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Transcription Initiation Mediated by Initiator Binding Protein in *Saccharomyces cerevisiae*

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Many instances of the initiator element in the core promoter of protein-coding genes have been reported in mammalian cells and their viruses, but only one has been reported in the yeast *Saccharomyces cerevisiae* at the *GAL80* gene. The initiator element of *GAL80* directs transcription by itself and interacts with a nuclear protein designated yeast initiator binding factor (yIF). Here we show that yIF in a partially purified sample binds the sequence from −18 to +10 of *GAL80* . By employing a selected and amplified binding procedure, we have determined the preferred sequence for yIF binding to be −2 CACT*N* +3 (*N* indicates any nucleotide). Binding affinity of selected sequences to yIF correlated with their initiator-directed transcription *in vivo,* suggesting that the yIF–initiator interaction mediates transcription from the initiator in yeast. We also suggest that sequences flanking the preferred sequence affect both yIF binding and initiator activity.

Most eukaryotic genes transcribed by RNA polymerase II contain several cis-elements that assure the accurate initiation of transcription. Initiator is a discrete element that functions alone or in concert with the TATA box and upstream activating elements to direct transcription initiation (1–3). The initiator element was first reported in the murine terminal deoxynucleotidyl transferase gene (4). Since then, many instances of initiator have been found in mammalian cells and their viruses (2, 3), and its consensus sequence has been determined as −2 YYANA/TYY +5 (Y indicates pyrimidine and nucleotide positions are respective to the transcription initiation site at +1) by mutation analyses (5, 6). It has been shown that initiator-dependent transcription is mediated by interaction between the initiator and various proteins (2, 3) including YY1 (7), TFII-I (8), USF (8), RNA polymerase II (9), and/or components of the TATA box-binding protein associated factors (10).

In the yeast *Saccharomyces cerevisiae,* only one case of initiator has so far been reported at the *GAL80* gene (11), despite a large body of information on the transcription mechanism as well as the completion of the whole genome sequencing in this organism. The *GAL80* gene encodes a negative regulatory protein for the galactose-inducible genes (12, 13). The initiator element of *GAL80* directs transcription by itself, thus maintains the constitutive expression to a certain basal level, and thereby represses expression of the galactose-inducible genes in the absence of galactose. We previously demonstrated that the *GAL80* initiator interacts with a nuclear protein designated yeast initiator binding factor (yIF) (11). The *GAL80* gene also contains the TATA box that mediates inducible transcription, and thus this gene has two alternative pathways in transcriptional initiation (11). Here we show that the yIF-initiator interaction is correlated to initiator-mediated transcription, and suggest that yIF is required for transcription from the initiator in yeast.

MATERIALS AND METHODS

Plasmids. Plasmid pG80-47 was created by subcloning the *Alu*I-*Bgl*II fragment of *GAL80* (nucleotide positions from −47 to +84 with respect to the transcription initiation site at +1) (14) into the *Sma*I and *Bgl*II sites of pSK142 (11). A *GAL80-GAL7* hybrid fragment was prepared by replacing the sequence from −3 to +9 of *GAL80* with the corresponding sequence of *GAL7* (−3 AAAACAGTTGAA +9) in pG80Im-47 (11). Plasmid pSK250 contained the *HIS3* open reading frame (ORF) in a *TRP1*-marked centromeric plasmid (11). Plasmids pSK329 (Ini_{G80}-*HIS3*) and pSK333 (Ini_{G7}-*HIS3*) contained the *GAL80* initiator and the hybrid initiator described above, respectively, upstream of the *HIS3* ORF of pSK250 (11). A *Bam*HI site was intro-

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duced 95-base pairs upstream to the *ARG5,6* ORF in pCB4 (15) by polymerase chain reaction (PCR) to create pCB4B. Plasmid Ini_{ARG}-*HIS3* bearing a fusion of *ARG5,6* promoter region and *HIS3* ORF was constructed by subcloning the *Bam*HI–*Bgl*II fragment (from −95 to −28) of pCB4B into the *Bam*HI site of pSK250.

Gel retardation assay. Gel retardation assay was carried out as described (11). Probe DNA and protein fraction used are indicated in the figure legends.

Partial purification of yIF. Yeast nuclear extract was prepared from BJ2168 (*MATa ura3-52 leu2 trp1 prb1-1122 pep4-3 prc1-407 gal2*) cells as described (11). Presence of yIF was monitored by gel retardation assay using a ^{32}P-labeled *Pst*I–*Bgl*II fragment of pG80-47 that contained the region from −47 to +84 of *GAL80*. The nuclear extract (4 ml, 10 mg protein/ml) was loaded on a 5-ml Bio-Rex 70 (Bio-Rad) column equilibrated with buffer A (20 mM Tris–acetate pH 7.9, 1 mM EDTA, 20% glycerol, 1 mM dithiothreitol, and protease inhibitors) containing 0.1 M potassium acetate. After washing the column with the same buffer, bound proteins were eluted step-wise with 0.3, 0.5, and 1.0 M potassium acetate in buffer A. The 1.0 M potassium acetate fraction containing yIF (5 mg protein) was dialyzed against buffer A plus 0.1 M potassium acetate and loaded on a 2-ml Q Sepharose (Pharmacia) column equilibrated with the same buffer. After washing the column, bulk of the yIF activity eluted with 0.3 M potassium acetate in buffer A (0.36 mg protein) was dialyzed against buffer A plus 0.1 M potassium acetate.

DNase I footprint and methylation interference analyses. Either the top or bottom strand of the *Bam*HI–*Bgl*II fragment of pG80-47 was 5′ end-labeled with ^{32}P. Partially purified yIF fraction (1.5 μg of protein) was incubated with 0.5 ng of probe DNA in the presence of 100 ng of poly (dI-dC) for 20 min at room temperature (11). After digestion with 10 ng DNase I for 1 min, DNA was purified and subjected to electrophoresis on a 6% polyacrylamide–7 M urea gel (11). For methylation interference analysis, the end-labeled DNA fragment was partially methylated by dimethylsulphate (16). The binding reaction was performed as gel retardation assay except that the reaction mixture was scaled up 5-fold. The sample was applied on a native 4% polyacrylamide gel to separate yIF-bound and unbound DNA fragments. After autoradiography, DNA fragments recovered from the gel were cleaved with piperidine and electrophoresed on a 6% polyacrylamide–7 M urea gel (11, 16).

Selected and amplified binding (SAAB) procedure. The SAAB procedure was performed as described by Purnell *et al.* (17). Two oligonucleotides were synthesized; one corresponds to the top strand of *GAL80* from −14 to −7, to which restriction sites for *Bam*HI and *Eco*RV are introduced at the 5′ end. The other corresponds to the bottom strand from −14 to +14 and contains restriction sites for *Eco*RI and *Eco*RV at the 5′ and 3′ ends, respectively. The latter contains randomized sequences at 5 nucleotides between −2 to +3. Two oligonucleotides were annealed, and the single-stranded regions were filled up with Klenow fragment. The resultant DNA molecules (see Fig. 2A) were amplified by PCR with 5′-GGATCCGATATCTAC-3′ and 5′-GGGAATTCGCAAGGACC-3′ as primers. Portion of the amplified fragment (1.5 ng) was end-labeled and used for gel retardation assay. DNA fragments in the complex were recovered from the gel and amplified by PCR as above. After three cycles of selection and amplification, selected fragments were digested with *Eco*RV and *Eco*RI, and cloned into the *Sma*I and *Eco*RI sites of pSK250 to create pGAL80-*HIS3* derivatives. Cloned fragments were sequenced by using 7-DEAZA Sequencing Kit ver. 2 (TAKARA Shuzo, Kyoto).

Assay of in vivo initiator activity. The *HIS3* fusion derivatives were introduced into a *his3* mutant strain NOY396 (*MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100*) (11). The transformants were streaked on a plate of histidine-lacking medium containing 20 or 50 mM 3-aminotriazole (3-AT), and the cell growth was evaluated after incubation for 3 to 4 days at 30°C to determine the expression of *HIS3* as described (11).

RESULTS

Determination of yIF-binding region. Interaction between yIF and the initiator of *GAL80* was analyzed by DNase I footprint technique using a DNA fragment containing the sequence from −47 to +84 of *GAL80* and the protein fraction enriched for yIF. As shown in Fig. 1A, protection against DNase I digestion was observed in the initiation region between −18 and +6 of *GAL80* [from −17 to +6 of the top strand (compare lanes 1 and 2) and from −18 to +3 of the bottom strand (compare lanes 8 and 9)]. When a binding mixture received an excess amount of unlabeled probe fragment, the observed footprint was diminished (lanes 3 and 10). By contrast, addition of the control fragment, in which the sequence between −3 and +9 was replaced with the corresponding sequence of *GAL7* (11), did not affect the footprint pattern (lanes 4 and 11); the fragment was previously shown not to inhibit formation of yIF–initiator complex in gel retardation assay (11). The observed protection against DNase I by this protein sample was therefore ascribed to specific binding of yIF to the sequence.

We also analyzed the yIF-initiator interaction by methylation interference method (18). As shown in Fig. 1B, guanine residue at −4 on the top strand was undermethylated in the yIF–DNA complex (compare lanes 2 and 3). On the bottom strand, guanine residues at −12, +1, and +9 were undermethylated (compare lanes 5 and 6). Slight undermethylation was also observed at +7 (top strand) and +10 (bottom strand). Based on the results of DNase I footprint and methylation interference analyses as summarized in Fig. 1C, we conclude that yIF binds the sequence from −18 to +10 of the *GAL80* initiator.

Isolation of preferred sequences for yIF binding. To determine the nucleotide sequence required for recognition by yIF, we adopted a SAAB procedure (17, 18). We generated a library of *GAL80* initiator variants with randomized sequences in 5 bases around +1 (Fig. 2A). By means of gel retardation assay with a yeast nuclear extract, yIF-bound DNA molecules were separated from unbound DNA and amplified by PCR. After three cycles of selection and amplification, selected fragments were cloned in an appropriate plasmid and sequenced. Nucleotide sequences of 18 clones are listed in Fig. 2B. We identified −2 CACTN +3 sequence as the preferred sequence for yIF-binding, which was also found in the authentic initiator of *GAL80* (−2 CACTG +3). Sequences of seven clones matched with the preferred sequence (clones 1–7). The TCACT sequence (clone 8), in which the preferred sequence was located one base downstream from the authentic initiator, was also obtained. (From now on, nucleotides identical with

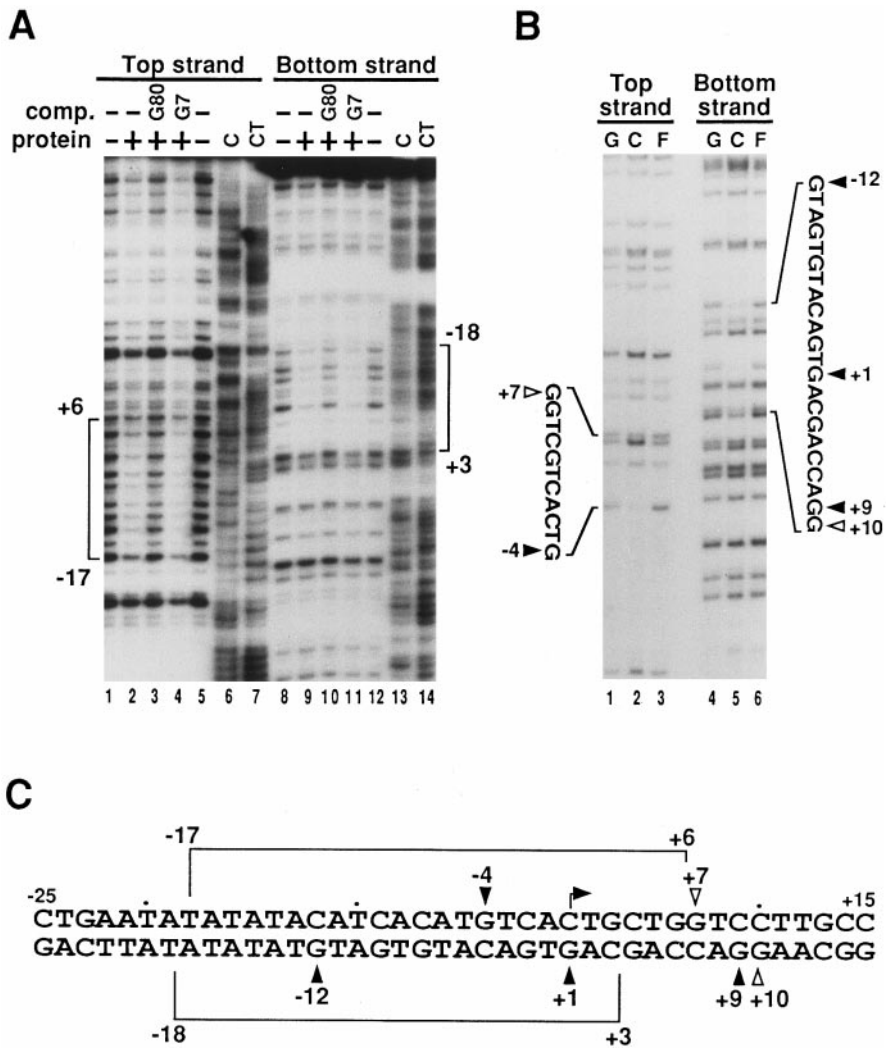
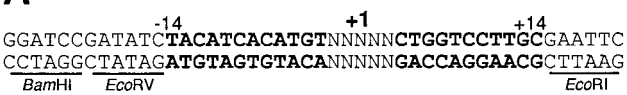


FIG. 1. Binding of yIF on *GAL80* core promoter. (A) DNase I footprint analysis. Probe DNA was labeled at 5′ end of either the top (lanes 1–7) or bottom strand (lanes 8–14). Binding reaction was performed in the absence (lanes 1, 5, 8, and 12) or presence (lanes 2–4 and 9–11) of the partially purified yIF. Tenfold molar excess of the unlabeled probe (G80, lanes 3 and 10) and the *GAL80–GAL7* hybrid fragment (G7, lanes 4 and 11) were also added to the binding mixtures. Products of chemical sequencing reaction were co-electrophoresed as markers (C cleavage reaction, lanes 6 and 13; C+T cleavage reaction, lanes 7 and 14). Protected regions from DNase I digestion are indicated by brackets. (B) Methylation interference analysis. Probe DNA was labeled at 5′ end of either the top (lanes 1–3) or bottom strand (lanes 4–6). Piperidine cleaved fragments prepared from the yIF–DNA complex (C, lanes 2 and 5), unbound DNA (F, lanes 3 and 6), and probe DNA (G, lanes 1 and 4) were electrophoresed. Undermethylated G residues are shown by filled (strong undermethylation) and open (slight undermethylation) triangles. (C) Summary of yIF binding region in *GAL80* core promoter. Transcription initiation site is shown by arrow. Brackets indicate the region protected from DNase I digestion. Guanine residues involved in the yIF-binding are shown by filled (strong interference) and open (slight interference) triangles.

the preferred sequence are underlined.) Note that this clone was deleted of one of two G residues at +6 and +7. Selected sequences CTCTG (clone 9), CCCTC (clone 10), CAATT (clone 11), and GACTA (clone 13) contained one base-alterations from CACTN.

Relationship between yIF–DNA interaction and initiator activity. The yIF-binding affinity of the selected sequences was evaluated by gel retardation assay (Fig. 3A and summarized in Fig. 2B). A labeled fragment containing the sequence TCACT (clone 8) was recognized by yIF as efficient as the initiator fragment of *GAL80* (CACTG) (compare lanes 2 and 3). The CTCTG (clone 9) or CTCCG (clone 12) sequence also formed yIF–DNA complex with an efficiency of approximately 80% of that of the authentic sequence (lanes 4 and 5). The CAATT sequence (clone 11), which contained an alteration of the conserved C residue at +1 to A, was efficiently recognized by yIF (lane 6). By contrast, yIF-binding affinity was significantly reduced by C to G change at −2 [GACTA (clone 13), see lane 8]. These results suggest that the −2 CACTN +3 sequence is preferred for the binding of yIF, and that C at −2 is

A



B

Clone	Sequence of isolated fragments							yIF-binding activity	Initiator activity
	-5	-2	-1	+1	+2	+3	+8		
1.	tgt	**C**	**A**	**C**	**T**	G	ctggt	100%	++
2.	tgt	**C**	**A**	**C**	**T**	G	ctggt	100%	++
3.	tgt	**C**	**A**	**C**	**T**	G	ctggt	100%	++
4.	tgt	**C**	**A**	**C**	**T**	A	ctggt	NT	++
5.	tgt	**C**	**A**	**C**	**T**	A	ctggt	NT	++
6.	tgt	**C**	**A**	**C**	**T**	C	ctggt	NT	++
7.	tgt	**C**	**A**	**C**	**T**	C	ctggt	NT	++
8.	tgt	T	**C**	**A**	**C**	**T**	ct-gt	99%	++
9.	tgt	**C**	T	**C**	**T**	G	ctggt	79%	++
10.	tgt	**C**	C	**C**	**T**	C	ctggt	NT	++
11.	tgt	**C**	**A**	A	**T**	T	ctggt	93%	++
12.	tgt	**C**	T	**C**	C	G	ctggt	81%	++
13.	tgt	G	**A**	**C**	**T**	A	ctggt	24%	+
14.	tgt	T	C	**C**	**T**	C	ctggt	NT	+
15.	tgt	T	T	**C**	G	T	ctggt	29%	+
16.	tgt	G	T	**C**	**T**	A	ctggt	19%	+
17.	tgt	**C**	G	**C**	A	T	ctggt	NT	+
18.	tgt	**C**	C	G	**T**	C	ctggt	12%	+
GAL7	tga	A	A	A	C	A	gttga	2%	-
	G:	2	1	1	1	5			
	A:	0	**9**	2	1	4			
	T:	3	4	0	**14**	4			
	C:	**13**	4	**15**	2	5			
		-2	-1	+1	+2	+3			
preferred sequence:		**C**	**A**	**C**	**T**	N			

FIG. 2. Isolation of preferred sequences for yIF-binding. (A) The probe fragment for SAAB analysis contained the *GAL80* promoter region from −14 to +14 (boldface) with randomized sequences at 5 nucleotides between −2 and +3 (N), and restriction sites. (B) Nucleotide sequences of 18 isolated fragments, base frequencies, and preferred sequence are shown. Selected and flanking sequences are shown by uppercase and lowercase letters, respectively. Nucleotide sequences which match with the preferred sequence are indicated by boldface. Note that clone 8 was deleted of one of two G residues at +6 and +7. The yIF-binding ability of the selected fragments was analyzed by gel retardation assay (Fig. 3A), and the binding affinity relative to the *GAL80* initiator (CACTG) was determined by densitometric analysis of the autoradiogram. NT, not tested. The initiator activity was analyzed as Fig. 3B and expressed as strong (++, growth on 50 mM 3-AT medium) or weak (+, growth on 20 mM 3-AT medium). The *GAL80–GAL7* hybrid fragment (*GAL7*, bottom of the selected sequences) supported neither formation of the yIF–DNA complex (2%) nor growth of yeast on 20 mM 3-AT medium (shown by −).

critical for the yIF–DNA interaction. Somewhat less efficient formation of yIF–DNA complex was observed with fragments containing TTCGT (clone 15), GTCTA (clone 16), or CCGTC (clone 18), presumably due to a low affinity to yIF (lanes 7, 9, and 10).

The initiator activity of the selected sequences was assayed *in vivo* by a gene fusion technique. Each of the selected sequences was placed immediately upstream the *HIS3* ORF in a centromeric-type plasmid, and the plasmid was examined for the ability to complement the growth defect of a *his3* yeast on a histidine-lacking plate (11). The plate also contained 3-aminotriazole (3-AT), a competitive inhibitor of *HIS3*-encoded enzyme, so that the growth behavior unequivocally correlates with the expression of *HIS3* (19). Transformants harboring initiator variant-*HIS3* fusions successfully grew on a histidine-lacking plate containing 20 mM 3-AT (Fig. 2B and data not shown). By contrast, transformants harboring *HIS3* ORF alone or *GAL7* initiation site-*HIS3* fusions failed to grow on that plate (Figs. 3B and 4C). Thus, the selected yIF-binding sequences directed transcription of *HIS3* in the cell. Plasmids containing sequences with a high affinity to yIF [CACTN, TCACT, CTCTG, CCCTC, CAATT, or CTCCG (clones 1-12)] supported the growth of *his3* yeast on the plate even if the concentration of 3-AT was increased to 50 mM (Figs. 2B and 3B). That the yIF-binding was closely correlated with the *in vivo* initiator activity suggested that the yIF–DNA interaction was necessary for transcription in yeast.

Analysis of CACTN sequence in ARG5,6 gene. The entire sequence of the whole genome is now known in *S. cerevisiae,* and yet genome-wide search for the initiator sequence has been hampered. This is partly because the transcription initiation site has been determined only in a minor fraction of the genes. We then searched for the tentative consensus sequence of initiator in some 20 genes whose initiation sites have been determined, which include those for galactose metabolism, amino acid biosynthesis, cell division cycle, and radiation sensitivity. We found that the *ARG5,6* gene (15), which encodes acetylglutamyl-*p* reductase and acetylglutamate kinase, contained a CACTA sequence overlapping one of its initiation sites (Fig. 4A). To test whether the initiation region of *ARG5,6* interacts with yIF, we carried out gel retardation assay. As shown in Fig. 4B, a fragment containing the CACTA sequence of *ARG5,6* failed to interfere with the formation of yIF–*GAL80* initiator complex (lanes 4 and 5). When the *ARG5,6* fragment was used as a probe, DNA–protein complex showing similar mobility with the yIF–initiator complex was not observed (lanes 6–10). Furthermore, a plasmid bearing a fusion of the *ARG5,6* fragment and *HIS3* (Ini_{ARG}-*HIS3*) could not complement the *his3* strain for the histidine requirement (Fig. 4C).

DISCUSSION

Here we have shown that yIF binds the sequence from −18 to +10 of *GAL80* and that the −2 CACTN +3 sequence is preferred for the binding. Furthermore, the yIF-binding affinity of the initiator variants directly

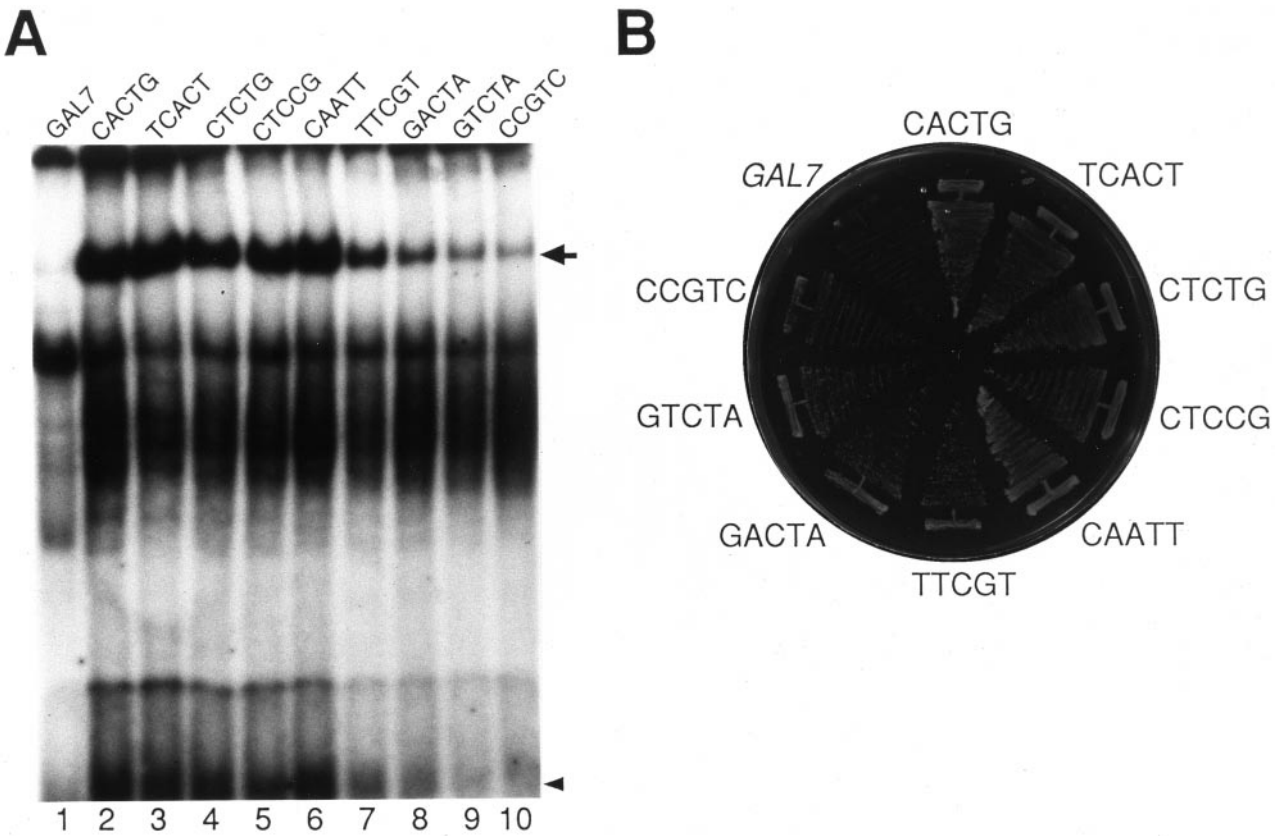


FIG. 3. Characterization of initiator derivatives. (A) Interaction with yIF. Each of the end-labeled fragments containing the indicated nucleotide sequences was prepared from pGAL80-*HIS3* derivatives by digestion with *Bam*HI and *Eco*RI, and used for gel retardation assay with the nuclear extract (lanes 2–10). The *GAL80-GAL7* hybrid fragment was used as a control (*GAL7*, lane 1). Arrow and arrowhead indicate yIF–DNA complex and probe DNA, respectively. (B) *In vivo* initiator activity. Each of transformants harboring the indicated pGAL80-*HIS3* derivatives or Ini_{G7}-*HIS3* (*GAL7*) was streaked on 50 mM 3-AT medium.

correlated with their transcription-directing ability in the cell judged by a gene fusion technique with *HIS3* ORF. We suggest that the yIF–DNA interaction mediates transcription in yeast. However, the CACTA sequence isolated from the promoter region of *ARG5,6*, which overlaps one of the initiation sites, did not bind yIF. We assume that unknown sequence flanking the proposed initiator sequence was also involved in the yIF-binding. This hypothesis is supported by the fact that CACT was localized at a fixed position from −2 to +2 in most of the initiator variants isolated by SAAB, and that clone 8 containing that sequence at positions from −1 to +3 was deleted of one of two G residues at +6 and +7. We also attempted the SAAB screening within the nucleotides from −7 to −3 or from +4 to +8 of *GAL80*, but obtained no preferred sequence after three rounds of selection (data not shown). The CACTN sequence from *ARG5,6 per se* did not function as the initiator in our *in vivo* assay, suggesting that another sequence required for yIF binding as mentioned above was also involved in the yIF-mediated transcription. Notwithstanding such an unknown sequence, it may be safely concluded that the CACTN sequence proposed in the present work is important in the initiator-directed transcription in yeast.

We previously demonstrated that constitutive and inducible transcription of *GAL80* is mediated by the initiator and the TATA box, respectively, and suggested that each element provides a site for assembly of different preinitiation complex (11). In support of this idea, we also showed that global transcription regulator Gal11, a component of RNA polymerase II holoenzyme, is required for full expression of TATA-dependent genes but not for initiator-dependent transcription of *GAL80* (20, 21). In addition, the general transcription factor TFIIE, which is required for transcription of many genes in yeast, is partially dispensable for transcription from the *GAL80* initiator (22). Recently, certain mutations of the general transcription factor TFIIA have been reported to cause differential effects on transcription of various genes in yeast depending on the core promoter element (23). Thus, in the TFIIA mutants, the TATA-dependent transcription of *GAL80* decreases significantly, whereas its initiator-dependent transcription increases several fold. Since TFIIA interacts with TBP to mediate transcription ac-

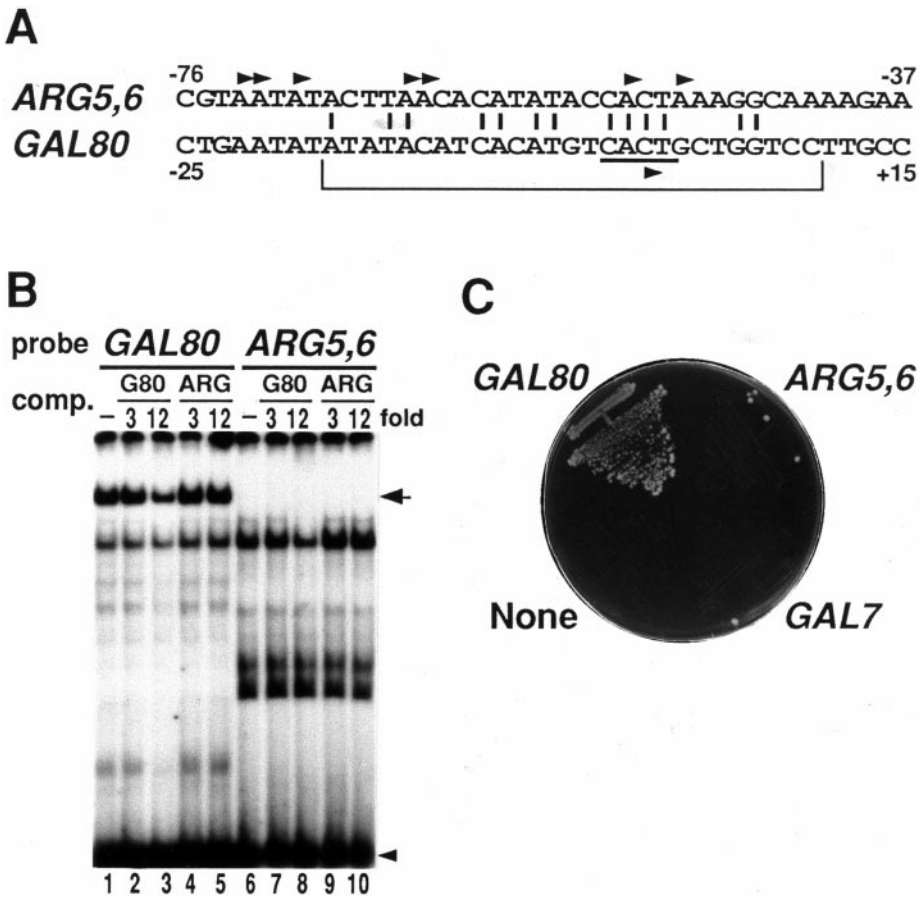


FIG. 4. Analysis of initiation region of *ARG5,6*. (A) Nucleotide sequences of the initiation regions of *ARG5,6* and *GAL80*. Numbers indicate nucleotide positions respective to the first ATG of *ARG5,6* or to the transcription initiation site of *GAL80*. Transcription initiation sites and the CACT sequence are shown by arrowhead and underline, respectively. Bars indicate the identical nucleotides between *ARG5,6* and *GAL80* in the yIF-binding region (bracket). (B) Interaction with yIF. The *Eco*RV–*Bgl*II fragment of Ini_{G80}-*HIS3* (*GAL80*, lanes 1–5) and the *Bam*HI–*Bgl*II fragment of pCB4B (*ARG5,6*, lanes 6–10) were used for gel retardation assay with the nuclear extract. Unlabeled probes were added to the reaction mixtures at the amount indicated by fold (*GAL80*, lanes 2, 3, 7, and 8; *ARG5,6*, lanes 4, 5, 9, and 10). Arrow and arrowhead indicate yIF–DNA complex and probe DNA, respectively. (C) *In vivo* initiator activity. Transformants harboring gene fusions of *HIS3* were streaked on 20 mM 3-AT medium. Fusion genes are Ini_{G80}-*HIS3* (*GAL80*), Ini_{ARG}-*HIS3* (*ARG5,6*), and Ini_{G7}-*HIS3* (*GAL7*). None contains only *HIS3* ORF.

tivation *in vitro*, these findings further strengthen the idea that the yIF-initiator interaction is crucial for initiator-dependent transcription whose mechanism is different from that of TATA-dependent pathway.

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