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Two Types of TATA Elements for the *CYC1* Gene of the Yeast *Saccharomyces cerevisiae*

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Functional TATA elements in the 5′ untranslated region of the *CYC1* gene in the yeast *Saccharomyces cerevisiae* have been defined by transcriptional analysis of site-directed mutations. Five sites previously suggested to contain functional TATA elements were altered individually and in all possible combinations. The results indicated that only two elements are required for transcription at the normal level and the normal start sites. The two functional TATA elements are located at sites −178 and −123, where the A of the ATG start codon is assigned nucleotide position +1. They direct initiation within windows encompassing −70 to −46 and −46 to −28, respectively. Only when both of the upstream TATA sites were rendered nonfunctional were the third and fourth downstream TATA-like sequences activated, as indicated by the presence of low levels of transcription starting at −28. The two upstream functional TATA elements differed in sequence. The sequence of the most 5′ one at site 1, denoted β-type, was ATATATATAT, whereas that of the second one at site 2, denoted α-type, was TATATAAAA. The following rearrangements of the β-type and α-type elements at two sites (1 and 2) were examined: site1β-site2α; site1α-site2β; site1α-site2α; and site1β-site2β. When different types were at different sites (site1β-site2α and site1α-site2β), both were used equally. In contrast, when the same type was present at both sites (site1α-site2α and site1β-site2β), only the upstream element was used. We suggest that the two TATA elements are recognized by different factors of the transcription apparatus.

The organization of the functional elements of RNA polymerase II promoters in the yeast *Saccharomyces cerevisiae* is similar to that of higher eucaryotes. In general, yeast promoters have several basic regulatory sequences. These include TATA elements, upstream activation sequence (UAS) elements, and initiators (for review, see references 11, 12, 37, 38). Because of the nature of the A+T-rich 5′ noncoding region, several TATA-like sequences usually can be found in a yeast gene. In addition, unlike a single mRNA initiation site, as is usually seen in higher eucaryotic genes, multiple mRNA initiation sites, sometimes spanning a 100-bp or longer region, can be found in some yeast genes.

Studies of promoter mutations have revealed several features of yeast TATA elements. First, yeast promoters occasionally have redundant functional TATA elements. However, it is clear that not all putative TATA-like sequences are functional in normal cells (9, 17, 24, 26; this study). Second, a yeast TATA element controls transcription initiation in a window between 40 and 120 bp downstream. However, the width of each window is different in different genes, even though some genes have the same TATA sequences. These results indicate that the sequences in the vicinity of TATA elements are also important for controlling transcription initiation. Because the TATA element controls transcription in a window, the common phenomenon of multiple start sites can be explained by more than one initiator in the window creating more than one start site. Third, TATA elements are essential for transcriptional initiation of many yeast genes (see, as examples, references 28 and 35). Fourth, TATA elements are binding sites for the transcription factors. Recently, a yeast TATA-binding protein (yeast TFIID) has been purified and cloned (3, 4, 15, 19, 20, 30). The yeast TATA-binding protein can specifically bind to TATA boxes of both yeast and mammalian genes and can substitute for the mammalian TFIID factor in a reconstituted in vitro transcription system (3, 4). Functional studies showed that this gene, *SPT15*, was essential for growth and that *spt15* mutants had altered transcription initiation in vivo (6). Recent studies also suggest the possible existence of multiple TATA-binding proteins (5). Fifth, several lines of evidence have shown that different sequences of TATA elements have distinct functions. In higher eucaryotes, the TATA box of the *hsp70* promoter contains a TATA box with the sequence TATAAA. It is capable of induction by both heat shock and E1A factor (a positive transcription regulation protein). However, when the normal *hsp70* promoter TATA box sequence TATAAA is replaced by the simian virus 40 (SV40) TATA box sequence TATT TAT, heat shock response still occurs but E1A response is lost (34). Another study also revealed a different response to the muscle-specific enhancer (MSE); the myoglobin TATA-box TATAAAA can respond to the MSE but the SV40 TATA-box TATTTAT cannot (41).

In yeast cells, studies of the *HIS3* gene have also led to the idea that there are two kinds of TATA elements, constitutive (T_C) for basal-level expression and regulatory (T_R) for induction of this gene under amino acid starvation (36). Further studies indicated that different T_R sequences had distinct functions in responding to the activator proteins GCN4 and GAL4 (18). One interesting observation is that not all redundant TATA elements are functional in a single promoter; in a construction containing identically duplicated TATA elements in the *HIS4* gene, only the upstream TATA element was utilized (26). In fact, no promoter containing two identical TATA elements has been identified as yet.

The promoter of the *CYC1* gene, encoding iso-1-cytochrome *c*, exhibits most of the features discussed above. Transcription of the *CYC1* gene is regulated by oxygen,

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TABLE 1. List of the oligonucleotides

Strand[a]	Purpose	Oligonucleotide	Gene	Sequence[b]
Coding	In vitro mutagenesis	OL87-77	*CYC1*	(−185)TACAGGCATA**CGCG**TATGTGTGCG(−162)
		OL87-81	*CYC1*	(−131)TCTGTATGTA**GCGC**AAACTCTTGT(−108)
		OL87-75	*CYC1*	(−101)CTTTTCTCTA**GGCC**TTCTTTCCTT(−78)
		OL87-76	*CYC1*	(−87)TTCTTTCCTT**GCG**CATTAGGTCC(−65)
		OL87-82	*CYC1*	(−64)TTTGTAGCAT**CCGC**TACTATACTT(−41)
		OL88-78	*CYC1*	(−185)TACAGGCATATATA**AAA**GTGTGCGACG(−159)
		OL89-79	*CYC1*	(−185)TACAGGCATATATATATGTGTGCG(−162)
		OL88-77	*CYC1*	(−131)TCTGTATGTATATA**TATA**TCTTGTTTTC(−104)
		OL88-56	*CYC1*	(−131)TCTGTATGTATATAAAACTCTTGT(−108)
	Sequencing of mutations	OL87-78	*CYC1*	(−211)GGCCAGGCAACTTTAGTGC(−193)
		OL87-80	*CYC1*	(−144)GGCATGCATGTGCTCTG(−128)
Anticoding	Northern blot hybridization	OL88-67	*CYC1*	(+80)TCCACGGTGTGGCATTGTAGACATCTAGTCTTGA(+47)
		OL88-68	*ACT1*	(+426)TCTTGGTCTACCGACGATAGATGGGAAGACAGCA(+393)
	Primer extension	OL88-193	*CYC1*	(+48)GAAAAGTGTAGCACCTTTCTTAGCAGAACCGGCC(+15)

[a] Strand of duplex DNA. The coding strand refers to the strand having the same sequence as the mRNA, and the anticoding strand is the template for transcription of mRNA.

[b] Oligonucleotide sequences are given from the 5′ to 3′ direction. The numbers in parentheses at both ends are the corresponding nucleotide positions of the gene. Altered bases are underlined and boldfaced.

heme, and carbon source via the *CYC1* UAS elements (13, 14, 22). The wild-type *CYC1* mRNA initiates at five major sites, −70, −62, −57, −46, and −38 (where the A of the ATG translation initiation codon is numbered +1). Some of the initiation sites appear as clusters of several adjacent nucleotides. There are as many as five different TATA-like sequences at nucleotides −178, −123, −93, −78, and −56. These TATA-like sequences are close to or within the mRNA initiation region. Early studies on defining functional *CYC1* TATA elements by using promoter deletions led to the conclusion that at least the most upstream three, or as many as five, of the TATA-like sequences are functional in a normal cell (7, 17, 24). However, it is known that large deletions bring new DNA sequences into the region of study and alter the spatial relationship between *cis* functional elements.

In order to precisely define the number of functional TATA elements of the *CYC1* gene, we have made substitutions of a small number of bases in the middle of the putative TATA elements. This strategy results in minimal alteration of DNA sequences in the promoter region and ensures that the distances between the TATA, the UAS, and the transcription initiation sites do not vary. The effects of these alterations on transcription initiation have been studied in vivo. Our results indicate that only two of the most upstream TATA elements are required for normal expression of the *CYC1* gene. They are the β-type, ATATATATAT, at −178 and the α-type, TATATAAAA, at −123. In order to function simultaneously, these two TATA elements have to be of different types. If both locations contain the same type of TATA sequence, only the upstream TATA element is functional. This finding suggests the existence of two or more sequence-specific regulatory proteins.

MATERIALS AND METHODS

Genetic nomenclature. The wild-type allele encoding iso-1-cytochrome *c* is denoted *CYC1* or *CYC1*$^+$. Mutant alleles that produce either normal or decreased levels of *CYC1* mRNA are designated *cyc1*, followed by the allele number, e.g., *cyc1-918*, *cyc1-919*.

Plasmids. Plasmid pAB458 (8), used for in vitro site-directed mutagenesis, has the Amp^r marker for bacterial selection, the f1(IG) sequence for preparation of plasmid single-stranded DNA (ssDNA), and the yeast *URA3*$^+$ gene for yeast transformation selection. The mutation-containing plasmids derived from pAB458 are designated pAB, followed by the mutation number.

Oligonucleotides. The oligonucleotides used in this study (Table 1) were synthesized with an Applied Biosystems Synthesizer model 380A, and the amine-protecting group was removed according to a procedure suggested by the manufacturer. The oligonucleotides were further manipulated according to how they were to be used. The oligonucleotides were used for in vitro mutagenesis, mutational screening, and DNA sequencing without further purification. The oligonucleotides used for Northern (RNA) blot hybridization and primer extension analysis were further purified in a sequencing-sized gel containing 15% polyacrylamide and 7 M urea. Oligonucleotides of the correct size were cut out of the gel, eluted in 0.5 M ammonium acetate–0.01 M magnesium acetate buffer, precipitated in 70% ethanol, and resuspended in distilled water. For Northern blot hybridization, the oligonucleotides were 5′ end labeled with [γ-^{32}P]ATP. For the primer extension reaction, the oligonucleotides were 5′ end labeled with [γ-^{35}S]ATP (Amersham SJ318) by a modified phosphorylation reaction procedure. A typical reaction was carried out in a 20-μl volume containing 30 pmol of oligonucleotides, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 100 μCi of [γ-^{35}S]ATP, and 20 to 40 U of T4 polynucleotide kinase. The mixture was incubated at 37°C for 2 to 3 h, and the reaction was terminated by adding 1 μl of 0.5 M EDTA. The labeled oligonucleotides were then purified with a Sephadex G-25 spin column (23), ethanol precipitated, and resuspended in 20 μl of distilled water.

In vitro mutagenesis and mutation screening. Oligonucleotide-directed in vitro mutagenesis was performed as described by Kunkel et al. (21) with minor modifications. Multiple oligonucleotide primers were used in the reactions in order to generate different combinations of mutations at

several sites. Because of the overlapping sequences of OL87-75, for mutating site 3, and OL87-76, for mutating site 4, both oligonucleotides were not used in the same multiple-primer reaction. Single-stranded uracil-containing plasmid DNA was isolated from *Escherichia coli* CJ236 [*dut-1 ung-1 thi-1 relA*(pCJ105[Cm^r])] (21) in the presence of helper phage R408 (29). To select multiple mutations from one multiple-primer reaction, a highly efficient screening method was used in which competent JM103 was directly transformed with the in vitro-synthesized heteroduplex plasmid DNA. A total of 30 individual transformants were subcloned, and a single colony from each subclone was inoculated onto an LB-Amp (LB medium with ampicillin [25]) master plate in a pattern corresponding to the rod locations of a 32-rod inoculator. A 32-well immunological tray was filled with 0.5 ml of 10^{-4}-diluted R408 phage stock in each well, and cell suspensions were made by transferring the cells from the master plate to each corresponding well of the immunological tray with a sterilized 32-rod inoculator. The cell suspensions were then inoculated onto several LB-Amp plates with the same inoculator. The plates were then incubated at 37°C overnight until each cell spot was completely lysed. A Colony/Plaque Screen (New England Nuclear) hybridization filter disk was laid onto the surface of each plate and soaked for 5 min before being carefully peeled off. The filter was submerged in 0.1 N NaOH–1.5 M NaCl solution for 30 s, neutralized in 0.2 M Tris-HCl (pH 7.5)–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 s, and then air dried at room temperature. Subsequently, differential hybridization screening was performed (44). All the oligonucleotides that were used in the in vitro DNA synthesis were individually labeled and hybridized to separate filters. The type of mutation exhibited by each individual colony was determined by comparing the autoradiographs of the filters. Putative mutations were sequenced by standard methods (29a).

Construction of yeast strains by gene replacement. The standard culture and genetic procedures have been described previously (32). The yeast strain B-7056 (also designated S260-11B; *MATα cyc1::CYH2^s cyc7-67 ura3-52 leu2-3 leu2-112 cyh2^r* [1]) was transformed with the *CYC1*-containing plasmids to Ura$^+$ as described before (2). The Ura$^+$ phenotype of the transformants indicated that the plasmid had integrated at the chromosomal *CYC1* locus. The Ura$^+$ transformants were then grown on FOA medium (42) to obtain Ura$^-$ colonies, from which the plasmid was excised. FOA medium was used for further subcloning and genotype testing of these Ura$^-$ colonies. A successful gene replacement of *cyc1::CYH2^s* in the chromosome by the *CYC1* or mutant alleles in the plasmid was demonstrated by growth on medium containing cycloheximide (Cyh^r) and a nonfermentable carbon source such as glycerol (Nfs$^+$) and by lack of growth on medium lacking uracil (Ura$^-$). One strain of each type of mutant was selected for further study.

Determination of cytochrome *c* content. The total amount of cytochrome *c* was estimated in intact cells at −196°C with a Hartree low-dispersion spectroscope (31). More accurate determinations of the iso-1-cytochrome *c* content in intact cells were also made by low-temperature (−196°C) spectroscopic recordings with a modified Cary 14 spectrophotometer (33).

Yeast RNA preparation. Yeast strains were grown in 250 ml of the repressing medium YP3%D (1% yeast extract, 2% peptone, 3% glucose) or the derepressing medium YPE. Cells were harvested at about 2×10^7 to 3×10^7/ml, washed in 5 ml of cell washing buffer (0.1 M LiCl, 0.1 M Tris-HCl [pH 7.5], 0.1 mM EDTA), and resuspended in 2 ml of cell lysis buffer (0.1 M LiCl, 0.1 M Tris-HCl [pH 7.5], 0.1 mM EDTA, 0.5% sodium dodecyl sulfate [SDS]). An equal volume of phenol-chloroform-isoamyl alcohol (PCI, 25:24:1) and 2 g of glass beads (0.45 mm) were added, and the mixture was vortexed three to four times for 15-s intervals, with cooling on ice between each interval. The lysate was centrifuged at 10,000 × *g* for 10 min, and the supernatant was extracted three times with an equal volume of PCI. The RNA was then precipitated by adding 2.5 volumes of ethanol. The pellet was washed with 70% ethanol and dissolved in an appropriate volume of distilled water, usually 0.2 to 0.4 ml. The concentration of the RNA samples was determined from the A_{260} at a 1:1,000 dilution with an extinction coefficient of 38 μg/ml/OD unit.

Northern blot analysis. The agarose gels for the Northern blot transfer were made with minor modifications of a procedure described by Fourney et al. (10). A 1.5% agarose gel with 0.1 μg of ethidium bromide per ml was used to fractionate RNA. After transfer to nitrocellulose and baking at 80°C in a vacuum oven, the filter was prehybridized at 42°C overnight in 50% formamide–50 mM $NaHPO_4$ (pH 6.5)–5× SSC–1× Denhardt's solution–0.25 mg of sheared and denatured calf thymus DNA per ml (40). ^{32}P-end-labeled oligonucleotide probes were added to the above prehybridization solution to a final concentration of 5×10^5 cpm/ml. Hybridization was performed at room temperature for 16 to 36 h. The filter was then washed two times with 2× SSC–0.1% SDS and two times with 1× SSC–0.1% SDS at room temperature. Autoradiography was performed at −70°C with an intensifying screen.

Quantitative analysis of mRNA. For analysis of *CYC1* mRNA levels, *ACT1* mRNA was used as an internal control (1, 43), since the expression of *ACT1* is not affected by growth with different carbon sources (21a, 39). In addition, a series of *CYC1* mRNA dilutions were made by mixing various amounts of total cellular RNA from a wild-type *CYC1* strain (B-7969) and a *CYC1* deletion strain (B-7056). The total amount of RNA in each dilution was kept at 20 μg so that the amount of *ACT1* mRNA would remain constant. The amount of *CYC1* mRNA was determined as a ratio of the densities of the *CYC1* and *ACT1* mRNA bands. An LKB 2222-010 UltroScan XL laser densitometer was used to determine the relative densities, which were presented by the computer as the relative area of each band on the autoradiograph of the Northern blot. The amount of *CYC1* mRNA in each mutant was determined by reference to the standard dilution curve, with that produced by the normal *CYC1* allele in the derepressed growth condition defined as 100%. Each value shown in Tables 2 and 3 is an average from at least two experiments after values for the lanes with degraded, overloaded, or underloaded RNA samples were discarded.

Primer extension mapping of *CYC1* mRNA initiation sites. A total of 50 to 100 μg of total cellular RNA, 1×10^5 to 5×10^5 cpm of 5′-^{35}S-labeled oligonucleotides, and 2.5 μl of 10× reverse transcriptase buffer (0.5 M Tris-HCl [pH 8.3], 60 mM $MgCl_2$, 0.1 M dithiothreitol, and 1.0 M NaCl) were mixed in a 20-μl volume. Annealing was carried out at 42°C for 2 h. Then, dATP, dCTP, dGTP, and TTP were added to a final concentration of 0.5 mM each, and 25 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals) was added, resulting in a final volume of 25 μl. The cDNA extension was performed by incubating the reaction mixture at 42°C for 90 min. At the end of the incubation, 1 μl of 0.5 M EDTA was added to stop the

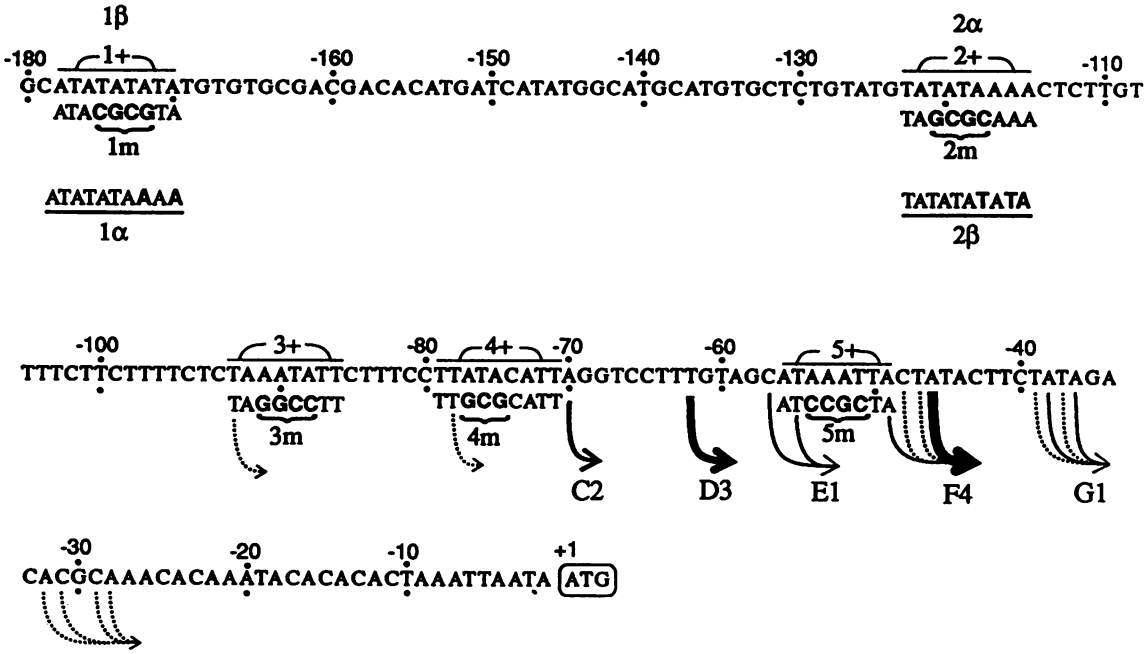

FIG. 1. *CYC1* gene promoter. The A residue of the translation initiation codon ATG is numbered +1. The five wild-type TATA-like sequences are overlined and labeled 1+, 2+, etc., above the lines. The symbols 1β and 2α are equivalent to 1+ and 2+, respectively. The mutant (m) and rearranged types of TATA element (α or β) sequences are shown below the normal sequences. The wild-type mRNA initiation sites (A to H) are shown by arrows under the DNA sequence. The thickness of each arrow denotes the abundance of initiation at that site; the dotted arrows represent very weak initiation sites that are discussed in the text but not presented in Table 2.

reaction. A total of 1 μl of 1-mg/ml RNase A was added, and the mixture was digested at 37°C for 30 min. A total of 100 μl of 2.5 M ammonium acetate was added, and the mixture was extracted once with an equal volume of PCI. Then 2.5 volumes of ethanol were added to the upper aqueous phase to precipitate the cDNA. The pellet was resuspended in 4 μl of TE (pH 8.0)–5 μl of formamide loading dye. The samples were then heated at 90°C for 2 min and cooled to 0°C immediately, and 4 μl was loaded on an 8% acrylamide sequencing gel according to the standard method (23).

RESULTS

Construction of mutations of TATA-like sequences in the *CYC1* gene. Five TATA-like sequences of the *CYC1* gene were inferred to be functional (24) and are located at −178 (ATATATATAT), −123 (TATATAAAA), −93 (TAAAT ATT), −78 (TATACAT), and −56 (ATAAATTA) from the ATG translation initiation codon. (These sites are referred to as sites 1, 2, 3, 4, and 5, respectively [Fig. 1]; normal and mutated sequences are denoted, for example, site 1+ and site 1m, respectively.) To avoid changes in distance between elements or the introduction of foreign sequences, such as occurs with large deletions, site-directed mutagenesis in vitro was used to produce a limited number of base-pair changes at each TATA-like sequence. To ensure elimination of the function of each TATA element, three to four base-pair changes of A or T to G or C were altered at each TATA-like sequence (Fig. 1). To examine the function of the five elements, all 32 possible combinations of alterations of the five TATA-like sequences were examined, i.e., either wild type or mutated sequence. Site-directed mutagenesis was carried out with as many as four primers in order to efficiently generate the desired 31 alterations. The high efficiency of the mutation hybridization screening method described in the Materials and Methods section allowed detection of different combinations of multisite mutations from a minimal number of in vitro reactions. Although the mutagenesis efficiency of each oligonucleotide was lower in the mixture than in a single primer reaction, efficiency was still high enough to allow easy detection of a variety of multisite mutations. A result from a master plate of one of the mutagenesis reactions is shown in Fig. 2. All the mutations were confirmed by DNA sequencing.

All the mutations were introduced into the yeast strain B-7056 by replacing the *cyc1*::*CYH2*^{s} allele, which is located at the chromosomal *CYC1* locus. This allele, *cyc1*::*CYH2*^{s}, contains a *CYC1* deletion from the *Sma*I site at −380 to the *Kpn*I site at +250, including the entire promoter region and a large portion of the protein-coding region. The *CYH2*^{s} gene fragment replacing the deleted region of *CYC1* encodes a dominant cycloheximide-sensitive (Cyh^s) allele (1). Because the integrating plasmids had the *URA3*$^+$ gene, Ura$^+$ transformants with integration at the *CYC1* locus would be selected. Strains with a single copy of *cyc1* were obtained by selecting strains lacking the plasmid. The *URA3*-containing plasmid was excised from the transformant in two ways. One of these recombinational excisions could occur at the region where the integration had occurred, resulting in a strain with the original parental phenotype that did not grow on medium lacking uracil (Ura$^-$), on medium containing cycloheximide (Cyh^s), or on glycerol medium (Nfs$^-$). The other possibility is recombinant excision on the opposite side of the *cyc1* gene from the site of integration, resulting in a Ura$^-$ Cyh^r Nfs$^+$ phenotype that indicated gene replacement. The Ura$^-$ colonies contained approximately equally numbers of the parental type (Cyh^s Nfs$^-$) and the gene-replaced type (Cyh^r Nfs$^+$).

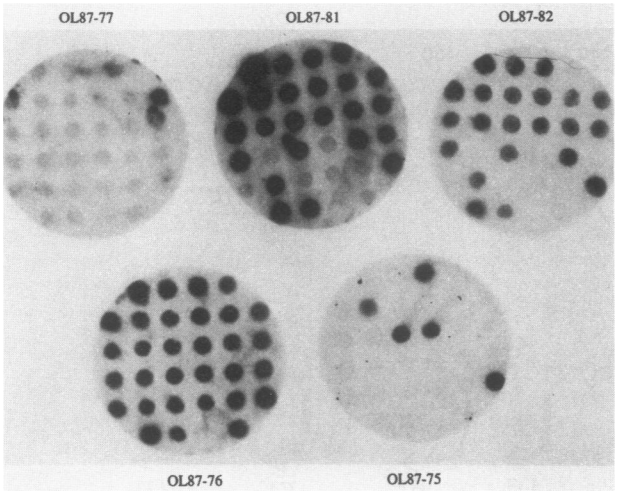

FIG. 2. Hybridization screening of multiple-site mutants. The autoradiograph is from a multiple-primer in vitro mutagenesis reaction (procedure described in Materials and Methods section). The template in the reaction was single-stranded pAB674, which has mutated site 4m, to avoid using the two overlapped primers, OL87-75 and OL87-76, in the same in vitro mutagenesis reaction. The primers that were used in the reaction were OL87-77, OL87-81, OL87-82, and OL87-75, for mutating sites 1, 2, 3, and 5, respectively. The original master plate of the JM103 transformants was replicated to five plates in the presence of helper phage R408 and transferred to filter disks after being grown overnight. All four oligonucleotides used as primers in the in vitro reaction and OL87-76 were individually labeled and hybridized to the filter separately. From this autoradiograph, many combinations of multisite mutations can be found.

The TATA mutations of the *CYC1* gene were analyzed by three methods. Each strain was first tested for iso-1-cytochrome *c* content by low-temperature spectroscopy, since this method was rapid and efficient for screening large numbers of strains for *CYC1* expression. *CYC1* mRNA steady-state levels and transcription initiation patterns were determined for all single-site mutations, all quadruple-site mutations, which had only one wild-type TATA sequence, and representative strains of each group of multisite mutants.

Two functional TATA elements in the *CYC1* gene. The expression of the wild-type *CYC1* was defined as 100% for both iso-1-cytochrome *c* level (Table 2) and mRNA level (Fig. 3). The 5′ transcription initiation sites present in both derepressed and repressed conditions were determined by primer extension analysis (Fig. 4, lanes 2 and 17, and Fig. 5, lane 1, respectively). In the ethanol-derepressed condition, the wild-type *CYC1* mRNA initiated at five cluster sites (Fig. 1, Table 2). The most abundant one was start site F, which was a cluster of several adjacent initiation sites with the strongest at the −46 position along with minor ones at −47, −48, and −49. The intermediate-strength site was found at −62 (start site D). The third most abundant site was at −70 (start site C). The two minor start sites occurred at or near the −57 region and the −38 positions. The −57 site (start site E) was a cluster of two initiations at −57 and −55, and the −38 site (start site G) was a cluster of several starts at −39, −38, −37, and −36. In the wild type, there were also some very minor initiation sites that could be observed on the autoradiograph film after a long exposure time. These sites were at −28 (start site H), −74, −78 (start site B), and −93 (start site A). Initiation sites downstream of start site H were not detected, a finding that was confirmed by primer extension with OL88-67 (data not shown). Under the repressing condition, transcription initiation was similar to that in the derepressed condition except that the −57 site was very weak (Fig. 5). A background band at −74 was observed with all strains (Fig. 5), including the *cyc1*::*CYH2* mutant, which lacks the *CYC1* gene.

All of the single TATA-like sequence mutations allowed production of normal protein levels (Table 2). However, the single TATA mutations at either site 1m or site 2m allowed *CYC1* mRNA levels about half that of the wild type, whereas single mutations at site 3m, 4m, or 5m allowed normal *CYC1* mRNA levels (Fig. 3). The relative abundances of mRNA initiation sites were determined by primer extension analysis for ethanol-grown derepressed conditions. When site 1 was mutated, initiation at all upstream sites, from −70 to −57, was greatly decreased while initiation at the downstream sites, between −46 and −28, was increased (Fig. 4, *cyc1-918*). In contrast, the site 2m mutation led to decreased initiation at downstream sites and increased initiation at upstream sites between −70 and −46 (Fig. 4, *cyc1-919*). Under glucose-repressed conditions, the site 1m and 2m mutations gave initiation patterns similar to that in derepressed conditions; the site 1m mutation showed decreased initiation in the upstream region and increased initiation in the downstream sites; the site 2m mutation showed increased initiation in the upstream region and diminished initiation in the downstream sites.

With the single mutations at sites 3m, 4m, and 5m, the overall initiation pattern was generally similar to that of the wild type and a few differences. In the site 3m mutant, the −93 site was enhanced (Fig. 4, *cyc1-920*); in the site 4m mutant, the −78 site and the −28 and the −38 region were also enhanced (Fig. 4, *cyc1-921*). In the site 5m mutant, the −60 site was used instead of the −62 (Fig. 4, *cyc1-922*). Each enhanced or new site was just one or two bases upstream of the mutated sequence; therefore, the local sequence changes facilitated the utilization of these weak or new sites.

Among the double TATA element mutants, only the site 1m and site 2m double mutation allele (*cyc1-923*) had a large effect on protein, mRNA, and transcription initiation (Table 2). The iso-1-cytochrome *c* level in this mutant was reduced fivefold and the *CYC1* mRNA level was decreased 10-fold. A dramatic change in the transcription initiation pattern was observed in the primer extension analysis of this strain; all the initiation sites disappeared except some very weak ones in the −28 region (Fig. 4, *cyc1-923*). Other double mutants had nearly normal iso-1-cytochrome *c* levels. *CYC1* mRNA levels were about 50% of the wild-type level when a mutation at site 3m, 4m, or 5m was combined with a mutation at either site 1m or 2m. A wild-type level was seen when the double-site mutation did not include at least one of the first two TATA sequences, site 1m and site 2m.

Among the triple-site mutants, only those alleles having at least both site 1m and site 2m mutated showed a significant reduction in the iso-1-cytochrome *c* content. The triple-site mutants with other combinations had nearly normal amounts of iso-1-cytochrome *c* (Table 2). The allele *cyc1-942* has all three of the downstream TATA-like sequences mutated. This mutant had a level of *CYC1* mRNA similar to that of the wild type, and the transcription initiation pattern was close to that of a combination of these three single-site mutations, i.e., it had enhanced initiation at −93, −78, −38, −28, and −60 sites and no initiation at −62 (Fig. 4, lane 11).

A quadruple-site mutation had four out of the five sites

TABLE 2. Summary of iso-1-cytochrome *c* levels, mRNA levels, and mRNA start sites in strains having normal and altered TATA elements at the five sites

Strain	Allele	TATA site[a]					Iso-1-cytochrome *c* (% of wild type)	mRNA (% of wild type)		Relative intensity[b] of start site:							
		1 (−178)	2 (−123)	3 (−93)	4 (−78)	5 (−56)		Glucose	Ethanol	A (−93)	B (−78)	C (−70)	D (−62)	E (−57)	F (−46)	G (−38)	H (−28)
Wild-type																	
B-7969	*CYC1*$^+$	+	+	+	+	+	100	20	100			9	24	13	50	4	
One-site mutants																	
B-7970	*cyc1-918*	m	+	+	+	+	100	20	50						27	16	7
B-7971	*cyc1-919*	+	m	+	+	+	100	14	55			9	16	11	19		
B-7972	*cyc1-920*	+	+	m	+	+	100	20	95	8		12	24	8	32	12	
B-7973	*cyc1-921*	+	+	+	m	+	100	20	100		7	7	17	3	33	20	13
B-7974	*cyc1-922*	+	+	+	+	m	100	20	100			8	19[c]	31	19	23	
Two-site mutants																	
B-7975	*cyc1-923*	m	m	+	+	+	20	5	14							3	11
B-7976	*cyc1-924*	m	+	m	+	+	95	20	60						32	20	8
B-7977	*cyc1-925*	m	+	+	m	+	95	20	45								
B-7978	*cyc1-926*	m	+	+	+	m	95										
B-7979	*cyc1-927*	+	m	m	+	+	95	12	65	4		9	17	13	22		
B-7980	*cyc1-928*	+	m	+	m	+	95	10	65								
B-7981	*cyc1-929*	+	m	+	+	m	95										
B-7982	*cyc1-930*	+	+	m	m	+	95	20	95	4	15	15	15		22	15	11
B-7983	*cyc1-931*	+	+	m	+	m	95										
B-7984	*cyc1-932*	+	+	+	m	m	95										
Three-site mutants																	
B-7985	*cyc1-933*	m	m	m	+	+	15										
B-7986	*cyc1-934*	m	m	+	m	+	15										
B-7987	*cyc1-935*	m	m	+	+	m	18										
B-7988	*cyc1-936*	m	+	m	m	+	95	20	50						20	20	10
B-7989	*cyc1-937*	m	+	m	+	m	95										
B-7990	*cyc1-938*	m	+	+	m	m	95										
B-7991	*cyc1-939*	+	m	m	m	+	90	9	50	2	11	11	14		12		
B-7992	*cyc1-940*	+	m	m	+	m	95										
B-7993	*cyc1-941*	+	m	+	m	m	95										
B-7994	*cyc1-942*	+	+	m	m	m	95	20	100	19	19	13	13[c]	6	3	19	6
Four-site mutants																	
B-7995	*cyc1-943*	+	m	m	m	m	70	4	55	12	18	6	12[c]		6		
B-7996	*cyc1-944*	m	+	m	m	m	95	10	60							40	20
B-7997	*cyc1-945*	m	m	+	m	m	10	2	10							3	6
B-7998	*cyc1-946*	m	m	m	+	m	10	2	10							3	6
B-7999	*cyc1-947*	m	m	m	m	+	5	2	2								
Five-site mutant																	
B-8000	*cyc1-948*	m	m	m	m	m	5	2	2								

[a] The locations of the five TATA-like sequence sites are indicated and are shown in Fig. 1. See text for explanation of + and m.

[b] Intensity of the *CYC1* mRNA start sites was estimated from the densitometer scan of primer extension gels. The relative intensities have been assigned values so that their sum equals the values for the percent mRNA from ethanol-grown derepressed cells, as shown in the column indicating mRNA (% of wild type) for ethanol. Blank spaces indicate very weak or undetectable values.

[c] The initiation at this site is at −60, not −62.

altered and one normal site; the quintuple-site mutation had all five of the TATA-like sequences mutated. The mutant with only the wild-type site 1+ (*cyc1-943*) had a slightly lower iso-1-cytochrome *c* level, about 70% of the wild-type level, approximately one-half of the wild-type *CYC1* mRNA level, about 70% of the wild-type level, approximately one-half of the wild-type *CYC1* mRNA level, and normal initiation in the upstream initiation region but greatly reduced at the downstream site. The mutant that had only wild-type site 2+ (*cyc1-944*) had a nearly normal protein level, half the mRNA level, and initiation of transcription in the downstream sites mainly in the −38 and −28 sites (Fig. 4, lane 13). The allele that had only wild-type site 3+ (*cyc1-945*; Fig. 4, lane 14) or only wild-type site 4+ (*cyc1-946*; Fig. 4, lane 15) had less than 10% of the wild-type *CYC1* mRNA and the same transcription initiation sites as the

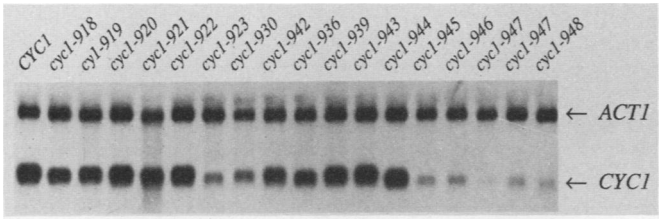

FIG. 3. Northern blot hybridization of representative TATA mutants after ethanol-derepressed growth. The probes are ^{32}P-end-labeled OL88-67 (for *CYC1* mRNA) and OL88-68 (for *ACT1* mRNA). The *cyc1* alleles, shown on the top, are described in Table 2. As mentioned in the Materials and Methods section, two lanes in this figure were discarded, degraded *cyc1-921* and underloaded *cyc1-930*. In addition, the two lanes of *cyc1-947* were loaded with RNA samples that were isolated from two independent yeast transformants.

double-site (site 1m, site 2m) mutant (*cyc1-923*) (Fig. 4, lane 8). The *cyc1-947* mutant, which contained four mutated upstream TATA sequences, and the *cyc1-948* mutant, which had all five TATA sequences mutated, had about 5% of the wild-type iso-1-cytochrome *c* levels, *CYC1* mRNA levels at about 2 to 3% that of the wild type, and mRNA initiations were undetectable.

Therefore, our results suggest that site 1 and site 2 are the only functional TATA elements in the *CYC1* gene. Site 3 and site 4 are not required in normal cells, since mutations of these two elements did not affect *CYC1* gene expression. However, when both site 1m and site 2m were mutated, the

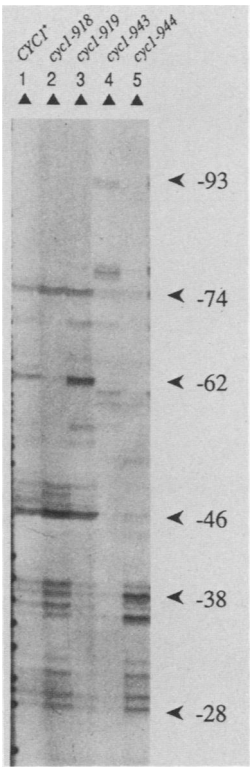

FIG. 5. Patterns of initiation sites by primer extension of glucose repressed strains. See Fig. 4 legend for description.

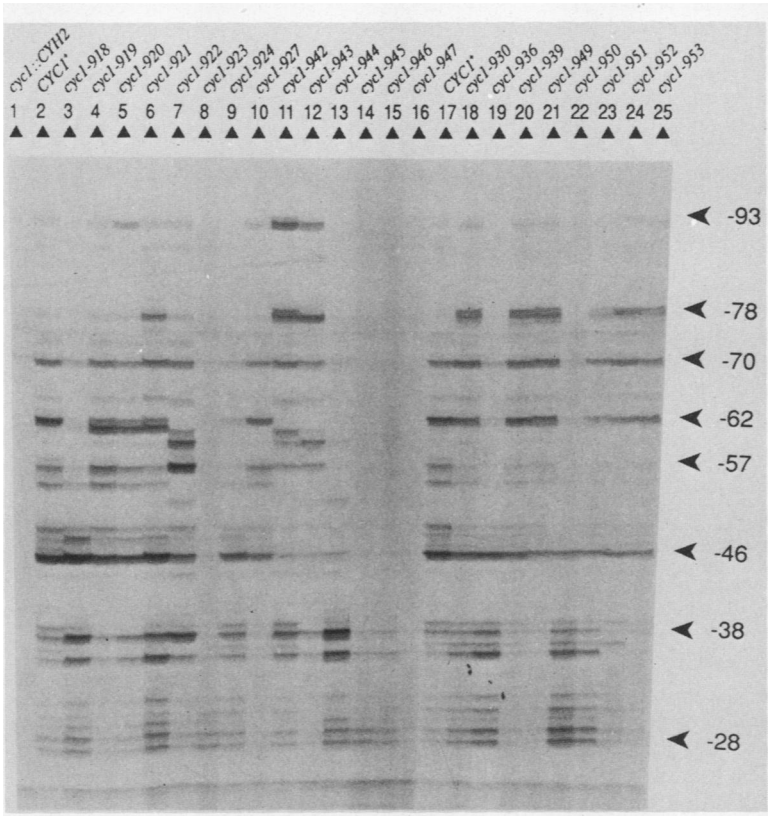

FIG. 4. Patterns of initiation sites by primer extension of ethanol-derepressed strains. ^{35}S-5′-end-labeled OL88-193, which hybridizes to the *CYC1* mRNA coding region, was used as the primer. RNA was extracted from ethanol-grown derepressed cells. The major initiation site of each cluster is numbered at the right. The *cyc1::CYH2* strain (lane 1) contains a *CYC1* deletion and was used as a negative control.

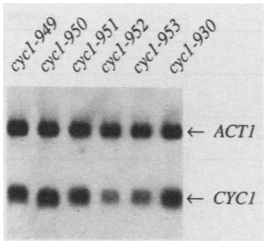

FIG. 6. Northern blot hybridization of rearranged TATA element alleles after ethanol-derepressed growth. The probes are described in the Fig. 3 legend. The *cyc1* alleles, shown on the top, are described in Table 3.

functions of site 3 and site 4 were elevated, as seen in mutants carrying alleles *cyc1-923*, *cyc1-945*, and *cyc1-946*. These mutants had protein and mRNA levels above the basal level and barely detectable amounts of initiation in the downstream initiation region. Site 5 does not seem to be a functional element in terms of controlling transcription initiation in the *CYC1* gene even in the absence of upstream TATA elements.

Two functionally distinguishable TATA elements. From the above results, it is clear that the *CYC1* gene has only two functional TATA elements. The two active TATA elements of the *CYC1* gene are 55 bp apart, site 1 at −178 and site 2 at −123, yet both TATA elements are used to an almost equal extent. The two functional TATA elements, however, have slightly different sequences, ATATATATAT, which we will refer to as β-type, for site 1 and TATATAAAA, which we will refer to as α-type, for site 2. We constructed mutants that revealed that these two TATA elements of the *CYC1* gene actually represent two distinct types of elements, each acting independently, and that two copies of the same TATA elements did not both function together.

These questions were addressed by using the in vitro mutagenesis and yeast transformation techniques described above to make a set of isogenic yeast strains with all nine possible rearrangements of these two types of TATA elements, with each site 1 or 2 containing either the α-type, the β-type, or the mutant type. Since site 1+ is defined as site 1β and site 2+ is defined as site 2α, the following four of the nine rearrangements were discussed above, the site1β-site2α, site1m-site2α, site1β-site2m, and site1m-site2m combinations.

All strains of this set had mutated site 3m and site 4m. There were two reasons for this choice. First, it eliminated any potential transcriptional initiation activation by these two downstream TATA-like sequences. Second, transcription initiation from downstream sites (−38 and −28 region) resulting from initiation stimulated by site 2+ was slightly increased in the site3m-site4m double-site mutants. This allows a better comparison of site 2+ TATA element function in the presence of the functional site 1+ TATA element.

The iso-1-cytochrome *c* and *CYC1* mRNA levels (Fig. 6) and the *CYC1* mRNA initiation patterns (Fig. 4) were determined for all strains. The results of the complete analysis of these strains are summarized in Table 3. When the native β-type site 1, allele *cyc1-939* (site1β-site2m), was replaced with an α-type TATA element, allele *cyc1-949* (site1α-site2m), *CYC1* protein and mRNA levels were the same, i.e., 95% of iso-1-cytochrome *c* level and 50% of the mRNA level of the wild type, and transcription initiations were alike (Fig. 4, lanes 20 and 21). Similarly, little change was seen when the native α-type site 2 of the *cyc1-936* mutant (site1m-site2α) was replaced by a β-type TATA element as seen in the *cyc1-950* mutant (site1m-site2β) (Fig. 4, lanes 19 and 22). When the order of the site1β-site2α of the *cyc1-930* mutant was switched to site1α-site2β (*cyc1-951* mutant), the *CYC1* protein and the mRNA levels and initiation sites were still similar to those of the control strain *cyc1-930* (Table 3; Fig. 4, lanes 18 and 23). Most strikingly, when both site 1 and site 2 were of the same type, either both α-type (site1α-site2α, *cyc1-952*) or both β-type (site1β-site2β, *cyc1-953*) the iso-1-cytochrome *c* contents were nearly normal, the mRNA levels were about half that of wild-type *CYC1*, and transcription was initiated in the upstream initiation region between −93 and −46 (Table 3; Fig. 4, lanes 24 and 25). These results were same as those found with the alleles *cyc1-939* (site1β-site2m) and *cyc1-949* (site1α-site2m), which had a functional site 1 (either β-type or α-type) and a mutated site 2m.

In summary, these results showed that when two identical TATA elements were adjacent within a certain distance, only the upstream TATA element would function. In contrast, both TATA elements were functional when the two TATA elements were of different types. The results suggest that these two TATA elements are functionally distinguishable.

TABLE 3. Summary of iso-1-cytochrome *c* levels, mRNA levels, and mRNA start sites in strains having α-type, β-type, and altered TATA elements at the five sites

Strain	Allele	TATA site[a]					% of wild-type level			Relative intensity[b] of start site:							
							Iso-1-cytochrome *c*	mRNA									
		1	2	3	4	5		Glucose	Ethanol	A (−93)	B (−78)	C (−70)	D (−62)	E (−57)	F (−46)	G (−38)	H (−28)
B-7982	*cyc1-930*	β	α	m	m	+	95	20	95	4	15	15	15		22	15	11
B-7988	*cyc1-936*	m	α	m	m	+	95	20	50						20	20	10
B-7991	*cyc1-939*	β	m	m	m	+	90	9	50	2	11	11	14		12		
B-8001	*cyc1-949*	α	m	m	m	+	70	4	45	2	10	10	14		9		
B-8002	*cyc1-950*	m	β	m	m	+	95	20	55						17	22	17
B-8003	*cyc1-951*	α	β	m	m	+	95	20	100	4	12	15	12		19	19	19
B-8004	*cyc1-952*	α	α	m	m	+	70	18	45	2	10	14	15		9	7	
B-8005	*cyc1-953*	β	β	m	m	+	70	10	50	2	11	11	15		11		

[a] The α-type is TATATAAAA and the β-type is ATATATATAT. In this table, the two normal functional TATA elements, site 1+ and site 2+, are given by α and β instead of the + symbol. m, Altered.

[b] See Table 2, footnote *b*.

DISCUSSION

A detailed mutational analysis of the *CYC1* TATA-like sequences in *S. cerevisiae* is reported in this article. Superficially, the *CYC1* promoter appears to control mRNA initiation indiscriminately, because as many as five functional TATA elements with different sequences have been proposed, because of the many transcription initiation sites spanning a region of more than 60 bp, and because some of the TATA-like elements overlap the mRNA initiation region. However, our results indicate that only two of the five TATA-like sequences in this gene are functional in normal cells. They are the β-type at site 1 (−178 bp) and the α-type at site 2 (−123 bp). Thus, the functional TATA element region is separated from the transcription initiation region.

Among all of the TATA sequence mutants, only the ones with a mutated site 1 (β-type) or site 2 (α-type) or both showed significant changes in *CYC1* mRNA levels and transcription initiation patterns. Mutations at either site 1 or site 2 caused diminished transcription, to about 50% of wild type; mutations of both site 1m and site 2m caused a diminution to less than 15% of the normal *CYC1* mRNA level and less than 20% of the iso-1-cytochrome *c* level. Site1m-site2+ strains showed significantly decreased initiation in the upstream initiation region, whereas site1+-site2m strains had diminished initiation in the downstream initiation region. Mutations of other TATA-like sequences at sites 3m, 4m, or 5m, did not cause such effects, although mutations of these three sites were responsible for enhanced initiation at some mRNA start sites. These results indicate that the β-type TATA element at site 1 regulates transcription initiation in a window between −70 and −46, which covers the upstream initiation region, and the α-type TATA element at site 2 controls transcription initiation in a window of the downstream initiation region between −46 and −28. Sites 1 and 2 appear to be approximately equal in directing transcription. No systematic difference was uncovered by comparing eight pairs of site1+-site2m and site1m-site2+ mutants, with each pair having identical alterations at other sites (Tables 2 and 3).

Most genes that have been studied so far have only a single TATA element. Although there are genes, especially in *S. cerevisiae*, that have more than one functional TATA element, it is rare for a gene to have two functionally independent inducible TATA elements. Whether there is any biological significance to this type of transcription regulation is not clear at present.

The site 3 (at −93 bp), site 4 (at −78 bp), and site 5 (at −56 bp) TATA-like sequences, encompassed in the upstream transcription initiation region, are barely functional in the presence of both or either of the functional site 1+ and site 2+. However, when both site 1 and site 2 are mutated, the functions of site 3 and site 4 are slightly increased, as indicated in the *cyc1-923* mutant, which contained 20% of the iso-1-cytochrome *c* and 14% of the *CYC1* mRNA of the wild type (Table 2). Because the site3m-site4m *cyc1-930* mutant and the site3m-site4m-site5m *cyc1-942* mutant contain normal or nearly normal amounts of iso-1-cytochrome *c* and *CYC1* mRNA (Table 2), sites 3, 4, and 5 do not appear to be functioning in the normal cell. Furthermore, because transcription initiates at start sites −38 and −28 in the *cyc1-923* mutant (site1m-site2m strain), and because transcription actually slightly increased at these −38 and −28 start sites in the *cyc1-921*, *cyc1-930*, and *cyc1-942* mutants (site1+-site2+ strains with site 3m, 4m, and/or 5m mutations), it is possible that the transcriptional contributions of sites 3, 4, and 5 may actually be zero. Although we have not rigorously excluded that sites 3, 4, and 5 are responsible for a low level of transcription in the normal cell, this low basal level would be minor and probably not physiologically significant. The wild-type *CYC1* gene has similar transcription initiation patterns between glucose-repressed and ethanol-derepressed growth conditions. This result indicates that these two TATA elements of site 1 and site 2 are functional in both repressed and derepressed growth conditions.

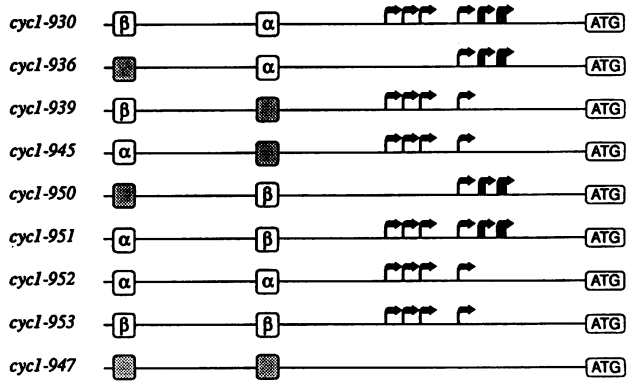

FIG. 7. Schematic representation of the regulation of the two types of TATA elements in the *CYC1* gene. The locations of the two functional TATA elements at site 1 and site 2 are shown by boxes. α and β denote, respectively, the α-type TATA element TATAAA and the β-type TATA element ATATATATAT. Shaded boxes are mutated, nonfunctional sequences. *CYC1* mRNA initiation sites are shown by arrows, with single and multiple vertical lines for single sites and multiple sites respectively. The translation initiation codon ATG is shown at the right. This figure is drawn to scale.

The α-type and β-type TATA elements appear to be complementary, as schematically represented in Fig. 7. When site 1 and site 2 have different types of TATA elements, either site1β-site2α or site1α-site2β, both TATA elements are functional. However, when both locations have the same type of TATA element, either α-type or β-type, only the upstream element is functional. Although sequences adjacent to a particular TATA element may influence function, several previous studies have demonstrated that the TATA core sequences per se mediate distinct transcription activation in response to different enhancers or UAS elements (18, 34, 41). Also, although the sizes of the α-type and β-type elements have not been delineated in this investigation, previous studies have indicated that core TATA elements may be restricted to 6 to 8 nucleotides (5, 18).

The results presented in this study suggest the existence of different regulatory factors acting on the two types of sequences or the same regulatory factor interacting with the two types of TATA elements differently. It has been proposed that different TATA-binding proteins recognize T_C and T_R elements of the yeast *HIS3* gene (5) or different TATA-binding proteins recognize TATTTAT (SV40 type) and TATAAA type of mammalian TATA box sequences (34, 41). One of the possible candidates could be the TATA-binding protein TFIID. This transcription factor specifically binds to TATA elements in promoters and plays a major role in transcription initiation (27). There may be multiple TATA-binding factors, and each factor may bind to one or a group of related TATA elements. However, recent studies have shown that the yeast TFIID binds to both consensus and

nonconsensus TATA sequences (16). In addition, TFIID is highly conserved in structure and function between yeast and mammalian cells and is encoded by a single-copy gene (3, 4, 15, 19, 20, 30). Maybe the TFIID factor is, in fact, a general transcription factor which binds all TATA elements with consensus or related sequences. Therefore, there may be regulatory factors other than TFIID that specifically recognize different but closely related TATA sequences, such as the α-type and β-type of the yeast *CYC1* gene.

Nevertheless, it is unknown why only the 5′ TATA element is functional when the promoter region contains two elements with the same sequence. Perhaps the greater proximity to UAS elements plays a role in this selection. Considering the results of this study, we can speculate upon the mechanism of transcription regulation for the *CYC1* gene. The UAS complex of the *CYC1* gene may involve two recognition sites, one specific for α-type TATA-binding factor and the other specific for β-type TATA-binding factor. If the *CYC1* gene contains two different types of TATA elements that are bound by different TATA-binding factors, both factors can interact with the UAS complex, and therefore both TATA elements can be functional simultaneously. However, if the *CYC1* gene has two of the same type of TATA elements at both site 1 and site 2, the UAS complex tracks along the DNA from the upstream direction, finds the first TATA element, and activates transcription from this TATA element. The UAS complex cannot interact with the second TATA element since it has only one site for this particular type of TATA element; therefore, the second TATA element is not used.

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