae2* gene or the 1.2-kilobase *Cla*I fragment of the *S. cerevisiae ACT1* gene.

thermore, the TGACGT sequence at nt −175 and the TGACGA sequence at nt −102 resemble the binding site for the mammalian activating transcription factor ATF/CREB (cAMP response element binding) (28). ATF/CREB is activated through phosphorylation by the cAMP-dependent kinase A (29) and was reported to bind as a dimer to the GTGACGTACAG consensus sequence (30).

Deletion and Mutation Analysis of Putative Regulatory Elements in mae2 Promoter—Eighteen unidirectional deletion fragments of the *mae2* promoter were fused in-frame with the *lac*Z ORF (pMZ1 to pMZ18 in Table I) to evaluate their ability to support transcription. Transcription from plasmids pMZ1 to pMZ12 were unaffected as determined by β-galactosidase assays (only the results for pMZ12 are shown in Fig. 3). Removal of the sequences upstream of nt −218 decreased the activity by more than 50% (pMZ15), whereas deletion of the adjacent 36 nucleotides in pMZ16 restored the wild type levels of activity. Deletion of the sequences upstream of nt −73 in pMZ17 removed the putative TATA element at nt −88 and reduced transcription to only 4%. Smaller deletions within the *Sph*I-*Ava*II region (nt −472 to −122) showed decreased transcription, especially for pMZ48 and pMZ71. In contrast, deletions within the *Xho*I-*Ava*II region increased transcription 4-fold for pMZ47, 7- to 8-fold for pMZ77, and 8- to 9-fold for pMZ79. This suggested the presence of positive regulatory sequences between nt −362 and −284 and negative regulatory sequences between nt −218 and −182.

Site-directed mutations of nine putative *cis*-acting elements

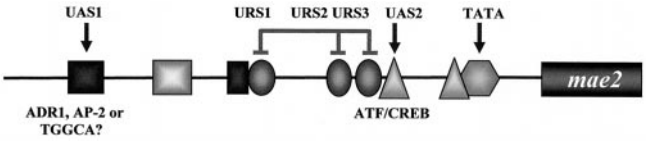

FIG. 5. **Proposed model for the regulated expression of the *S. pombe* malic enzyme gene.** UAS1 and UAS2 serve as activator sites and have homology with the binding sites for eukaryotic transcription-activating factors as indicated. The novel URS1, URS2, and URS3 elements act cooperatively to repress transcription of the *mae2* gene. The putative TATA element indicated is required for wild type levels of expression.

were introduced with polymerase chain reaction primers (Fig. 1). Three of these elements were identified as conserved elements in the *mae1* and *mae2* promoters, whereas the other elements were identified based on homology with recognition binding sites for eukaryotic regulatory proteins. Mutation of the putative TATA element at nt −88 (pMZ59) decreased transcription to only 10%, confirming an essential function in transcription of the *mae2* gene. Mutation of the CCAAT-like element at nt −298 (pMZ53), ADR1 at nt −251 (pMZ292), or ATF/CREB at nt −102 (pMZ293) did not affect transcription significantly (Fig. 3). However, mutation of TCCCCTGGCA at nt −359 (homology with the ADR1, AP-2, and TGGCA binding sites) in pMZ88 and TGACGT at nt −175 (homology with ATF/CREB binding site) in pMZ291 resulted in only 53 and 20% of wild type levels, respectively. These elements therefore seem to serve as upstream activating sequences that regulate the expression of the *mae2* gene and are designated UAS1 and UAS2, respectively, in further discussions (Fig. 5).

Mutation of TCATTCATT at nt −244 (pMZ54), AAATTGCGAG at nt −202 (pMZ55), and TGGGCTAAT at nt −186 (pMZ86) increased transcription to 154, 170, and 224%, respectively (Fig. 3). These elements seem to function as upstream repressor sequences and were designated URS1, URS2, and URS3, respectively (Fig. 5). Furthermore, pMZ61 (mutated URS1 and URS2) and pMZ89 (mutated URS1, URS2, and URS3) showed increased levels of transcription of approximately 3- and 4-fold, respectively, suggesting that URS1, URS2, and URS3 may confer a cooperative repression of *mae2* expression.

DISCUSSION

Transcription of *S. pombe* class II promoters is typical of higher eukaryotes where TFIID is the general transcription factor that binds to the TATA box and initiates the assembly of the transcription complex (31). However, the promoter structure of *S. pombe* genes in general differ from that of *S. cerevisiae* and in some cases more closely resembles that of mammalian promoters (32). Our results showed that mutation of the putative TATA element 37 bp upstream of the transcription initiation site reduced transcription to only 10% (pMZ59, Fig. 3). The distance between the putative TATA box and the initiation start site is typical for *S. pombe* (32) and within the 35 to 180 nt reported for *S. cerevisiae* (33). However, the *S. pombe mae2* promoter was not functional when expressed in *S. cerevisiae* as measured by malic enzyme activity and β-galactosidase assays. This lack of promoter function could be because of the inability of the *S. cerevisiae* transcription complex to recognize the *mae2* promoter structure or the absence of essential transcription factors.

The *S. pombe* malic enzyme has a high substrate affinity (K_m of 3.2 mM) (34) and decarboxylates L-malate to pyruvate and CO_2 with the concurrent reduction of NAD^+ to NADH. Under anaerobic conditions, the pyruvate is further metabolized to ethanol and CO_2 with the re-oxidation of NADH to NAD^+. The degradation of L-malate in *S. pombe* was reported not to be subject to substrate induction or glucose repression (35). Our results confirmed that expression of *mae2* was not affected by 0.2% L-malate (Fig. 3), but induced levels of *mae2* transcription were obtained when grown in 8% glucose or under anaerobic conditions (Table IV). A 2- to 3-fold increase in transcription under anaerobic conditions was reported for the *S. cerevisiae* malic enzyme gene (12), and a role in the provision of intramitochondrial NADPH or pyruvate under anaerobic conditions was proposed for the NADP-dependent enzyme. Unlike the *S. cerevisiae* malic enzyme, the DNA sequence of the *mae2* gene (6) did not indicate the presence of a mitochondrial signal sequence, suggesting that the enzyme is located in the cytosol.

Little information is available on the regulation of enzymes involved in carbon metabolism in *S. pombe* cells. Expression of the *S. pombe fbp1* gene, encoding fructose 1,6-bisphosphatase, was reported to be repressed in 8% glucose (36) because of regulation by a glucose-induced cAMP signal (37). The increased expression of *mae2* observed in high concentrations of glucose (and therefore high cAMP levels) suggested that cAMP induction may be involved in this response. We have found two positive *cis*-acting elements, UAS1 and UAS2, that show homology with binding sites for cAMP-dependent regulatory proteins. UAS1 contains overlapping recognition sites for the transcriptional activator proteins ADR1, AP-2, and TGGCA-binding protein (Fig. 5), whereas UAS2 has homology with the transcriptional activator ATF/CREB. ADR1 is inactivated by a cAMP-dependent kinase, whereas the activity of AP-2 and ATF/CREB are induced in the presence of cAMP (24–30). Mutation of UAS1 (pMZ88) and UAS2 (pMZ291) decreased the levels of transcription to 53 and 20%, respectively (Fig. 3). It would be of interest to determine whether these mutations also affect the induced expression of *mae2* under fermentative conditions.

Deletion and mutation analysis of the *mae2* promoter indicated the presence of three negative-acting elements, URS1, URS2, and URS3. Mutation of URS3 (pMZ86) had a stronger effect than that of URS1 (pMZ54) or URS2 (pMZ55). However, mutation of either URS1 plus URS2 (pMZ61) or all three elements (pMZ89) resulted in higher levels of transcription than for any one of the individual elements, suggesting that they function cooperatively to repress transcription of the *mae2* gene (Fig. 5). Homologous copies of the URS1 and URS2 elements are also present in the promoter of the *S. pombe* malate transporter gene, *mae1,* indicating possible co-regulation of enzymes involved in malate degradation in *S. pombe*. Further analysis of these elements and the regulatory proteins that bind them is essential to elucidate the transcriptional regulation of the *mae2* gene and perhaps also that of the *mae1* gene.

Because cells of *S. pombe* do not utilize L-malate as the only source of carbon or energy, the physiological importance of the active transport and strong intracellular degradation is intriguing. The oxidative decarboxylation of malate coincides with the reduction of NAD^+ to NADH, suggesting that the malic enzyme may play an important role in maintaining the redox balance under aerobic conditions. Under anaerobic conditions, however, the pyruvate produced during this reaction is further metabolized to ethanol with the concomitant oxidation of NADH to NAD^+. Because there is no net gain or loss in NADH during the conversion of malate to ethanol, the increased expression of the malic enzyme under fermentative conditions should not affect the redox balance.

The *S. pombe* cells may use the malic enzyme to provide pyruvate for essential anaplerotic reactions under fermentative conditions. Pyruvate plays an essential role in the provision of α-ketoglutarate and oxaloacetate for the biosynthesis of amino acids and nucleotides. Both these precursors are synthe-

sized in the mitochondria and transported to the cytosol for biosynthetic reactions; therefore alternative pathways have to be utilized for the synthesis of these precursors when the mitochondria are not functional (38). These anaplerotic reactions include the carboxylation of pyruvate to oxaloacetate via pyruvate carboxylase, the oxidation of malate to pyruvate via the malic enzyme, and the production of succinate via the glyoxylate cycle. The induced expression of the *S. pombe* malic enzyme under fermentative conditions may therefore serve as an important auxiliary pathway for the production of pyruvate for other metabolic requirements.

To our knowledge, this is the first report on the glucose-induced expression of a malic enzyme gene in yeast. Results presented here indicate the differential expression of the *S. pombe mae2* gene under fermentative conditions, *i.e.* 8% glucose and anaerobic conditions. We therefore propose that the NAD-dependent malic enzyme from *S. pombe* may provide cytosolic pyruvate for anaplerotic pathways under fermentative conditions. A function in the provision of NADH for reductive biosynthesis and maintenance of the redox balance is, however, not excluded. Further research is required to elucidate the physiological role of the malic enzyme in *S. pombe* and the importance of the various UASs and URSs in the regulated expression of the *mae2* gene.

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