Two Alternative Pathways of Transcription Initiation in the Yeast Negative Regulatory Gene *GAL80*

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The yeast *GAL80* gene, encoding a negative regulatory protein of galactose-inducible genes, shows both constitutive and galactose-inducible expression. The inducible transcription is under the control of Gal4p, a common activator for the galactose-inducible genes, which binds to an upstream activation sequence, called UAS_G, spanning between –105 and –89 in the 5'-flanking region of *GAL80*. Here we demonstrate that the constitutive transcription started at +1, whereas the inducible transcription occurs from a set of downstream sites at +37, +47, +56, and +67. Both transcriptions were enhanced 10-fold by another UAS, whose 5' boundary is located between –195 and –185. Gal4p stimulated transcription, which depends on the TATA box located at –20, from all the downstream sites. By contrast, the constitutive transcription depended on a small region of less than 16 bp long encompassing the +1 site, which directed transcription even in the absence of both the TATA box and the UASs. When a fragment covering that region was inserted immediately upstream of the open reading frame of *HIS3*, the resulting gene fusion, if introduced into a *his3* yeast strain, supported growth on histidine-lacking medium. We detected by gel retardation assay a protein specifically interacting with this fragment. All the transcriptions observed in the *in vivo* experiments were faithfully reproduced in a cell-free transcription system. From these results, we suggest that initiation of *GAL80* transcription involves two alternative pathways; one is initiator dependent, and the other is Gal4p regulated and TATA dependent.

Transcriptional regulation of eukaryotic genes involves interactions between regulatory proteins and the basal transcriptional machinery. The regulatory proteins, which confer the response to extra and intracellular signals, bind to specific DNA sequences termed enhancers or upstream activation sequences (UASs). These specific factors appear to control transcriptional efficiency either positively or negatively (9, 21, 41). Their regulatory signals are transmitted to the basal transcriptional machinery formed on core promoter elements by mechanisms that are not understood (8, 25, 38, 40, 41). Two distinct elements have been identified as constituents of the core promoter, the TATA box and the initiator. The TATA box, which is required for accurate and efficient transcription, helps ordered assembly of general transcription factors and RNA polymerase II into the preinitiation complex (PIC) (56). However, some genes function in the absence of the TATA box. A TATA-less promoter contains an initiator element that overlaps the transcription start site. The initiator contains all the information required for transcription initiation and directs accurate initiation by itself (51, 55). Initiator-dependent transcription may involve a PIC that is different from the PIC formed on the TATA box (39, 40, 42, 55, 57).

The regulatory circuit controlling the expression of galactose-inducible genes (collectively called *Gal* genes, hereafter) of the yeast *Saccharomyces cerevisiae* is a paradigm of eukaryotic gene expression with a remarkable generality. Transcriptional activation of *Gal* genes takes place within several minutes after addition of galactose into a yeast culture growing on nonfermentable carbon sources. The rapid expression involves an interplay of three regulatory proteins, Gal4p, Gal80p, and Gal3p (22). All the *Gal* genes have one or more sets of a

cis-acting element called UAS_G, which is recognized by Gal4p, which functions as a transcription activator. Gal4p interacts with the negative regulatory protein Gal80p *in vivo* (26) as well as *in vitro* (6, 28, 36). In the absence of galactose, the activation function of Gal4p is inhibited by direct interaction with Gal80p, while Gal4p remains bound to the UAS_G (27, 47). In the induced state, the activation domain of Gal4p is thought to be exposed either by dissociation from Gal80p (35) or by a conformational change of the Gal4p-Gal80p complex (26). A precise balance of intracellular concentrations of these proteins appears to be crucial for the regulated expression of *Gal* genes. Thus, overproduction of Gal4p (17, 24) results in constitutive expression of *Gal* genes, and concomitant overproduction of Gal80p along with Gal4p alleviates the constitutive expression (17). Furthermore, overproduction of Gal3p has recently been demonstrated to cause constitutive expression of *Gal* genes (2).

To fully understand the regulatory circuit controlling the expression of the *Gal* genes, we must know how the expression of these regulatory genes themselves is regulated. Recent studies have revealed that the weak constitutive expression of *GAL4*, which is subject to the glucose repression, is mediated by two positive *cis* elements (13, 14). By contrast, transcription of *GAL80* is refractory to the glucose repression but is regulated positively by Gal4p and negatively by Gal80p itself (19, 49). A set of UAS_Gs was found in the 5'-flanking region of *GAL80* (31), to which Gal4p was shown to bind *in vivo* (27) as well as *in vitro* (3). *GAL80* is expressed constitutively to a significant level under the control of its upstream region (19), but no further information had been available. In this report, we studied the expression of *GAL80* by *in vivo* as well as *in vitro* analyses. These experiments have revealed new *cis* elements and a new factor and have provided evidence that transcription of *GAL80* is regulated through two different pathways; one involves the TATA box, whereas the other involves an initiator.

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MATERIALS AND METHODS

Yeast strains and media. The yeast strains used in this study were as follows: NOY396 (*MAT α ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100*) was kindly supplied by Y. Nogi. HS18 (*gal4::HIS3*) was a *gal4* null strain of NOY396. BJ2168 (*MAT α ura3-52 leu2 trp1 prb1-1122 pep4-3 prc1-407 gal2*) was used for preparation of yeast nuclear extract.

YPGlyLac medium consisted of 1% yeast extract, 2% Bacto Peptone, 3% glycerol, and 2% sodium lactate. YPGlyLacGlu and YPGlyLacGal media contained 2% glucose and 2% galactose, respectively, in the YPGlyLac medium. Enriched synthetic medium (ESGlyLac) contained 3% glycerol and 2% sodium lactate as the sole carbon sources in a solution of 0.66% yeast nitrogen base, 0.5% Casamino Acids, 0.002% adenine sulfate, 0.002% uracil, and 0.002% tryptophan. ESGlyLacGlu and ESGlyLacGal media contained 2% glucose and 2% galactose, respectively, in ESGlyLac. The growth of transformants harboring plasmids containing initiator-*HIS3* fusions was assayed on a plate containing 20 mM 3-aminotriazole in SC medium (48) lacking histidine and tryptophan.

Plasmid construction. Plasmid pG80-1400 was constructed as follows: Plasmid pTF8005 carrying *GAL80* (31) was digested with *HindIII* (at nucleotide position -1,400 with respect to the transcription initiation site at +1 [31]), blunt ended by klenow fragment, and digested with *BglII* (at +84). The resulting 1.5-kb fragment was cloned into the *SmaI* and *BglII* sites of pSK142, a derivative of pUC119 with modification at the *SacI* site which had been converted to a *BglII* site. Derivatives of *GAL80* with various deletions in the 5'-flanking region were created from pG80-1400 by digestion with *DraI* (at -218), *SspI* (at -136), *HpaII* (at -106), *MnII* (at -72), or *AflIII* (at -7). The resulting fragments were blunt ended by Klenow fragment or by T4 DNA polymerase and digested with *BglII* (at +84). Each of the liberated fragments containing *GAL80* with various lengths of the 5'-flanking region was cloned into the *SmaI* and *BglII* sites of pSK142. These plasmids were referred to as pG80-218, pG80-136, pG80-106, pG80-72, and pG80-7, respectively. Plasmids pG80-208, pG80-195, and pG80-185 were constructed by exonuclease III digestion (18) from pG80-218, using a Kilo-sequencing deletion kit (TAKARA Shuzo Co., Ltd., Kyoto, Japan). To construct pG80Tpm, the TATA sequence at -20 of pG80-218 was altered by PCR with the primer 5' +1 GTGACATGTGATGTAGATATCTTCAGGT TC -29 3' (changed nucleotides were underlined). To construct pG80Tdm, a TATA-like sequence lying at -68 of pG80-218 was altered by PCR with the primer 5' -45 GAGC TTTTGTTATTGTTAACGTAGCAGAC -74 3'. Plasmid pG80Im, in which the sequence from -3 to +9 was changed to the corresponding sequence of *GAL7* (30), was also constructed by PCR with template pG80-218 and the primer 5' +33 AGATTGTATACGCTGGTCGGCAAGTTCACACGT TTTTCATG -7 3'.

Reporter plasmids containing *GAL80-lacZ* fusions were constructed as follows: plasmid pLG669Z (15), a YE24 plasmid harboring a *CYC1-lacZ* fusion, was digested with *SalI* and *BamHI* to remove the *CYC1* promoter region. The resulting vector fragment was ligated with the *SalI-BglII* fragment of the pG80 series plasmid to yield the pEZ series.

Tester genes of the pIG80 series were constructed as follows: plasmid pTF8005 (31) was digested with *EcoRI* (at +390), blunt ended by Klenow fragment, and ligated with *BamHI* linker. This was digested with *HindIII* (at +1,700), and the liberated 1.3-kb *BamHI-HindIII* fragment carrying part of the open reading frame (ORF) of *GAL80* was cloned into the *BamHI* and *HindIII* sites of YIp5 to yield pIG80V. The

SalI-BglII fragment of the pG80 series was cloned into the *SalI* and *BamHI* sites of pIG80V.

Plasmids containing initiator-*HIS3* fusions were constructed as follows: the *EcoRI* (at -8)-*XhoI* (at +880) fragment of pBM252 (23), which contains the *HIS3* ORF but does not carry the *HIS3* promoter, was cloned into the *EcoRI* and *XhoI* sites of pRS314 (50) to yield pSK250. The *XbaI-BglII* fragment of pSP72 (Promega) containing a multicloning site was cloned into the *XbaI* and *BamHI* sites of pG80-7 to yield pSK157sp. Plasmid pSK332sp containing the *GAL7*-type +1 region was constructed by PCR with template pSK157sp and the primer as in pG80Im above. Plasmid pSK157sp or pSK332sp was digested with *TaqI* (at +35), blunt ended by Klenow fragment, and digested with *SacI*. Each of the fragments was cloned into the *SacI* and *SmaI* sites of pSK250 to yield pSK329 and pSK333, respectively. Plasmid pSK328 was created from pSK157sp by digestion with *AvaII* (at +9) and constructed as pSK329.

Assay of β -galactosidase activity. Yeast strains were transformed with the pEZ series plasmids by lithium acetate methods (20). Each of the transformants was grown in ESGlyLac medium lacking uracil to an optical density at 600 nm (OD_{600}) of 0.2 to 0.3. The culture was supplemented with 2% galactose and further incubated for 4 to 6 h such that cell density reached an OD_{600} of 1.0. The activity of β -galactosidase was assayed as previously described (15, 45).

Preparation of yeast RNA. Plasmids of the pIG80 series were digested with *NsiI* and integrated into the *URA3* locus of NOY396. The transformants were grown as described above in ESGlyLac lacking uracil medium supplemented with either 2% glucose or 2% galactose. NOY396 and HS18 were grown in YPGlyLac medium. Total RNA prepared as previously described (5) was further purified by precipitation with 2 M LiCl. RNA was dissolved in 2 M ammonium acetate, extracted with phenol-chloroform (1:1), and recovered by ethanol precipitation.

S1 nuclease mapping analysis. S1 nuclease mapping analysis was carried out as previously described (46) by using the probe DNA described in the legend to Fig. 3B. S1 nuclease-protected fragments were separated on a 5% polyacrylamide-7 M urea gel and visualized by autoradiography.

DNase I footprint analysis. Yeast recombinant TATA-binding protein (TBP) was kindly supplied by H. Handa. *GAL4*-VP16 (44) was purified as previously described (45). The probe DNA was a 330-bp *PstI-BglII* fragment of pG80-218, 5' end labeled at the *BglII* site (at +88). The binding reaction was carried out in 10 μ l of binding buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH (pH 7.6), 50 mM potassium acetate, 5 mM $MgCl_2$, 0.6 mM EDTA, 0.6 mM dithiothreitol, 0.06% Nonidet P-40, 6% glycerol] containing 0.5 ng of probe DNA for 30 min at 25°C. Recombinant TBP (50 ng of protein) or *GAL4*-VP16 (30 ng of protein) was incubated in a reaction mixture containing 100 ng of poly(dG-dC) or 100 ng of poly(dI-dC), respectively. After the binding reaction, 10 μ l of DNase I solution (1 μ g/ml; dissolved in binding buffer containing 10 mM $CaCl_2$ and 1 mg of bovine serum albumin per ml) was added, and the mixture was incubated for 2 min on ice. The reaction was halted by the addition of 200 μ l of stop solution (0.3 M sodium acetate [pH 5.2], 0.5% sodium dodecyl sulfate, 5 mM EDTA). Nucleic acids were precipitated by ethanol and separated on a 6% polyacrylamide-7 M urea gel.

Gel retardation assay. Probe DNAs were 125-bp *EcoRI* fragments of pSK157sp or pSK332sp. Extract was prepared from yeast nuclei of strain BJ2168 as previously described (45). The binding reaction was performed, as described above, in the

presence of 7 μ g of nuclear protein and 500 ng of poly(dI-dC). The binding reaction mixtures were electrophoresed on a 4% polyacrylamide gel equilibrated with a buffer containing 25 mM Trizma base, 190 mM glycine, and 1 mM EDTA.

In vitro transcription. Yeast nuclear extract was prepared from strain BJ2168 as previously described (45). The transcription reaction was performed for 90 min at 25°C in a 20- μ l reaction mixture as previously described (45) except that the reaction mixture contained 200 ng of template DNA and a 0.4 mM concentration of each of the four ribonucleoside triphosphates. The reactions were terminated by the addition of 5 μ g of DNase I and 2 μ g of tRNA. After incubation at 25°C for 10 min, 0.2 ml of the stop solution containing 40 μ g of proteinase K was added, and the mixtures were further incubated at 30°C for 20 min. The solution was extracted twice with phenol-chloroform, and the nucleic acids were precipitated by the addition of 2.5 volumes of ethanol. The precipitate was subjected to the S1 nuclease mapping as described above.

RESULTS

Analysis of initiation sites of *GAL80* mRNA. At first, we analyzed the transcription initiation sites of *GAL80* mRNA by S1 nuclease mapping. Samples of RNA were prepared from wild-type yeast cells cultured in an uninducing medium containing glycerol and lactate as the sole carbon sources (YPGlyLac), to which glucose (YPGlyLacGlu) or galactose (YPGlyLacGal) was supplemented for uninducing or inducing conditions, respectively. Probe DNA for S1 mapping was a fragment between nucleotide positions -218 and +172 of *GAL80* with 5' end labeling at +172. As shown in Fig. 1, when yeast cells were grown in YPGlyLac, transcription of *GAL80* was initiated at site +1 (lane 1), the site which was previously identified as the initiation site of the *GAL80* mRNA (31). In agreement with the previous experiment (49), the amount of the *GAL80* mRNA was not affected by the presence of glucose in the growth medium (lane 2), indicating that *GAL80* expression is not subject to glucose repression. When yeast cells were grown in YPGlyLacGal, the transcription from +1 increased twofold over the uninduced level. In addition, transcription was also initiated at multiple sites: +37, +47, +56, and +67 (lane 3). The transcripts initiated at the downstream sites were not previously observed (31), probably because the probe DNA used in those experiments was labeled at +88 and was unable to hybridize efficiently with the downstream transcripts (data not shown). Densitometric analysis revealed that the total amount of *GAL80* mRNA increased approximately 10-fold over the uninduced level by the addition of galactose as was observed by Northern (RNA) analysis previously (49). Next we analyzed the effect of *gal4* null mutation on the expression of *GAL80*. In yeast cells grown in YPGlyLacGlu, transcription was initiated at +1 as efficiently as in the wild type (compare lanes 2 and 4). Addition of galactose in place of glucose in the medium had no effect on the profile of *GAL80* transcription (lane 5). These results indicated that the twofold enhancement of transcription from +1 and the transcription from the downstream sites were fully dependent on Gal4p under inducing conditions and that Gal4p did not affect the constitutive transcription from +1 under uninducing conditions.

Determination of binding sites of GAL4-VP16 and TBP. To locate *cis*-acting sequences recognized by TBP or Gal4p in the upstream region of *GAL80*, we employed DNase I footprint analysis. As shown in Fig. 2, lane 3, and also as summarized in Fig. 3A, recombinant TBP bound to the region between -23 and -7 containing a typical TATA sequence at -20 TATAT

FIG. 1. Transcription initiation sites of the *GAL80* gene. The wild-type (WT) yeast (NOY396) (lanes 1 to 3) or its isogenic *gal4* null strain (HS18) (lanes 4 and 5) were grown in the YPGlyLac medium (GlyLac, lane 1), and then glucose (+DEX, lanes 2 and 4) or galactose (+GAL, lanes 3 and 5) were added to the culture to a final concentration of 2%. Total RNA was prepared from the yeast cells, and 20 μ g of RNA was subjected to S1 nuclease mapping. Probe DNA was the *Dra*I (at -218)-*Fok*I (at +172) fragment, with 5' end labeled at the *Fok*I site. Fragments protected against S1 digestion were separated on a 6% acrylamide-7 M urea gel and visualized by autoradiography. Numbers to the right of the panel indicate transcription start sites (in nucleotide position) corresponding to the respective S1-protected fragments (31).

ATA -13. The region from -72 to -54 was weakly recognized by TBP. Two AT-rich regions, from -203 to -181 and from -147 to -126, were also protected against DNase I digestion. These sequences may be too far upstream from the initiation sites to function as the TATA box of *GAL80*, since functional TATA boxes are usually located between 40 and 120 bp upstream of the initiation sites in *S. cerevisiae* (29, 53). The binding site of Gal4p had been studied with partially purified Gal4p by Bram et al. (3). We reinvestigated the Gal4p binding site by using recombinant GAL4-VP16, a fusion protein composed of the DNA-binding domain of Gal4p and the transcription activation domain of herpes simplex virus protein VP16 (44). As shown in Fig. 2, GAL4-VP16 bound to the region spanning from -111 to -88 which contains the consensus UAS_G; the result was in perfect agreement with the previous report of Bram et al. (3).

Determination of *cis*-acting elements in upstream region of *GAL80*. To further analyze the *cis*-acting elements in the 5'-flanking region of *GAL80*, we constructed reporter genes bearing various lengths of the 5'-flanking region of *GAL80* fused to *lacZ* and introduced them into the wild-type or *gal4* null yeast strains. The transformants were grown in the inducing medium, and expression of the *GAL80-lacZ* was estimated by determining the β -galactosidase activity (Table 1). Deletions extending through -195 did not affect the expression of the reporter gene. By contrast, deletion through -185 resulted in a 10-fold reduction from that of pEZ-195. When the UAS_G was deleted from the upstream region (pEZ-72), the activity of

FIG. 2. Binding sites of TBP or Gal4p. Probe DNA was the fragment spanning from -218 to $+88$ of *GAL80*, 5' end labeled at $+88$. The probe DNA was incubated in the absence (lanes 1 and 4) or presence of GAL4-VP16 (lane 2) or TBP (lane 3). The reaction mixture was treated with DNase I, and the DNA fragments protected against nuclease digestion were analyzed by gel electrophoresis and autoradiography. Lanes C and CT, cleaved fragments of the probe DNA were coelectrophoresed.

β -galactosidase became undetectable. In a *gal4* null yeast strain, the β -galactosidase activity was lowered by a factor of 20 compared with the wild-type level. Deletion from -195 to -185 also led to a 10-fold decrease in expression of the reporter, however. These results suggest that an element lying between -195 and -185 was required for efficient expression of *GAL80* in addition to UAS_G . This element was designated

UAS_{GAL80} (upstream activation sequence of *GAL80*) in this work.

To study role of the *cis* elements of *GAL80* in selection of the initiation sites, we constructed a tester gene bearing a deletion in the ORF of *GAL80* (Fig. 3B) and integrated it into the *URA3* locus. The truncated *GAL80* produces a nonfunctional protein presumably because of a lack of the nuclear localization signal (32). When transcripts were analyzed by S1 mapping, only a part of the probe DNA should hybridize with mRNA from the *GAL80* locus, generating a shorter S1-protected fragment than those with mRNA from the tester gene (Fig. 3B, and Fig. 4, band C). Band C therefore served as an internal control in the S1 mapping experiments. As shown in Fig. 4A, RNA from glucose-grown transformants harboring pIG80-1400 yielded an S1-protected band only at $+1$ (lane 1), indicating that transcription started only at that site. Addition of galactose in place of glucose led not only to enhancement of the $+1$ transcription by a factor of 2 but also to the appearance of transcripts initiated at $+37$, $+47$, $+56$, and $+67$ (lane 2). Thus, the initiation pattern of pIG80-1400-directed transcription was almost identical to that of the authentic *GAL80*-directed transcription observed in Fig. 1. We therefore concluded that expression of the tester gene faithfully reflected that of the authentic *GAL80* gene. We then studied transcription of tester genes having various deletions in the 5' region of *GAL80*. A deletion extending through -218 did not affect transcription of the tester gene, as was observed in the *GAL80-lacZ* fusion (lanes 3 and 4). Deletion through -136 which removes UAS_{GAL80} resulted in a decrease of transcripts in glucose-grown yeast cells (compare lanes 3 and 5). The addition of galactose did not enhance transcription from $+1$ but activated transcription from $+37$, $+47$, $+56$, and $+67$ (lane 6). The transcription initiation patterns of pIG80-106 were similar to those of pIG80-136 in both glucose- and galactose-grown yeasts (lanes 7 and 8). When UAS_G was deleted, the addition of galactose did not activate transcription from $+37$ and $+67$ (lane 10), and yet, transcripts from $+47$ and $+56$ were still observed in a small amount, irrespective of the presence or absence of galactose in the medium. These transcriptions were presumed to be stimulated by an unknown sequence in the vector region (transcripts from $+47$ were also seen in lane 7). Surprisingly, transcription from $+1$ remained unaffected by deletion extending through -7 that caused loss both of the TATA sequence and of the other upstream elements (lanes 11 and 12).

In light of the above results, we suggest that the 5'-flanking region of *GAL80* has an additional unrecognized activating sequence, UAS_{GAL80} located around -190 . The UAS_G , which had been previously identified, is located between -105 and -89 . The UAS_{GAL80} appeared to be capable of activating transcription independently of Gal4p, since this region showed a stimulatory effect in the uninducing conditions (compare lanes 3 and 5 of Fig. 4A) as well as in *gal4* null yeast cells (Table 1). We also found that the weak transcription from $+1$ did not require any other elements located upstream of -7 .

Effects of mutations in the TATA box or initiation sites on initiation. Next we studied the role of the promoter elements in utilization of the initiation sites of *GAL80* transcription (Fig. 4B). Transcripts of the tester gene having a mutated TATA sequence were analyzed by S1 nuclease mapping. Constitutive transcription of the tester gene was initiated at $+1$ as efficiently as in the control gene with the wild-type TATA box (compare lanes 1 and 3 of Fig. 4B). The addition of galactose resulted in activation of transcription more remarkably from $+1$ and less remarkably from the downstream sites in the mutant TATA-bearing gene compared with the control gene (lane 4). A

FIG. 3. Structure of 5'-flanking region of *GAL80* and a tester gene. (A) Nucleotide sequence of the 5'-flanking region of *GAL80*. Figures on the sequence indicate nucleotide positions with respect to the transcription initiation site at +1 (31). Arrows under the sequence indicate positions of the 5' end of deletions. Segments protected by TBP or Gal4p from DNase I footprint analysis are indicated by brackets. Mutated nucleotides in the TATA sequence or the initiator region are expressed in small letters. (B) Structure of the tester gene and probe DNA. The uppermost line is a scale showing nucleotide positions with the most upstream start site of transcription being +1. Boxes in the second and third rows represent *GAL80* ORF in the chromosome (upper) or in the tester gene at the *URA3* locus (lower). The shaded area in the *GAL80* ORF indicates the deleted region in the tester gene. The probe DNA used for S1 mapping is shown under the tester gene, which represents the *Bam*I-*Bgl*II fragment of pIG80-218, 5' end labeled at the *Bgl*II site (asterisks). Five lines with asterisks at the right ends represent five S1-protected fragments produced through hybridization of transcripts from the tester gene (see the text) with the probe DNA. They are approximately of 380, 340, 330, 320, and 310 nucleotides, which correspond to initiation sites at +1, +37, +47, +56, and +67, respectively. The bottom line with an asterisk at the end represents an S1-protected fragment produced through hybridization of transcripts from the *GAL80* locus (see the text) with the probe. The size of the fragment is 290 nucleotides long between the *Eco*RI at +390 and *Bgl*II at +680 sites.

script was detected at the +1 position under either uninducing or inducing conditions. By contrast, galactose-induced transcription occurred from the downstream sites, +67 in particular and additionally +78 (lane 6). These results suggest that the TATA box lying at -20 is required for Gal4p-activated transcription from the downstream sites and further that the +1 region is involved, independently of the other elements, in constitutive as well as induced transcription of *GAL80*.

TABLE 1. Expression of *GAL80-lacZ* fusions with various deletions in the 5' upstream region

Deletion end	β -Galactosidase activity ^a	
	<i>GAL4</i>	<i>gal4</i>
-1400	82	NT
-218	75	4.0
-208	86	4.1
-195	87	4.1
-185	8.2	0.5
-136	7.7	0.5
-106	8.8	NT
-72	1.0	NT

^a Plasmids of the pEZ series were introduced into NOY396 (*GAL4*) or its *gal4* null derivative, HS18 (*gal4*). Transformants were grown in ESGlyLacGal medium lacking uracil, and the β -galactosidase activity was assayed. The specific activity of β -galactosidase is expressed as the amount of orthonitrophenyl- β -D-galactopyranoside hydrolyzed per minute per cell mass in arbitrary units, which is calculated by the following formula (15): $OD_{420} \times 1,000 / (OD_{600} \times \text{time in minutes})$. NT, not tested.

The +1 region of *GAL80* directs transcription by itself. To examine whether the +1 region of *GAL80* per se is capable of directing transcription in the absence of other *cis* elements, we constructed a plasmid having a fragment from -7 to +35 of *GAL80* immediately upstream of the *HIS3* ORF. The plasmid was introduced into a *his3* yeast strain, and growth of the transformants was tested on a histidine-lacking plate containing 20 mM 3-aminotriazole. As shown in Fig. 5, the transformants with the *HIS3* ORF bearing the +1 region of *GAL80* grew on the plate (*Ini_{G80}-HIS3*), whereas the control yeast strain harboring only the *HIS3* ORF could not grow on the plate (*HIS3*). When 12 nucleotides from -3 to +9 within the +1 region of *GAL80* in the chimeric gene were changed to the corresponding sequence of *GAL7* (Fig. 3A), no growth of the transformants was observed on the histidine-lacking plate (*Ini_{G7}-HIS3*). The minimal element sufficient to produce the *HIS3*⁺ phenotype was localized between -7 and +9 (*Ini_{16bp}-HIS3*). These results indicated that the 16-nucleotide sequence of +1 region of *GAL80* was able to initiate transcription by itself without any other *cis* elements and therefore meets the criteria of an initiator (51, 55).

Initiator interacts with a nuclear protein. It has been known that in some cases initiator is recognized by a specific DNA-binding protein to initiate transcription (55). To test whether the *GAL80* initiator interacts with specific proteins, we carried out a gel retardation assay. The probe DNA (*GAL80* probe) was a fragment spanning from -7 to +88 of *GAL80*. We also used a control probe, in which the sequence from -3 to +9 in the *GAL80* probe was changed to the corresponding sequence of *GAL7* (*GAL7* probe [Fig. 3A]). Each probe was incubated with extract from yeast nuclei, and the mixture was fractionated on an acrylamide gel in the native state (Fig. 6). A DNA-protein complex was detected with the *GAL80* probe but not with the *GAL7* probe (compare lanes 3 and 4). To rule out the possibility that the observed complex was due to nonspecific interaction of nuclear protein with the *GAL80* probe, competition experiments were carried out. The addition of increasing amounts of the unlabeled initiator fragment of *GAL80* inhibited the formation of the complex (lanes 5 and 7), whereas the fragment containing the +1 region of *GAL7* failed to do so (lanes 9 and 11). These results indicated that the initiator element of *GAL80* interacted specifically with a protein(s) contained in the yeast nuclear extract.

Transcription of *GAL80* in a cell-free system. We exploited a cell-free transcription system composed of yeast nuclear

FIG. 4. S1 nuclease mapping of transcripts of *GAL80* with deletions (A) or mutations (B) in the 5'-flanking region. Twenty micrograms of an RNA sample was subjected to S1 nuclease analysis. Probe DNA was prepared from pIG80-218 (see Fig. 3B) except for the experiment in lanes 5 and 6 in panel B, in which it was prepared from pIG80Im. Numbers to the right, transcription initiation sites corresponding to the respective fragments. The letter C, transcripts from the *GAL80* locus, which serves as an internal control. The RNA samples were prepared from transformants grown in ESGlyLac medium supplemented with 2% glucose (D [odd lanes]) or 2% galactose (G [even lanes]). (A) Tester plasmids are pIG80-1400 (lanes 1 and 2), pIG80-218 (lanes 3 and 4), pIG80-136 (lanes 5 and 6), pIG80-106 (lanes 7 and 8), pIG80-72 (lanes 9 and 10), and pIG80-7 (lanes 11 and 12). (B) Test plasmids are pIG80-218 (lanes 1 and 2), pIG80Tpm (lanes 3 and 4), and pIG80Im (lanes 5 and 6).

extracts to analyze the effects of mutations in various regulatory elements on transcription of *GAL80* in vitro. Figure 7 shows the results of S1 nuclease mapping analyses of in vitro transcripts. When template DNA containing the fragment between -218 and +84 of *GAL80* was transcribed in vitro, transcription was initiated at +1 and +47 by RNA polymerase

FIG. 5. Growth of transformants harboring various gene fusions of *HIS3* on histidine-lacking agar. Transformants were streaked on a plate of histidine-lacking minimal medium containing 20 mM 3-aminotriazole. Plasmids containing the *HIS3* ORF fusions are *HIS3* (pSK250), *Ini_{G80}-HIS3* (pSK329), *Ini_{G7}-HIS3* (pSK333), and *Ini_{16bp}-HIS3* (pSK328).

FIG. 6. Interaction of protein with the +1 region of *GAL80*. Each of the probe DNAs having the +1 region of *GAL80* or the corresponding region of *GAL7* was incubated with yeast nuclear extract, and the mixture was analyzed on a native gel by electrophoresis. Lanes 1 and 2 contain only the probe DNAs. The arrow indicates a DNA-protein complex formed, which is seen only with the *GAL80* probe. The probe DNAs used are *GAL80*-type +1 region (80 [odd lanes]) and *GAL7*-type +1 region (7 [even lanes]). Unlabeled probes (comp.) were added to the reaction mixture at the amount indicated by fold (*GAL80* type, lanes 5 to 8; *GAL7* type, lanes 9 to 12).

II, whose synthesis was α -amanitin sensitive (compare lanes 1 and 3). GAL4-VP16 enhanced transcription slightly from +1 and efficiently from +37, +47, +56, and +67 (lane 2), the sites which have just been shown to be responsive to galactose induction in vivo. Deletion of UAS_{*GAL80*} (pG80-136) resulted in a decrease of transcription from +1 (lanes 4). On this template only transcription from the downstream sites was activated by GAL4-VP16 (lane 5). In the absence of UAS_G (pG80-72), GAL4-VP16 could activate transcription neither from +1 nor from the four downstream sites (lane 8). The initiator-dependent transcription from +1 observed in vivo was detected on a template having a deletion extending through -7 (lane 10). When the TATA box at -20 was mutated, transcription from +1 was rather enhanced (compare lanes 1 and 12). In the template with the mutated TATA box, GAL4-VP16 stimulated transcription from +1 but did not do so from the downstream inducible sites (lane 13). Thus, the results of in vitro transcription experiments qualitatively paralleled with those of the in vivo experiments: UAS_{*GAL80*} was required for efficient transcription of *GAL80*, especially from +1 in vitro. The UAS_G-bound GAL4-VP16 also stimulated transcription from +1 as well as from the downstream sites. The activation from the downstream sites was observed only in the presence of the TATA box. The initiator of *GAL80* directed transcription in the absence of both the TATA box and the UASs.

DISCUSSION

Our in vivo and in vitro analyses have revealed a number of interesting features of the control of transcription of the yeast negative regulatory gene *GAL80*. Among them, the most notable is that *GAL80* has two elements in the core promoter, the TATA box and the initiator, and that these elements are used differentially, depending on the environmental conditions. Under uninducing conditions, transcription starts at the

FIG. 7. In vitro transcription analysis of *GAL80*. A transcription reaction was carried out with yeast nuclear extract, and transcripts were analyzed by S1 nuclease mapping. Probe DNA was the *Pst*I-*Nar*I fragment of pG80-218, 5' end labeled at the *Nar*I site. Numbers to the sides of the panel indicate the positions of the initiation sites shown in Fig. 1. The reaction was carried out in the presence of 1 pmol of GAL4-VP16 (lanes 2, 5, 8, and 13) or 20 μ g of α -amanitin (lanes 3, 6, 9, 11, and 14) per ml. Template DNAs are pG80-218 (lanes 1 to 3), pG80-136 (lanes 4 to 6), pG80-72 (lanes 7 to 9), pG80-7 (lanes 10 and 11), and pG80Tpm (lanes 12 to 14). The α -amanitin-sensitive strong bands of different mobilities are readthrough transcripts.

+1 site, which is mediated by the initiator element. By contrast, transcription under inducing conditions starts at multiple downstream sites and is mediated by the TATA box. Analogous to the *GAL80* gene, the yeast *HIS3* gene also contains two initiation sites: one directs constitutive expression, whereas the other directs Gcn4p-regulated expression. Although the regulated expression requires the canonical TATA sequence (T_R), the constitutive expression is mediated by a TATA-like sequence (T_C) but not by initiator (52).

In mammalian cells, transcription initiation occurs approximately 30 bp downstream from the TATA box, and the TATA box determines the start site of transcription. Certain mammalian genes that lack the TATA sequence, especially so-called housekeeping genes, contain initiator elements which are sufficient for accurate transcription in vivo as well as in vitro (55). In *S. cerevisiae*, however, the distance between the TATA box and initiation site is not strictly defined. Instead, preferential sites for initiation are found within a "window" spanning from 40 to 120 bp downstream of the TATA box (29, 53). Hahn et al. (16) suggested that consensus sequences of the initiation site are TCRA or RRYRR. Despite that most of the elements and factors that constitute the basal transcription machinery are well conserved from yeast to mammalian cells (25), no clear description had been made of the initiator element in yeasts. From these considerations, one might have thought that yeast genes have no sequences that function like mammalian initiators. Here we have demonstrated that the +1 region of *GAL80* possesses an initiator activity. (i) The TATA box of *GAL80* at -20 is too close to regulate transcription from the +1 site (29, 53). The sequence encompassing the +1 site has no obvious similarity to the consensus sequences of proposed

by Hahn et al. (16). (ii) 5' deletion analyses revealed that the sequence downstream from -7 is sufficient for transcription from $+1$ both in vivo and in vitro. (iii) When the $+1$ region was changed to the RRYRR sequence found in the $+1$ region of the *GAL7* gene, transcription initiation did not occur, at least within that region. (iv) Finally, the fusion of the $+1$ region of *GAL80* to the *HIS3* ORF produced a His^+ phenotype in a *his3* yeast strain. This result indicates that the $+1$ region of *GAL80* directs transcription by itself.

Ogden et al. (33) reported that the yeast *PGK* gene is transcribed in the absence of the TATA sequence. This result suggests that *PGK* has an initiator element, although we could not find obvious homology between *PGK* and *GAL80* in the sequence around the initiation sites. The *HIS4* gene also exhibits TATA-independent transcription under certain conditions. A low-level transcription of *HIS4* observed under amino acid-sufficient conditions is maintained by Bas1p and Bas2p, and the transcription is enhanced to a high level by Gcn4p under amino acid-deficient conditions (1). Such a low-level transcription is also seen in yeasts bearing mutations in the activation domain of Gcn4p under the amino acid-deficient conditions. Pellman et al. (37) showed that the TATA box is required for the high-level activation by wild-type Gcn4p but is dispensable for the low-level transcription conducted by Bas1p and Bas2p or by the mutant Gcn4p. Those authors assumed that the low-level transcription may be directed by PIC formed on the initiation site. Whatever the mechanism, the initiation site-dependent transcription of *HIS4* is distinctive from that of *GAL80*, since the former requires activator proteins, whereas the latter operates in their absence.

We have also shown that the initiator element of *GAL80* interacts with a nuclear protein(s). We assume that the observed binding is specific to the initiator, since the protein did not recognize the sequence RRYRR encompassing the initiation site of *GAL7*. The sequence of the mammalian initiator varies, and each sequence interacts with a specific DNA-binding protein (55). One of these proteins, TFII-I, binds to initiator elements in the genes encoding adenovirus major late protein, terminal deoxynucleotidyl transferase, and human immunodeficiency virus type 1 (43). The TFII-I protein is required for initiator-dependent transcription by generating the PIC through a pathway different from that of TATA-dependent initiation in vitro (42). We also speculate that the initiator-binding protein may be a transcription factor essential for formation of the PIC to direct initiator-dependent transcription of *GAL80*. We found that a mutation in the TATA box caused significant stimulation of transcription from $+1$ under the inducing conditions both in vivo and in vitro. Conversely, alteration of the $+1$ region resulted in higher activation from the downstream sites. These observations lead us to infer that there exists a competition between the TATA box- and the initiator-directed transcriptions. We imagine that this competition emerges via formation of PIC: in the induced state, formation of PIC should be initiated by binding of TBP at the TATA box, whereas PIC formation may be initiated by binding a protein like the one that we have shown in Fig. 6 at the initiator element. Since the distance between two elements is as short as 20 bp, the initiator-mediated PIC may interfere with formation of the TBP-initiated PIC by a steric hindrance. In support of this idea, O'Shea-Greenfield and Smale (34) reported that the terminal deoxynucleotidyl transferase initiator, when inserted downstream of the adenovirus major late protein TATA box at various distances in a synthetic promoter, appears to exhibit various initiator activities in vitro depending on the distance: the shorter the distance, the lower the initiator-mediated transcription.

FIG. 8. Schematic representation of *GAL80* transcriptional circuit. Unknown protein binds to *UAS_{GAL80}*, whereas Gal4p binds to *UAS_G*. TBP binds to the TATA box located at -20 . The protein designated YIF (yeast initiator binding factor) binds to the initiator element (Inr).

We have found a *cis*-acting element (*UAS_{GAL80}*) that is required for efficient expression of *GAL80* under both uninducing and inducing conditions. This element is located at around -190 and consists of an AT-rich sequence spanning from -198 to -180 , in which we could not find any consensus sequences recognized by known transcription factors, however. Lohr (27) has recently shown that the region containing *UAS_{GAL80}* and *UAS_G* are hypersensitive to nuclease digestion in both the uninduced and the induced states and suggested that nucleosomes are persistently absent from this region. This observation may suggest that *UAS_{GAL80}* provides sequence recognized by a factor like Grf2p, which creates a nucleosome-free region for facilitated binding of upstream activators and/or basal factors to DNA, resulting in stimulation of transcription (7, 12). Another possibility is that the AT-rich sequence may be recognized by Bas2p (1) (also known as Pho2p [54] or Grf10p [4]). Bas2p has a homeodomain and binds AT-rich sequences found in the upstream regions of various genes including *HIS4* (1), *ADE2* and *ADE5,7* (10), *PHO5* (54), and *HO* (4). It has been shown that Bas2p is required for full expression of these genes and for the removal of positioned nucleosomes from 5' upstream region of *PHO5* (11). Needless to say, many more experiments are needed to elucidate the structure and function of *UAS_{GAL80}*.

In summary, a possible model of the control circuit of *GAL80* may be drawn on the basis of currently available experimental results, as in Fig. 8. The transcription of *GAL80* is regulated by two upstream elements, *UAS_G* and *UAS_{GAL80}*, and two core promoter elements, the TATA box and the initiator. Under the uninducing conditions, constitutive expression starts from the $+1$ site. This transcription is maintained by the initiator activity and is further stimulated by an unknown factor(s) interacting with the *UAS_{GAL80}*. Galactose-induced transcription is fully dependent on Gal4p and *UAS_G*. *UAS_{GAL80}* is also involved to give high-level expression through Gal4p. The activation signal of Gal4p is transmitted to the transcription machinery formed on the TATA box to enhance transcription from the downstream sites. The initiator-dependent transcription is slightly stimulated by Gal4p only in the presence of the *UAS_{GAL80}*. Thus, the transcription of *GAL80* is regulated by two independent pathways; one is an initiator-dependent pathway which directs the constitutive

expression, while the other is a TATA-dependent pathway operating in the induced state that involves the Gal4p-UAS_G complex.

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