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Genetics

Upstream activation sites of the *CYC1* gene of *Saccharomyces cerevisiae* are active when inverted but not when placed downstream of the "TATA box"

(eukaryotic promoter/RNA polymerase entry site/enhancers/*lacZ* fusion/*rp51* intron)

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ABSTRACT **The ability of the upstream activation sites (UASs) of the yeast *CYC1* gene to function when inverted or when positioned downstream of the "TATA box" is investigated. Inversion of a 130-base-pair DNA fragment bearing the UASs leaves the activity of the sites almost completely intact. In contrast, positioning the sites downstream of the TATA box or in the intron of a *CYC1*–ribosomal protein 51–*lacZ* tribrid gene almost totally abolishes their activity. In the latter construct, the separation between the UASs and TATA box is roughly equivalent to that between the elements in the intact *CYC1* promoter region. The UASs are shown not to interrupt transcription or splicing in this construct since a *GAL10* UAS positioned upstream of the TATA box gives rise to galactose-inducible expression of the tribrid gene. The inability of the UASs to function in the intron is partly due to sequences between the intron and the TATA box that block the activation signal. However, a large component of the inactivity of the sites in the intron appears to be their downstream location. This result is discussed in light of possible mechanisms of upstream activation in yeast.**

In higher eukaryotes, RNA polymerase II typically initiates transcription 30–40 base pairs (bp) downstream of the canonical "TATA box" sequence (1, 2). The frequency of initiation, for many genes, is determined by sequences lying upstream of the TATA box, one class of which is found <100 bp away and regulates transcription in response to particular physiological signals (3–6). A second class of upstream sequences, called enhancers, lies in a more remote position and can mediate hormonal responsiveness or cell type-specific regulation in mammals (2, 7–11, 26). Several features of cloned enhancers are revealed when the sites are manipulated by recombination *in vitro*. For example, the sites can function when transposed to a location several kilobases away from the TATA box or when their orientation with respect to the transcriptional unit has been inverted. Although many enhancers naturally occur upstream of the TATA box, some are found to the 3′ side of the affected transcriptional unit (12–14). Many enhancers that are naturally found in an upstream location will function when positioned 3′ to the transcriptional unit (10, 15, 16).

In yeast, TATA boxes have been shown to be functionally important in several cases (17, 18). Sequences that lie upstream of the TATA box that activate transcription in response to particular physiological signals appear to occur frequently. Such upstream activation sites (UASs) have been found for genes involved in histidine biosynthesis, *HIS3* (17) and *HIS4* (19), a gene involved in galactose catabolism, *GAL10* (20), the *CYC1* gene, encoding the iso-1-cytochrome *c* (18, 21), and a gene involved in leucine biosynthesis, *LEU2* (22). These sites lie between −30 and −300 (from the transcriptional initiation site) and activate transcription in response to particular physiological signals (for review, see ref. 23). Genetic studies suggest that UASs are regulated by proteins encoded by specific regulatory loci (21).

The *CYC1* gene contains tandem sites, designated UAS1 and UAS2, which lie at about −275 and −225, respectively (21). These sites are differentially regulated as follows: both UASs are uninduced when cells are grown under conditions of deficiency of heme, the cytochrome cofactor; UAS1 is partly induced in cells grown under heme sufficiency in media with glucose as carbon source; both UAS1 and UAS2 are fully and equally active in cells grown under derepressing conditions in media containing a non-fermentable carbon source.

In several cases hybrid promoters have been constructed consisting of the UAS of one gene and the TATA box of another (20, 21). These hybrids direct transcription initiation at the correct site(s) specified by the TATA box region and are regulated in a manner determined by the UAS in *cis*. These and other experiments suggest that there is some flexibility in the spacing between UASs and TATA boxes. In this report, it is determined whether the *CYC1* UASs function when inverted with respect to the TATA box and when placed to the downstream side of that element. To aid in the design of the latter experiment, the effects of distance separating the UASs from the TATA box were also assessed. The results obtained strengthen the credibility of one possible molecular mechanism of UAS activation and weaken the credibility of another.

MATERIALS AND METHODS

Strains and Plasmids. Yeast strains BWG2-9A-1 (*Matα*, *his4-519*, *ade6*, *ura3-52*) and BWG1-7a (*Mat***a**, *leu2-2,2-112*, *his4-519*, *ade1-100*, *ura3-52*), TM1 (*hem1*, *ura3-52*), and LG1-2D (*MAT***a**, *trp1*, *ura3-52*, *gal80*) (21) were employed for all assays described. All plasmid constructs are derived from pLGΔ-312S (18).

Assays and Media. β-Galactosidase assays were performed as described (24). Units are according to Miller (25). Cells were grown in yeast nitrogen base without amino acids for β-galactosidase assays or RNA preparations.

DNA Manipulations. All methods including plasmid construction, DNA or RNA isolation, and S1 mapping were as described (18, 24).

Insertions. pLGΔ-312S was digested with *Sal* I and *Escherichia coli* DNA that had been cut with *Xho* I was inserted. The *Sal* I and *Xho* I ends are complementary. Insert sizes were determined by restriction analysis.

UAS inversion. pLGΔ-312 was digested with *Sma* I and *Xho* I, the *Xho* I end rendered flush with DNA polymerase,

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Abbreviations: UAS, upstream activation site; bp, base pair(s); kb, kilobase(s); E1 and E2, exons 1 and 2, respectively.

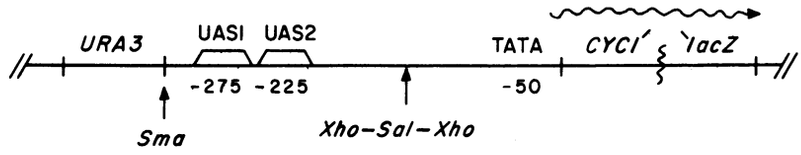

FIG. 1. The *CYC1* upstream region of pLGΔ-312S. Depicted is the upstream region of pLGΔ-312S (18). Numbering of UAS1, UAS2, and TATA is from the most upstream mRNA initiation site. The *Xho* I–*Sal* I–*Xho* I cluster of restriction sites was obtained by insertion of a *Sal* I linker into a filled-in *Xho* I site of pLGΔ-312 (18).

and the plasmid reclosed. Reinsertion of the *Sma* I–*Xho* I fragment was monitored, and orientation of the insert was determined by digesting the plasmid separately with three enzymes whose sites lay asymmetrically on the *Sma* I–*Xho* I fragment.

3′ construction. Plasmid pHZ18* (27) was cut with *Sal* I within the intron separating exons 1 and 2 (E1 and E2) of *rp51* and a 130-bp *Xho* I fragment bearing UAS1 and UAS2 (containing sequences in the *Sma* I–*Xho* I interval of pLGΔ-312) was inserted (Fig. 3). Inserts were verified and their orientations were determined by fine structure restriction analysis using several enzymes that have sites on the 130-bp fragment. Finally, to prove that the constructs were correct, the DNA sequence of E1 and across the intron–UAS junction was determined for candidates in both orientations. For this purpose, DNA was labeled by filling in a *Bam*HI site at the *CYC1*–E1 border with [α-^{32}P]dNTPs, subdigesting with *Acc* I, which cuts near the 3′ end of the intron (past the *Sal* I site), and performing chemical sequencing (28).

Construction of pLGcrl. A DNA fragment from pHZ18′ extending from an *Mbo* II site in the *CYC1* TATA box region to the *Sal* I site in the *rp51* intron was purified and its ends were rendered flush with DNA polymerase I. The fragment was then inserted into the *Xho* I site of pLG3L (21) that had been rendered flush. The *Mbo* II site lies just within the most upstream of the three *CYC1* TATA boxes (29).

RESULTS

The UASs Function in an Inverted Orientation. A test was performed to determine whether the UASs, like enhancers, could function in an inverted orientation. A DNA fragment of 130 bp extending from the *Sma* I site to the *Xho* I site of pLGΔ-312 and encompassing both UASs was inverted (see *Materials and Methods*; Fig. 1). The resulting plasmid, pLGΔ-312I, gave rise to expression that was properly regulated and whose levels were 60–70% those of the parental plasmid (Table 1). Since the vector is circular, it may be argued that the inverted UASs are propagating a signal around the 10 kilobases (kb) of the plasmid to approach the UASs from the 3′ side. However, the results from the following section make this possibility very remote.

Placement of the UASs 3′ to the TATA Box. (*i*) Effects of separation between the UASs and TATA box. Before adopting a strategy to position the UASs to the 3′ side of the TATA boxes, it was necessary to assess the distance over which the sites were capable of activating the TATA boxes from the 5′ side. Previous experiments indicated that the *CYC1* UASs functioned at 100% efficiency when brought closer to the TATA box by internal deletions between the elements or when moved 12 or 14 bp upstream of their normal position and functioned at 50% efficiency when displaced 150 bp upstream (18). To study in a more systematic manner the response of the UASs to separation from the TATA box, insertions of random *E. coli* DNA fragments into the *Sal* I site of pLGΔ-312 were made, and their effects on expression of a *CYC1–lacZ* fused gene were monitored (see *Materials and Methods*; Fig. 1; Table 2). Four insertions of between 50 and 150 bp reduced expression by a factor of about 4–6 in glucose but did not significantly affect derepressed levels of expression. One insertion of about 50 bp reduced both repressed and derepressed levels by a factor of about 3 and another insertion of about 350 bp did not affect expression in glucose or lactate. In all of these cases, heme deficiency abrogated expression, verifying that activity in heme-sufficient conditions is due to the UASs (Table 2). In contrast, large insertions of 0.8–9 kb either totally abolished expression or, in three of nine cases, gave rise to activity that was not correctly regulated. The activity of the latter class is probably due to sequences on the inserts that fortuitously activate transcription.

(*ii*) The UASs do not function when placed in the intron of a *CYC1–rp51–lacZ* tribrid gene driven by the *GAL10* UAS (UAS_{GAL10}). In order to determine whether the UASs functioned when positioned 3′ to the TATA box, it was necessary to place the sites downstream and close to the TATA box (because of the distance effects described above). To avoid any possible destabilization of a mature transcript, the UASs were inserted into an intron at a site about 200 bp downstream of the TATA box. For this purpose, we utilized a plasmid, pHZ18, which contains an E1–intron 1–E2 fragment of the yeast ribosomal protein gene *rp51* sandwiched between *CYC1* and *lacZ* (27). The coding sequences reading from *CYC1* into E1 and from E2 into *lacZ* are in frame. The intron is correctly spliced from the encoded mRNA and this splicing can be assayed by β-galactosidase production (27). Our approach, then, was to insert the UASs bounded on a 130-bp *Xho* I fragment (see *Materials and Methods*) into a *Sal* I site in the intron in pHZ18 that lies about 200 bp downstream of the *CYC1* TATA box (see Fig. 2).

The final concern was whether the UAS-bearing insert would interfere with splicing of the intron or fortuitously terminate transcription. Conveniently, upstream of the TATA box in pHZ18, the *CYC1* UASs have been replaced with the UAS_{GAL10} (20, 27), which renders transcription initiation tightly galactose inducible. The mRNA species induced by

Table 1. The UASs function when inverted

UAS	Without heme, glucose	With heme, glucose	Glycerol/ EtOH
Wild-type	<0.5	89	755
Inverted	<0.5	59	457

A 130-bp *Sma* I–*Xho* I fragment bearing UAS1 and UAS2 was inverted. β-Galactosidase was assayed in TM1 (without heme, glucose) or BWG2-9A-1 (with heme, glucose; glycerol/EtOH) bearing plasmids with wild-type or inverted UASs. TM1 grown in the presence of a heme supplement gave activities comparable to those given by BWG2-9A-1. Heme-deficient cells in this and other experiments were grown in medium supplemented with 1 μg of ergosterol per ml, 0.15% Tween 80, and 40 μg of methionine per ml. Minimal medium employed in this assay was yeast nitrogen base lacking amino acids and ammonia plus 40 μg of required amino acids per ml and a 2% carbon source. All assays in this and other experiments were performed at least twice with an error of <20%. Units in this and subsequent tables are according to Miller (25).

*The actual vector used is a derivative of pHZ18 with a *Bam*HI adapter at the E2–*lacZ* junction. The adapter adds one codon to the coding sequence (J. Teem, personal communication).

Table 2. Effects of insertions between the UASs and TATA box

Insert	Without heme	With heme, glucose	Lactate
—	<0.5	126	1006
(16) 0.05	<0.5	33	418
(12) 0.10	<0.5	21	1220
(125) 0.10	<0.5	22	796
(C) 0.15	<0.5	27	112
(105) 0.25	<0.5	16	679
(7) 0.35	<0.5	178	1138
(118) 0.8	<0.5	<0.5	<0.5
(2) 2.3	295	1189	2619
(119) 3.0	<0.5	<0.5	<0.5
(6) 3.3	6.4	30	81
(13) 3.8	<0.5	<0.5	<0.5
(116) 4.0	<0.5	<0.5	<0.5
(5) 4.5	<0.5	31	<0.5
(8) 8.0	<0.5	<0.5	<0.5
(4) 9.0	<0.5	<0.5	<0.5

Yeast strains employed for β-galactosidase assays are as for Table 1. Plasmids are derivatives of pLGΔ-312 (18) that bear inserts in the *Sal* I site of random *E. coli* DNA fragments whose approximate sizes are indicated in kb. The insert isolate numbers are in parentheses.

galactose from this hybrid promoter display the normal *CYC1* 5′ ends that are specified by the *CYC1* TATA boxes (27). Thus, when induced by galactose, the production of β-galactosidase activity in the recombinant construct would provide assurance that the UAS-containing fragment does not interfere with mRNA splicing or fortuitously terminate transcription. When grown on lactate or glucose, the *GAL10* UAS is not active and β-galactosidase production would be entirely dependent upon activity of the inserted UASs.

The derivatives of HZ18, pLGInt1 and pLGInt2, bearing the UAS fragment in the *Sal* I site in either orientation were introduced into yeast and β-galactosidase activity was assayed in cells grown on galactose or lactate. In galactose medium, the activity of plasmids bearing the UAS insertion in either orientation is 60–70% of the parent (Table 3). Thus, effects of the insertion on synthesis or splicing of mRNA induced by the UAS_{GAL10} are small.

In lactate, the parent, HZ18, displayed no detectable β-galactosidase activity, whereas the derivatives pLGInt1 and pLGInt2 displayed only about 2 units (<1% of the activity of the induced UAS_{GAL10}). In their normal 5′ location, the activity of the derepressed UASs is roughly 2-fold higher than that of the induced UAS_{GAL10} (Table 3). Thus, <0.5% of the

Table 3. Activity of UASs inserted in an intron 3′ to TATA box

Plasmid	Glucose	Lactate	Galactose
pHZ18′	<0.5	<0.5	274
pLGInt1	<0.5	1.2	196
pLGInt2	<0.5	1.6	209
pLGΔ-312	—	1006	—
pLGSD5	—	—	498

pLGInt1 and pLGInt2 bear the *CYC1* UASs inserted into the *Sal* I site of pHZ18′. The insertion is in an intron of *rp51*, the following exon of which is fused to β-galactosidase (Fig. 4). Upstream of the intron is the first exon of *rp51* preceded by the *CYC1* ATG and TATA box. The *CYC1* TATA box is preceded by the *GAL10* UAS rendering HZ18′ galactose inducible. Cells were grown in minimal medium with a 2% carbon source and β-galactosidase was assayed. All assays were performed four times with an error of <20%. pLGΔ-312 and pLGSD5 contain the *CYC1* and *GAL10* UASs upstream of the *CYC1* TATA box (18, 20).

normal activity of the UASs is observed when the sites lie downstream of the TATA boxes even when they are proximate.

Direct RNA determinations corroborated the β-galactose data (Fig. 3). The UAS_{GAL10} in pLGInt1 activated the production of transcripts initiating at the correct *CYC1* start sites (18), whereas the *CYC1* UASs did not give rise to any detectable transcripts.

(*iii*) The UAS_{GAL10} does not interfere with the *CYC1* UASs in *cis* in lactate medium. It seemed possible that the failure of the *CYC1* UASs to function when inserted into the *rp51* intron in pLGInt1 and pLGInt2 might be due to the presence of two UASs in proximity to one another. Interference by the UAS_{GAL10} might be most pronounced in glucose medium since *GAL10* is under glucose repression (30). To test whether such inhibition could occur, the *GAL10* UAS was inserted just upstream of the *CYC1* UASs in pLGΔ-312 (see *Materials and Methods*). The activity of the *CYC1* UASs in this construct was determined in glucose and glycerol/ethanol media.

Most importantly, the UAS_{GAL10} was found *not* to inhibit the *CYC1* UASs in glycerol/ethanol media (Table 4). Similar results were observed in lactate media (unpublished data). Under these conditions, the uninduced *GAL10* site apparently behaves as neutral DNA. Thus, the failure of the *CYC1* UASs in pLGInt1 and pLGInt2 to function in derepressing media cannot be due to inhibition by the UAS_{GAL10}.

In glucose medium, in contrast, the activity of the *CYC1* UASs was inhibited to about 1/20th by the presence of the *GAL10* sequences (Table 4). We presumed that this inhibition was due to sequences on the *GAL10* fragment that medi-

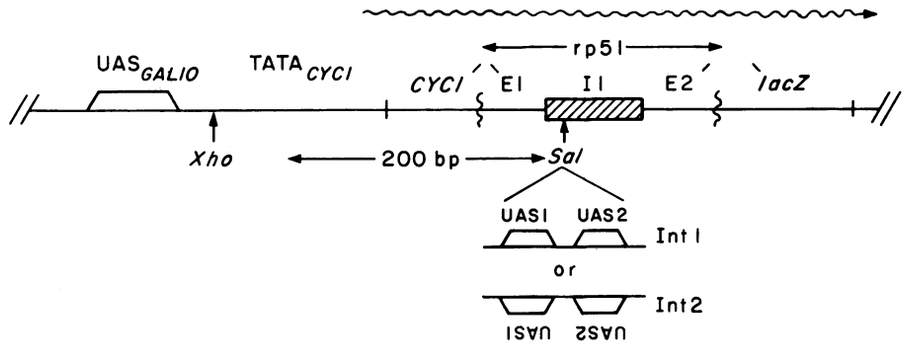

FIG. 2. Insertion of the UASs into the intron of a *CYC1–rp51–lacZ* tribrid gene. A DNA fragment bearing UAS1 and UAS2 extending from the *Sma* I site to the *Xho* I site of pLGΔ-312S was purified, rendered flush ended, and inserted into a filled-in *Sal* I site of pHZ18′ (28). Relevant features of pHZ18′ are the UAS from *GAL10*, the TATA boxes and 5′ leader from *CYC1*, one codon of *CYC1* fused to the first exon of *rp51* (E1), the *rp51* intron (I1), and the second exon of *rp51* (E2) fused to *lacZ*. Transcription of the tribrid gene is indicated by the wavy arrow. The recombinant plasmids bear UAS1 and UAS2 in their normal orientation (Int1) or inverted (Int2) and the sites are positioned roughly 250 bp from the TATA box region. This is the approximate distance between the UASs and TATA boxes in the intact *CYC1* promoter (21).

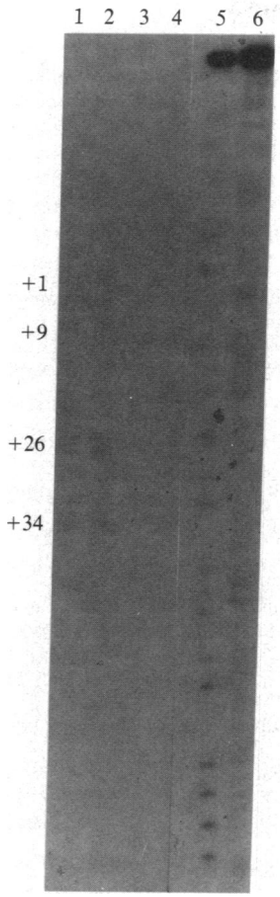

FIG. 3. S1 mapping of transcripts encoded by Int1 and Int2. RNA was isolated from BWG1-7a grown in galactose or lactate as described (18). A single-strand probe 5′ labeled at a *Bam*HI site at the *CYC1–lacZ* junction (Fig. 1) was isolated as described (18) and hybridized to 5 μg of total RNA, and S1 digestion and chemical sequencing were performed. Lanes 1 and 2, pLGInt1 and pLGInt2 galactose; lanes 3 and 4, pLGInt1 and pLGInt2 lactate; lanes 5 and 6, probe G, C + T.

ated glucose repression. To determine conclusively whether the UAS_{GAL10} fragment did encode glucose repression, we placed the site upstream of the TATA box of a *LEU2–lacZ* fusion. Similar constructs employing the *CYC1* UASs have been shown to place *LEU2* under control mediated by the UASs (21). The UAS_{GAL10}–$TATA_{LEU2}$ construct, unlike an intact *LEU2* promoter (21), clearly displayed repressibility by glucose (Table 4).

(*iv*) DNA sequences of *rp51* E1 and intron only partially block the activity of UASs situated upstream of the TATA box. Another possible reason why the UASs failed to function when inserted into the *rp51* intron could have been that a region of the DNA sequence between the TATA box and the *Sal* I insertion site of pHZ18′ blocks the propagation of the UAS activation signal. To test this possibility a DNA

Table 4. The *GAL10* UAS does not inhibit adjacent *CYC1* UASs in derepressing media

				Glucose	Glycerol/ EtOH
	UAS1	UAS2	$TATA_{CYC1}$	85	755
UAS_{GAL10}	UAS1	UAS2	$TATA_{CYC1}$	4	765
	UAS_{GAL10}		$TATA_{LEU2}$	122*	1550*

The UAS1 UAS2 $TATA_{CYC1}$ and UAS_{GAL10} UAS1 UAS2 $TATA_{CYC1}$ constructs were introduced into BWG2-9A-1 and β-galactosidase activity of the *CYC1–lacZ* gene was assayed as in previous experiments.

*The activity of the UAS_{GAL10} $TATA_{LEU2}$ construct was determined in strain LG1-2D, which expresses the GAL system constitutively due to a mutation in *gal80* (20). The activity of the *LEU2–lacZ* gene in this construct is a measure of UAS activity (21).

fragment extending from the TATA box region to the *Sal* I site of pHZ18 was inserted *between* the *CYC1* UASs in their normal, upstream location and the downstream *LEU2* TATA box in pLG3L (Fig. 4). The hybrid promoter region of pLG3L drives expression of a *LEU2–lacZ* fusion that is regulated by the *CYC1* UASs and depends on the *LEU2* transcription initiation region (21). Insertion of the fragment of HZ18 into the *Xho* I site of pLG3L in an inverted orientation requires UAS activation to move through the same sequences as in pLGInt1 and pLGInt2. The recombinant plasmid, pLGcrl, displayed a level of activity that was regulated by carbon source and was 10% that of pLG3L in lactate (Table 5). Thus, the sequences between the TATA box and intron of pHZ18 attenuate upstream activation by the UASs 10-fold in lactate when the sites are positioned 5′ to the TATA box.

DISCUSSION

In this report, two basic properties of the *CYC1* UASs have been established. First, the sites are functional when their orientation with respect to the TATA box has been inverted. Since the transcript upstream of the UASs in the yeast genome is not activated coordinately with *CYC1* (31), this bidirectionality is probably due to constraints on the mechanism of upstream activation rather than to a necessity to regulate divergent transcripts. Second, the sites are not functional when placed to the 3′ (or downstream) side of the TATA box (in either orientation) even when their separation from the TATA box is approximately that of the wild-type *CYC1* promoter.

In planning the optimal design of the "downstream" experiment, several considerations were addressed. First, the UASs had to be positioned at roughly their normal distance from the TATA box to avoid the possibility that the sites would fail to function because they were too far from the TATA box. Second, a convenient, precise assay of activation was desired. Third, and most importantly, a guarantee was needed that the UAS sequences were not serving to terminate transcription or destabilize the mRNA of which they were a part. In short, we wanted to be able to draw a strong

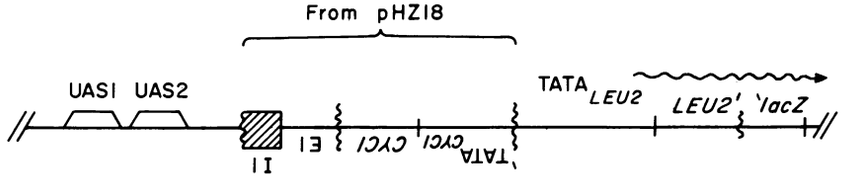

FIG. 4. Insertion of *rp51* and *CYC1* sequences between the UASs and a downstream TATA box. A DNA fragment from pHZ18′ extending from an *Mbo* II site in *CYC1* leader DNA to the *Sal* I site in the intron was purified and rendered flush ended. The fragment bears two of the three *CYC1* TATA boxes. This fragment was inserted into a filled-in *Xho* I site of pLG3L between UAS1 and UAS2 and the downstream *LEU2* TATA box. In pLG3L, the UASs activate transcription (wavy arrow) of a *LEU2–lacZ* fusion via the *LEU2* TATA box (21).

Table 5. Sequences in E1 of HZ18′ only partially block the activity of UASs positioned upstream of the TATA box

Plasmid	Glucose	Lactate
pLG3L	225	1482
pLGcrl	4.0	144

pLG3L (21), in which the *CYC1* UASs activate a *LEU2–lacZ* fusion, and pLGcrl were introduced into BWG2-9A-1 and β-galactosidase was assayed as in previous experiments.

conclusion from the possible negative result of no downstream activation.

To address these concerns, UAS1 and UAS2 were inserted into an intron of a *CYC1–rp51–lacZ* tribrid gene about 200 bp from the TATA box. Transcription of this gene was driven by a UAS_{GAL10}–$TATA_{CYC1}$ hybrid promoter that is under galactose inducibility. It has been shown that the intron is spliced efficiently from the *CYC1–rp51–lacZ* transcript, an event giving rise to β-galactosidase production when cells are induced by galactose (27). That the inserted UASs did not interfere with splicing or primary transcription was verified by the high levels of β-galactosidase (60–70% of the parent) of UAS-bearing plasmids when induced by galactose.

When cells bearing the construct were grown in lactate medium, which derepresses UAS1 and UAS2 to high levels when the sites reside upstream of the TATA box, little activity was observed (<0.5% of the activity observed when the sites are upstream of the TATA box). This result was obtained for both orientations of the sites. It is possible that this very low level of expression is due to mRNA initiation within the intron or E2 rather than at the *CYC1* initiation sites. Direct mRNA determinations indicated undetectable transcription in lactate medium. The UAS_{GAL10} did not interfere with the derepressed activity of the *CYC1* UASs in a construct in which both sites resided upstream of the TATA box.

The possibility that particular sequences between the TATA box and *rp51* intron cloning site blocked propagation of the UAS activation signal was investigated by inserting that region into a site between the UASs and a downstream TATA box. The inserted sequences imposed a considerable barrier to upstream activation (10-fold) as did some random sequences of *E. coli* DNA in the same size range (50–350 bp). (Larger insertions between the UASs and TATA box in the normal *CYC1* promoter (>800 bp) completely blocked UAS activation.) However, a substantial degree of activation was still observed to traverse the insert (10% of wild-type levels). This contrasts to the case in which the signal is unable to operate over the same sequences from a position 3′ to the TATA box (<0.5% wild-type levels).

Thus, it appears that the inactivity of the UASs situated in the intron of the *CYC1–rp51–lacZ* gene is largely due to their downstream location. A similar result has been obtained for the UAS of the *GAL10* gene (ref. 32; M. Lamphier and M. Ptashne, personal communication). The inability of the UASs to function in this location provides the first clue that these sites may differ mechanistically from certain enhancer sites. The immunoglobulin enhancer sites, for example, naturally occur in an intervening sequence between the exons encoding the variable and constant regions of the heavy chain of mouse immunoglobulin (12–14). If the UASs functioned by altering chromatin structure over a region, it is not obvious why the sites should fail to function when downstream and close to the TATA box. If, alternatively, the UASs comprised RNA polymerase entry sites, the sites would not be operable in a downstream location unless RNA polymerase were able to turn around at the TATA box. Thus, this model of upstream activation is favored from our results. Although the results in no way constitute proof of the model, they allow the formulation of more direct experiments that bear on the notion that RNA polymerase II or some other transcription factor enters the template at the UASs.

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