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No strict alignment is required between a transcriptional activator binding site and the "TATA box" of a yeast gene

(GAL4/*Saccharomyces cerevisiae*/lexA)

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ABSTRACT **GAL4 is a transcriptional activator of the galactose metabolism genes in the yeast *Saccharomyces cerevisiae*. We show that GAL4 expressed in yeast activated transcription equally well when a single GAL4 binding site was placed at any of nine positions upstream of the *GAL1* (galactokinase gene) "TATA box." We chose a sufficient number of positions for the binding site to ensure that, in several of these positions, GAL4 was on the opposite side of the DNA helix with respect to the TATA box. Smaller GAL4 derivatives were similar to wild-type GAL4 in that they also activated transcription in a manner independent of the side of the DNA helix they bound with respect to the TATA box. Unlike wild-type GAL4, however, these smaller GAL4 derivatives activated transcription better when we placed a binding site progressively closer to the TATA box over a distance of 34 base pairs.**

GAL4, an 881-amino acid protein, activates transcription of the galactose metabolism genes in the yeast *Saccharomyces cerevisiae* (1–4). The DNA-binding function of GAL4 lies within the first 74 amino acids (5), and portions of the remainder of the molecular bear the transcriptional-activation function (6, 7). The DNA-binding portion of GAL4 can be replaced with the DNA-binding domain of the *Escherichia coli* repressor LexA; this LexA–GAL4 hybrid protein expressed in yeast activates transcription of an appropriate *GAL1* (galactokinase gene) promoter. In all cases, activation of transcription by activators bearing either the GAL4 DNA-binding domain or the lexA DNA-binding domain require a GAL4 binding site or a *lexA* operator, respectively, upstream of the transcription start (4, 6). Ma and Ptashne (7) have shown that there are two regions of GAL4 each of which is sufficient to activate transcription when attached to the DNA-binding portion of GAL4. Also, many peptide sequences encoded by fragments of *E. coli* DNA can activate transcription when they are attached to the DNA-binding portion of GAL4 (8). One of the *E. coli*-encoded activating sequences activates transcription when attached to the DNA-binding domain of LexA (ref. 8; see also ref. 9). Giniger and Ptashne (10) have shown that a short peptide, designed to form an amphipathic α-helix with negative charges on one surface, is sufficient to substitute for the activation function of GAL4.

Several laboratories have shown that proteins bound to the same side of the DNA helix interact with each other much more readily than do proteins bound on opposite sides of the DNA helix both *in vitro* and *in vivo* (11–15). Electron microscopy has revealed that this protein–protein interaction, with concomitant formation of DNA loops, is favored when the binding sites for these proteins are aligned on the same side of the DNA helix (13, 16). Direct interaction between activators and "TATA box"-binding proteins through the formation of DNA loops may also be involved in activation of transcription in eukaryotes (17).

In this paper, we show that all transcriptional activators tested with the DNA-binding domains of either LexA or GAL4 function independently of their alignment to the 5′ TATAAA 3′ sequence in the *GAL1* promoter. Unlike wild-type GAL4, however, smaller derivatives of GAL4 activate transcription better when we placed a binding site progressively closer to the 5′ TATAAA 3′ sequence over a distance of 34 base pairs. We also show explicitly that the 5′ TATAAA 3′ sequence (the *GAL1* TATA box) is essential for proper transcription initiation.

MATERIALS AND METHODS

Strains and Media. Yeast strains DBY745(*leu2*, *ura3*, *ade1*) and GGY1(Δ*gal4*, Δ*gal80*, *ura3*, *his3*, *leu2*) have been described (18, 19). We constructed the following derivatives of DBY745 and GGY1, each of which has a *GAL1-lacZ* fusion plasmid with a single "17-mer" binding site (18) located upstream (by the indicated number of base pairs in parentheses) from the *GAL1* TATA box in single-copy integration at the *URA3* locus: GGY1::pMA60-12 and DBY745::pMA60-12 (33 base pairs), GGY1::pMA60-4 and DBY745::pMA60-4 (35 base pairs), GGY1:pMA60-8 and DBY745::pMA60-8 (37 base pairs), GGY1::pMA60 and DBY745::pMA60 (45 base pairs), GGY1::pMA60+4 and DBY745::pMA60+4 (49 base pairs), GGY1::pMA60X14 and DBY745::pMA60X14 (51 base pairs), GGY1::pMA23(Δ2-μm) and DBY745::pMA23 (Δ2-μm) (60 base pairs), GGY1::pMA60X26 and DBY745::pMA60X26 (63 base pairs), and GGY1::SV15(Δ2-μm) and DBY745::SV15(Δ2-μm) (67 base pairs).

We also constructed the following derivatives of GGY1, which have a *GAL1-lacZ* fusion plasmid with a single *lexA* operator located upstream (by the indicated number of base pairs in parentheses) from the *GAL1* TATA box in single-copy integration at the *URA3* locus: GGY1::Lex23 (55 base pairs), GGY1::Lex23(+4) (59 base pairs), GGY1::Lex23(+7) (62 base pairs), GGY1::Lex23(+10) (65 base pairs), GGY1::Lex20 (66 base pairs), GGY1::Lex20(+4) (70 base pairs), GGY1::Lex20(+7) (73 base pairs), GGY1::Lex20(+10) (76 base pairs), GGY1::Lex1 (112 base pairs), GGY1::Lex1(+4) (116 base pairs), GGY1::Lex1(+7) (119 base pairs), and GGY1::Lex1(+10) (122 base pairs). All distances in this paper are measured as described in Fig. 1. GGY1::Lex24 has a *GAL1-lacZ* fusion with a *lexA* operator and no TATA box. We made all 17-mer and *lexA*-operator *GAL1-lacZ* fusion plasmids using standard DNA-manipulation procedures. Details of their constructions are available upon request.

Yeast were grown in either yeast extract/peptone/dextrose-rich medium or minimal medium (20), and the carbon sources for β-galactosidase and RNA analyses were 2% (wt/vol) galactose, 2% (vol/vol) glycerol, and 2% (vol/vol) ethanol. All plasmid constructions were performed with

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either of the *E. coli* strains MM294 or JM101, and bacteria were grown in LB medium (21).

Yeast RNA Analysis, Yeast Transformation, and Assay of β-Galactosidase. Procedures of yeast RNA preparation and primer extension were as described (7, 22). Yeast cells were made competent for transformation by treatment with lithium acetate (23). We determined that integration of plasmids was in single copy in the yeast chromosome by Southern blot analysis (data not shown). Liquid β-galactosidase assays were performed as described (24, 25), and the standard error was less than 20%.

Replacing the *GAL1* TATA Box with Oligonucleotides. We had two oligonucleotides synthesized 5′ TCGAGTATAAA 3′ and 5′ TCGATTTATA 3′ (Harvard Microchemistry Facility). We hybridized and inserted these oligonucleotides into a *Xho* I site in either orientation, and one Xho I site was regenerated upstream of the 5′ TATAAA 3′ sequence. LRIΔ24 (ref. 25) and LRI(25-25, 24) were digested with *Xho* I, and the oligonucleotides were inserted to generate LR1(24)T$^+$, LR1(24)T$^-$, LR1(25-25, 24)T$^+$, and LR1(25-25, 24)T$^-$. The "+" and "−" indicate the orientation of the oligonucleotide, where + indicates the "forward," 5′ TATAAA 3′, orientation and − indicates the "reverse," 5′ TTTATA 3′, orientation relative to *GAL1*. The endogenous 5′ TATAAA 3′ sequence and the 5′ TATAAA 3′ sequence provided by the oligonucleotides are exactly the same distance from the *GAL1* transcription start. LR1(25-25, 24) (D.M.R., unpublished data) is identical to LR1Δ24 except that all DNA sequenced from position 405 (two base pairs downstream of the GAL4 binding-site 2; ref. 24) to the *Xho* I site are deleted. pRY131 contains *GAL1-lacZ* with a wild-type promoter (24). The *GAL1-lacZ* fusion genes described in this section are on high-copy-number episomes containing the yeast 2-μm origin of replication; in all other sections, we integrated the *GAL1-lacZ* plasmids into the yeast genome in single copy.

Plasmids That Encode GAL4 and LexA Derivatives. Plasmids GAL4-B3, GAL4-B17, GAL4-B42 (8) encode the first 147 amino acids of GAL4 attached to *E. coli*-encoded activation sequences, and pMA236 (7) encodes the GAL4 derivative containing activation region I, GAL4-(1–238). pMA457 (8) encodes the LexA-B42 fusion protein, which consists of the DNA binding domain of LexA-(1–87) attached to the *E. coli*-encoded activator sequence in GAL4-B42. pMA411 is a derivative of pRB1027 (6) and encodes the LexA–GAL4 fusion protein, which consists of the DNA binding domain of LexA, LexA-(1–87), attached to the activation region of GAL4, GAL4-(74–881). These plasmids also contain the yeast *HIS3* gene and the 2-μm origin of replication. Plasmid pLPK8 (5) encodes wild-type GAL4 and contains the yeast *Leu2* gene and the 2-μm origin of replication. We made B17Hi, the "*HIS3*-integrating" form of GAL4-B17, by inserting an *Xba* I 8-mer linker (New England Biolabs) into the *Sal* I site of GAL4-B17 (8) and inserting the

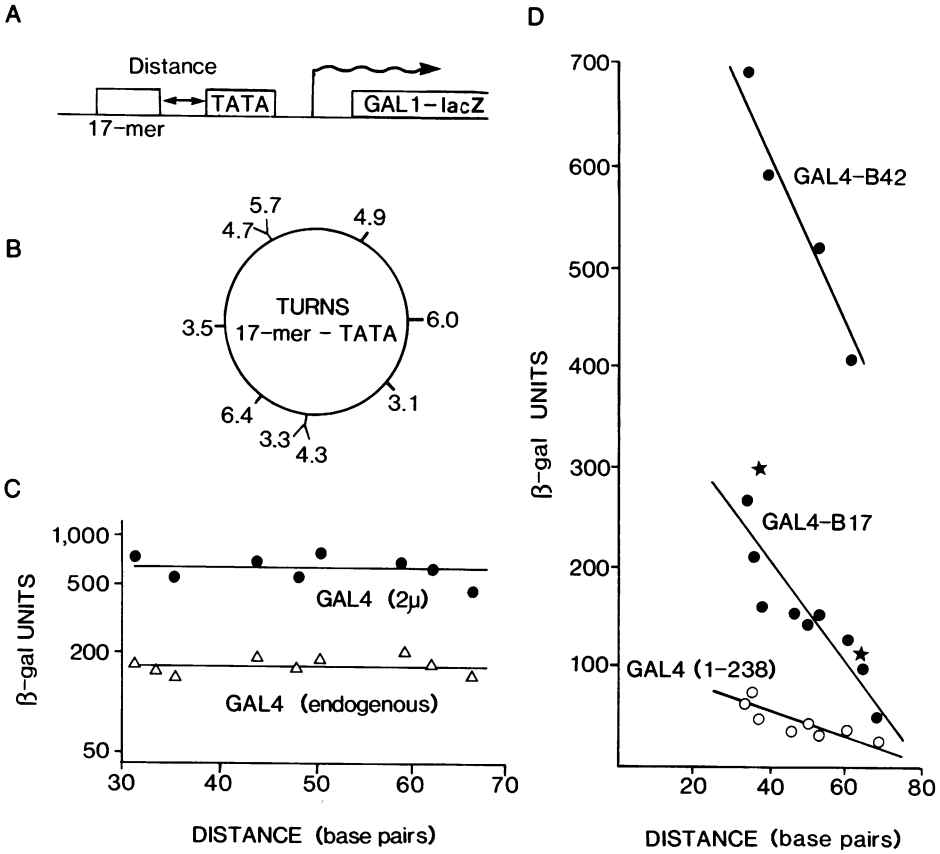

FIG. 1. Effects of changing the distance between a single GAL4 binding site, the "17-mer," and the *GAL1-lacZ* TATA box on transcription levels. (*A*) Diagram showing that the 17-mer GAL4 binding site is at several positions upstream of the *GAL1* 5′ TATAAA 3′ sequence; the distance is measured from the central base pair of the activator binding site to the first thymidine of the 5′ TATAAA 3′ sequence, inclusive. (*B*) A "helical wheel" shows that, if the DNA in the *GAL1* promoter is standard B-DNA with a periodicity of 10.5 base pairs per turn, the 17-mer GAL4 binding site is at several positions on the DNA helix with respect to the *GAL1* 5′ TATAAA 3′ sequence. (*C*) GAL4 activates transcription equally well when a 17-mer is placed from 33 to 67 base pairs upstream from the *GAL1* 5′ TATAAA 3′ sequence. GAL4(2-μm) indicates that GAL4 was produced from the high-copy-number episome pLPK8 in *gal4*-deletion strains, and GAL4 (endogenous) means that GAL4 strains were used. (*D*) When small GAL4 derivatives are made in *gal4*-deletion strains, *GAL1-lacZ* β-galactosidase enzyme levels increase progressively the closer a "17-mer" is placed to the *GAL1* 5′ TATAAA 3′ sequence. GAL4-B42 and GAL4-B17 contain the first 147 amino acids of GAL4 attached to *E. coli*-encoded activator sequences. The stars mark the β-galactosidase enzyme levels of GAL4-B17 (plasmid B17Hi) single-copy integrations into the chromosome in *gal4*-deletion strains that have a 17-mer 35 and 63 base pairs upstream of the *GAL1* 5′ TATAAA 3′ sequence.

Xho I–*Xba* I fragment from this plasmid into the *Xho* I to *Xba* I sites within *GAL4* of plasmid AA1 (A. Abeliovich, G. Gill, and M.P., unpublished data). AA1 has a 1.7-kilobase (kb) *Bam*HI fragment containing the *HIS3* gene inserted into the *Bam*HI site of pBR322 and the *ADH*(promoter)–*GAL4*–*ADH*(terminator) *Bam*HI fragment from pLKC15 (26) inserted into the *Pvu* II site (*ADH* = gene for alcohol dehydrogenase).

RESULTS

GAL4 Activated Transcription Equally Well from All Positions Upstream of the *GAL1* TATA Box. We placed a single GAL4 binding site (the 17-mer; ref. 18) at nine different positions that ranged from 33 to 67 base pairs upstream of the *GAL1* TATA box (see below). We assayed transcriptional activities of these promoters in both *GAL4* strains and *gal4*-deletion strains provided with *GAL4* on a high-copy-number episome (pLPK8 from ref. 5). In both cases, we observed no significant difference in transcriptional activity (β-galactosidase and mRNA levels) for any of these nine promoter-spacing mutants (Fig. 1*C* and Fig. 2, respectively). We observed higher β-galactosidase levels when *GAL4* was on a high-copy-number plasmid than when it was in the chromosome probably because the strain GGY1 is healthier than DBY745 and consistently gave higher β-galactosidase enzyme levels for *lacZ* fusion genes (D.M.R., unpublished observations).

Small Derivatives of GAL4 Activated Transcription Better When We Placed a Binding Site Progressively Closer to the TATA Box. We tested the transcriptional-activation abilities of small GAL4 derivatives in *gal4*-deletion strains. Again we used strains containing a single 17-mer placed between 33 and 67 base pairs upstream of the *GAL1* TATA box (Fig. 1*D*). In contrast to the results we obtained with wild-type GAL4, the small GAL4 derivatives activated transcription better when we placed a binding site progressively closer to the TATA box. The activators GAL4-B17 and GAL4-B42 contain *E. coli*-encoded activating sequences of 28 and 95 amino acids, respectively, fused to the DNA-binding portion of GAL4 (amino acids 1–147; ref. 8). GAL4-(1–238) (ref. 7; see also ref. 19) contains the DNA-binding portion of GAL4 and transcriptional activating region I.

The plasmids containing GAL4-B17 and GAL4-B42 are unstable as episomes in the yeast strains used in this experiment. As a control against possible artifacts caused by this instability, we integrated GAL-B17 into the chromosome in single copy (plasmid B17Hi, this paper) in each of two yeast strains containing a binding site in different positions upstream of the *GAL1* TATA box. GAL4-B17 activated transcription better when it bound closer to the TATA box both when it was produced from an unstable episome (Fig. 1*D*) and when it was stably produced from the chromosome (Fig. 1*D*, stars); therefore, we believe that the results presented with the GAL4 derivatives expressed on episomes are accurate. When no GAL4 derivative was present, the *GAL1-lacZ* genes produced <1 unit of β-galactosidase (data not shown).

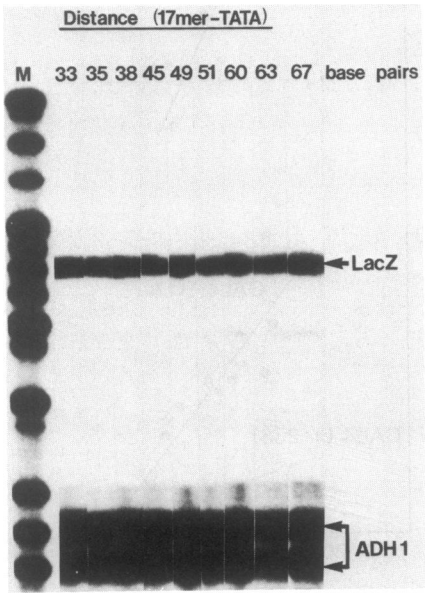

FIG. 2. There are no effects on GAL1-lacZ mRNA initiation when we vary the distance between a 17-mer and the 5′ TATAAA 3′ sequence in *GAL4* strains. Lane M is pBR322 cut with *Hpa* II DNA markers. We placed a single 17-mer GAL4 binding site 33, 35, 37, 49, 60, 63, and 67 base pairs upstream of the *GAL1* 5′ TATAAA 3′ sequence, as indicated. We grew the yeast in medium containing galactose as the carbon source, isolated the RNA, and performed primer extension analysis using single-stranded DNA primers complementary to lacZ mRNA and, as an internal control, to ADH1 mRNA. The bands labeled LacZ are the primer extension products of the GAL1-lacZ mRNA; the bands labeled ADH1 are the primer extension products of ADH1 mRNA.

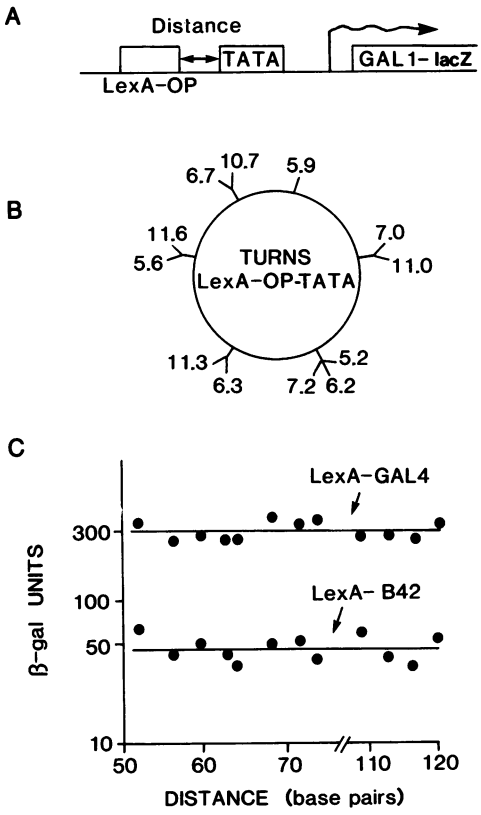

FIG. 3. There is no effect on β-galactosidase enzyme levels when we vary the distance between a *lexA* operator and the *GAL1-lacZ* 5′ TATAAA 3′ sequence in *gal4*-deletion strains provided with LexA fusion activators. (*A*) Diagram indicating that a single *lexA* operator is placed in several positions upstream of the *GAL1* 5′ TATAAA 3′ sequence. Distance is measured as in Fig. 1. (*B*) A "helical wheel" shows that, if the DNA in the *GAL1* promoter is standard B-DNA with a periodicity of 10.5 base pairs per turn, the *lexA* operator is at several positions on the DNA helix with respect to the *GAL1* 5′ TATAAA 3′ sequence. (*C*) LexA-GAL4 and LexA-B42 induce constant amounts of β-galactosidase enzyme levels when a *lexA* operator is placed 55–122 base pairs upstream from the *GAL1* 5′ TATAAA 3′ sequence. LexA-GAL4 and LexA-B42 consist of the DNA-binding domain of LexA-(1–87) attached to the transcriptional activation region of GAL4-(74–881) and to an *E. coli*-encoded activation sequence, respectively.

LexA Fusion Proteins Activated Transcription Equally as Well from All Positions Upstream of the *GAL1* TATA Box. We introduced the LexA-GAL4 (6) and LexA-B42 (8) fusion proteins into yeast strains that contained a single *lexA*-operator placed in 12 different positions that ranged from 55 to 122 base pairs upstream of the *GAL1* TATA box. These LexA fusion proteins contained the DNA-binding domain of LexA, LexA-(1–82), attached to GAL4-(74–881) and to the 95-amino acid *E. coli*-encoded activation region from GAL4-B42, respectively. As with wild-type GAL4, both of these LexA-fusion proteins activated transcription at identical levels from all positions upstream of the *GAL1* TATA box (Fig. 3). We detected no transcriptional activity when the *GAL1* TATA box was deleted, when no *lexA* operator was present, and when no LexA fusion protein was present (data not shown).

A TATA Box Was Required for Proper *GAL1* Transcription. Deletion of DNA including the sequence 5′ TATAAA 3′ in the *GAL1* promoter abolished GAL4-mediated transcription from the wild-type initiation site as measured by S1 nuclease and primer extension analyses (ref. 25 and Fig. 4 *Right*, respectively). GAL4-mediated transcription initiating at the wild-type site was restored when an oligonucleotide containing the sequence 5′ TATAAA 3′ was placed in the deleted region in the forward but not the reverse orientation (Fig. 4).

In the absence of a 5′ TATAAA 3′ sequence or when the 5′ TATAAA 3′ oligonucleotide was in the reverse orientation, there was no GAL4-mediated transcription from the wild-type initiation site, but there was GAL4-mediated induction of β-galactose activity (Fig. 4). We attribute this β-galactose activity to lower-efficiency "cryptic" TATA boxes in the *GAL1* promoter (25).

DISCUSSION

We presented several experiments that showed that no strict alignment is required between a transcriptional activator binding site and the TATA box of a yeast gene. Thus, we found that GAL4 activated transcription at nearly identical levels from nine positions that are 33 to 67 base pairs upstream of the 5′ TATAAA 3′ sequence in the *GAL1* promoter (Fig. 1*C* and Fig. 2). These positions of the binding site covered a range such that, in several of these positions, GAL4 was on the opposite side of the DNA helix with respect to this 5′ TATAAA 3′ sequence (Fig. 1*B*). We also tested the transcriptional activation function of two LexA fusion proteins on genes bearing a *lexA* operator in 12 positions between 55 and 122 base pairs upstream of the 5′ TATAAA 3′ sequence (Fig. 3*C*). Unlike GAL4, which is proposed to have a "Zn-finger" DNA-binding domain (27), LexA is proposed to have a helix-turn-helix DNA-recognition domain and presumably binds, as does phage λ repressor, to a single face of the DNA helix (28). Again, as with wild-type GAL4, the LexA fusion proteins activated transcription equally in all positions upstream of *GAL1*. Consistent with our results is the observation that cooperative DNA binding of GAL4 to two sites occurs even when the sites are on opposite sides of the DNA helix (29).

We showed that the region containing the 5′ TATAAA 3′ sequence contains the *GAL1* TATA box and that an oligo-

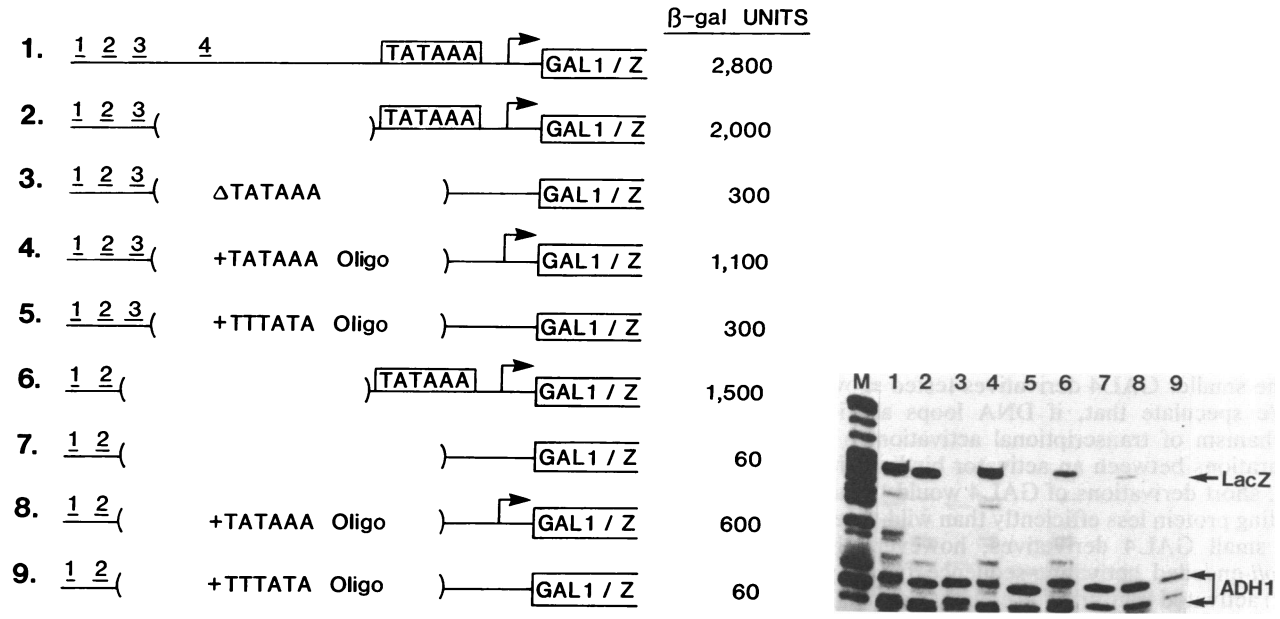

FIG. 4. The sequence 5′ TATAAA 3′ is the *GAL1* TATA box. (*Left*) Lanes: 1, pRY131 (*GAL1-lacZ* with a wild-type promoter: 1–4 represent the four GAL4 binding sites in the *GAL1* promoter in which site 4 is 199 base pairs from the TATA box); 2, LR1(Δ23) (*GAL1-lacZ* with sites 1–3 present in which, because of a deletion of *GAL1* promoter DNA, site 3 is 85 base pairs from the TATA box); 3, LR1(Δ24) (*GAL1-lacZ* with sites 1–3 present but with the 5′ TATAAA 3′ sequence deleted); 4, LR1(24)T$^+$ (same as lane 3 except that an oligonucleotide containing the sequence 5′ TATAAA 3′ is inserted in the forward (see *Materials and Methods*) orientation 50 base pairs downstream of site 3); 5, LR1(24)T$^-$ (same as lane 4 except that the oligonucleotide is in the reverse orientation); 6, LR1(25-25, 23) (*GAL1-lacZ* with sites 1 and 2 present in which, because of a deletion of *GAL1* promoter DNA, site 2 is 49 base pairs from the TATA box); 7, LR1(25-25, 24) (*GAL1-lacZ* with sites 1 and 2 present but with the 5′ TATAAA 3′ sequence deleted); 8, LR1(25-25, 24)T$^+$, same as lane 7 except that the 5′ TATAAA 3′ oligonucleotide is in the forward orientation 19 base pairs downstream of site 2); 9, LR1(25-25, 24)T$^-$, same as lane 8 except that the oligonucleotide is in the reverse orientation. (*Right*) A 5′ TATAAA 3′ sequence is sufficient for correct transcription initiation. The bands labeled LacZ are the primer extension products of the *GAL1-lacZ* mRNA; the bands labeled ADH1 are the primer extension products of ADH1 mRNA (see Fig. 2). Lanes: M, pBR322 cut with *Hpa* II DNA markers; 1–9, primer extension analyses of mRNA isolated from yeast containing the corresponding *GAL1-lacZ* plasmids as in *Left*. There is correct transcription initiation of *GAL1-lacZ* when the endogenous 5′ TATAAA 3′ sequence is present (lanes 1, 2, and 6) and when the 5′ TATAAA 3′ oligonucleotide is in the forward orientation (lanes 4 and 8). There is no correct transcription initiation of *GAL1-lacZ* when there is a deletion of the region containing the 5′ TATAAA 3′ sequence (lanes 3 and 7) and when the oligonucleotide is in the reverse orientation (lanes 5 and 9).

nucleotide containing this sequence substituted for the TATA box (Fig. 4, see also refs. 30–33). Selleck and Majors (34) have shown by photofootprinting analysis that a protein binds to the *GAL1* 5′ TATAAA 3′ sequence only when the gene is active. Also, several laboratories have shown that column fractions required for specific *in vitro* transcription contain proteins that bind to *Drosophila* (35, 36) and mammalian TATA box (37, 38).

Our results with yeast *GAL1* are in contrast to the results of Takahashi *et al.* (14), who showed that the simian virus 40 early promoter elements must be properly aligned for efficient transcription of the tumor antigen genes in HeLa cells. Our results are not peculiar to yeast, however, since Wirth *et al.* (39) and Chodosh *et al.* (40) demonstrated that the "octamer" transcriptional activator binding site and the major late transcription factor binding site, respectively, activated transcription to identical levels when they were placed at several closely spaced positions upstream of mammalian TATA boxes. How might we explain the lack of requirement for a strict alignment between an activator (e.g., GAL4) and its target (e.g., the TATA-binding protein or RNA polymerase II), while assuming that they contact each other through the formation of DNA loops? The observation might be explained if, when bound to DNA, the interacting regions of one or both of the proteins protrude laterally from opposite (or nearly opposite) sides of the DNA helix. In this case, any helical periodicity would be extremely difficult to detect. Alternatively, the interaction between the activator and its target might be sufficiently strong to supply the energy required to twist as well as bend the DNA; DNA supercoiling might decrease the energy required for this loop formation (see ref. 41). It is also possible that the activator target is highly flexible and can wrap around the DNA; the long heptapeptide-repeat sequence at the carboxyl terminus of the largest subunit of RNA polymerase II could provide such a function (42, 43).

In contrast to wild-type GAL4 and the LexA fusion proteins, smaller derivatives of GAL4 activated transcription in a distance-dependent manner over a distance of just 34 base pairs (Fig. 1*D*). When we moved a GAL4 binding site progressively closer to the TATA box, transcription increased in a roughly linear manner almost 5-fold for derivative GAL4-B17 and almost 2-fold for derivatives GAL4-B42 and GAL4-(1–238) (Fig. 1*D*). This increase in transcription was not dependent on the strength of the activator because all of the smaller GAL4 derivatives tested showed this effect.

We speculate that, if DNA loops are involved in the mechanism of transcriptional activation in yeast, at large separations between an activator binding site and a TATA box, short derivations of GAL4 would "reach" the TATA-binding protein less efficiently than wild-type GAL4. Unlike the small GAL4 derivatives, however, the small LexA *E.coli*-encoded activating-sequence fusion protein (LexA-B42) activated transcription equally well at all distances from the TATA box. This might indicate that LexA-B42 is more flexible or more extended than GAL4-B42.

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