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Properties of Promoters Cloned Randomly from the *Saccharomyces cerevisiae* Genome

GEORGE M. SANTANGELO,* JOANNE TORNOW, CALVIN S. McLAUGHLIN,† AND KIVIE MOLDAVE‡

Department of Biology, Portland State University, Portland, Oregon 97201-0751

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Promoters were isolated at random from the genome of *Saccharomyces cerevisiae* by using a plasmid that contains a divergently arrayed pair of promoterless reporter genes. A comprehensive library was constructed by inserting random (DNase I-generated) fragments into the intergenic region upstream from the reporter genes. Simple in vivo assays for either reporter gene product (alcohol dehydrogenase or β-galactosidase) allowed the rapid identification of promoters from among these random fragments. Poly(dA-dT) homopolymer tracts were present in three of five randomly cloned promoters. With two exceptions, each RNA start site detected was 40 to 100 base pairs downstream from a TATA element. All of the randomly cloned promoters were capable of activating reporter gene transcription bidirectionally. Interestingly, one of the promoter fragments originated in a region of the *S. cerevisiae* rDNA spacer; regulated divergent transcription (presumably by RNA polymerase II) initiated in the same region.

Transcription of several well-studied genes in *Saccharomyces cerevisiae* is thought to require at least two *cis*-acting regions of DNA: the proximal TATA element (12, 23, 32, 34) and the distal upstream activating sequence (UAS; 9, 14, 23, 25, 45, 46, 50). UASs are bidirectional elements: they function even when inverted (22, 36, 47). Transcription does not always require the presence of a TATA element; efficient expression of the *PGK* gene depends on UAS_{PGK} but does not require TATA sequences (36). Unlike mRNA synthesis in higher eucaryotes, sequences in the vicinity of each mRNA start site contribute to the specificity of transcription initiation (12, 32, 34). Interestingly, some DNA sequences may activate transcription by virtue of their unique structural properties: poly(dA-dT) homopolymer tracts can function as bidirectional promoter elements in the yeast genome (48). These regions have a helix repeat of 10.0 base pairs (bp) instead of the normal 10.6 bp (38, 40) and are associated with bends in DNA (28, 31).

Of primary concern when attempting to analyze the yeast genome comprehensively by using promoter libraries is the large number of yeast colonies (approximately 10^6 [43]) that must be screened. The use of two reporter genes (divergently flanking the cloning site) should reduce that number by 50% relative to libraries that use a single reporter gene. A vector that contains two reporter genes permits the detection of a unidirectional promoter regardless of its orientation in the cloning site. Also, if a promoter is bidirectional, it can be isolated from the library in a configuration that allows the simultaneous monitoring of transcription in both directions along the DNA helix. With this in mind, we have used a promoter-cloning vector (pPC0; Fig. 1) that contains a divergently arrayed pair of promoterless reporter genes, the *adh1* (alcohol dehydrogenase I [ADH]) gene of *S. cerevisiae* (3, 51) and the *lac4* (β-galactosidase) gene of *Kluyveromyces lactis* (10, 13). Both of these reporter genes contain an intact, uninterrupted coding region. The 53- bp upstream intergenic region lacks a UAS, poly(dA-dT) tract, or TATA element.

We created a comprehensive *S. cerevisiae* genomic library in pPC0 (43) by inserting random fragments into the *adh1-lac4* intergenic region. Filter colony assays were used to detect either ADH or β-galactosidase activity in yeast transformants. Genetic selection for ADH was used independently to identify promoter-containing fragments. To test this library, five library members that contained putative promoter elements were isolated at random. For all five of these plasmids, enzyme assays and S1 nuclease analyses were done on (i) transformants (containing each plasmid at high copy number) and (ii) integrants (containing an integrated copy of each plasmid). S1 nuclease analysis of $poly(A)^+$ genomic transcripts revealed that at least one RNA 5′ end mapped near each of the five randomly isolated inserts in its original genomic environment. Therefore, each promoter-containing fragment appeared to be active in its original chromosomal location. Poly(dA-dT) homopolymer sequences were present in three of the five randomly cloned fragments. With two exceptions, TATA elements were found within the expected range (40 to 100 bp [12, 34]) with respect to each RNA start site.

The data presented here support the idea that a screen of our comprehensive promoter library results in the isolation of bona fide yeast promoters. In addition, we report two relatively surprising findings: (i) although the five yeast promoters were isolated because they activated the expression of one reporter gene, they all have the capacity to activate transcription bidirectionally; and (ii) one of the randomly cloned promoters originated in a region of the rDNA spacer that contains RNA start sites for divergent and oppositely regulated transcripts.

MATERIALS AND METHODS

Nomenclature. Tne promoter-cloning vector used in this study was pPC0 (Fig. 1). Promoter library members (containing a random insertion at the *Xho*I site in the intergenic region) were named by adding the designation for the inserted fragment to the vector name, separated by a dash (e.g., pPC0-A7). The inverted orientation of each fragment (with respect to the original isolate) is indicated by append-

* Corresponding author.
† Present address: Department of Biological Chemistry, University of California, Irvine, CA 92717.
‡ Present address: Biology Board of Studies, University of California, Santa Cruz, CA 95064.

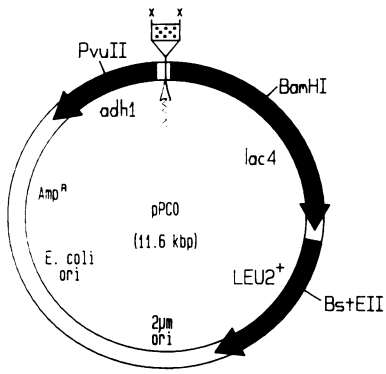

FIG. 1. The promoter-cloning vector pPC0. Both reporter genes (*adh1* and *lac4*) come complete with their own AUG codon but lack promoter elements. In addition to the reporter genes, this plasmid contains the *S. cerevisiae LEU2* gene (1), 2μm *ori* sequences, and the β-lactamase gene (Amp^R), as shown. Arrows indicate the direction of transcription and translation in yeast cells. The unique *Xho*I cloning site in the intergenic region (⟿▷) allows the insertion of either known or random fragments (▭) to test their promoter activity. Other unique restriction sites are *Pvu*II and *Bam*HI, used in S1 nuclease analysis of *ADH1* and *LAC4* transcripts, and *Bst*EII, used for integration (after deletion of 2μm sequences).

ing an "I" (for inverted; e.g., pPC0-A7I). Transformants were defined as yeast strains containing an episomally located plasmid at high copy number; integrants were those containing one or a few copies of a chromosomally integrated plasmid.

Strains and media. *S. cerevisiae* LL20 (*MATα can1 his3-11 his3-15 leu2-3 leu2-112* [Psi^+]) and DC45 (*MATα his4 leu2-2 leu2*-112 *adh1-Δ1 adm adr2*) were used in these experiments. All *S. cerevisiae* strains lack a gene encoding β-galactosidase. Yeast cells were grown in complex medium (2% peptone and 1% yeast extract, supplemented with either 2% glucose [YPD], 8% glucose [YP8D], or 3% ethanol [YPE]) or YNBD (minimal) medium (0.67% yeast nitrogen base, 1.25 mM NaOH, and 0.25 mM succinate buffer [pH 5.5], supplemented with 2% glucose and 40 mg of histidine per liter). Minimal medium containing 2% agar was used to select for $LEU2^+$ DC45 transformants and integrants. Selection for ADH was done in YPD or YPD agar to which 1 μg of antimycin A per ml was added (from a 10-mg/ml stock solution in ethanol).

Escherichia coli HB101 (7) was grown in SOB medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl). *E. coli* transformants were grown (in the presence of 100 μg of ampicillin per ml) in SOB medium or on SOB medium plus 1.5% agar.

Transformation and plasmid preparation. Transformation of *E. coli* HB101 was done by the procedure of Hanahan (26). Small-scale plasmid preparations were done on bacterial transformants by the alkaline extraction method of Birnboim and Doly (6). Large-scale bacterial plasmid preparations were performed by the alkaline extraction and CsCl-ethidium bromide gradient purification method of Garger et al. (20). Yeast transformations were done by using the lithium acetate procedure of Ito et al. (27). Plasmids were isolated from *S. cerevisiae* by the alkaline extraction method; Zymolyase (Seikagaku Kogyo Co., Tokyo, Japan) was substituted for lysozyme as suggested (6).

Construction of promoter-cloning libraries. We used a previously described cloning method to create a plasmid library of DNase I-generated small fragments of *S. cerevisiae* DNA (43). The use of DNase I, which digests DNA in an almost completely random fashion (4), allows similarly sized fragments to provide equal representation of the entire yeast genome in the pPC0 promoter library. This comprehensive genomic library contains small yeast DNA inserts (less than 600 bp) without a significant fraction of nonrecombinants (less than 12%) or double insertion events (less than 2.5% [43]). Enough clones (more than 10^6) were generated in the promoter library that each genomic fragment should be represented independently several times (43). This ensures that most yeast promoter elements are represented intact. The results of screening a small fraction of the promoter library are reported herein.

Enzyme assays. For in vitro assays, yeast transformants were grown in minimal medium to ensure retention of the plasmid; all cultures were harvested in logarithmic phase. The cells (a 1-ml portion) were pelleted; this pellet was suspended in 1 ml of Z buffer (33), after which the cells were permeabilized by the addition of 50 μl each of chloroform and 0.1% sodium dodecyl sulfate. Standard spectrophotometric assays for ADH (49) and β-galactosidase (24, 41) were done on this extract.

In vivo assays were done after yeast colonies had been transferred from minimal agar to nitrocellulose filters. These filters were freeze-thawed in liquid nitrogen by equilibration in an aluminum foil boat before immersion (to prevent cracking of the nitrocellulose [8]). Freeze-thawing was followed by incubation at 37°C on 9.0-cm Whatman filters soaked in 1.8 ml of ADH stain (0.1 M Tris hydrochloride [pH 8.5], 200 μg of Nitro Blue Tetrazolium per ml, 80 μg of phenazine methosulfate per ml, 1 mg of NAD^+ per ml, 0.1% ethanol [18]). ADH activity was indicated by a dark precipitate appearing on the Whatman filter directly beneath the colony. The same nitrocellulose filters were sprayed with β-galactosidase stain (2 mg of 4-methylumbelliferyl-β-D-galactoside per ml in dimethyl sulfoxide) to assay for β-galactosidase activity. Brief illumination with a short-wave UV lamp allowed visualization of β-galactosidase activity as fluorescence. Cultures could be prepared by inoculating minimal medium directly from colonies on these assayed filters.

Random isolation of promoters. Positive yeast transformants were picked on the basis of genetic selection and in vivo assays for ADH activity. One to five plasmid species were extracted from each yeast colony; these extracts were used to transform *E. coli*. For yeast strains that contained multiple plasmid species, this procedure resulted in the independent isolation of every plasmid, each of which was then used separately to transform DC45. The spectrophotometric ADH assay of these transformants identified, for each original yeast colony, the plasmid that was responsible for the restoration of ADH expression. The randomly cloned yeast DNA insert was isolated from each of these positive plasmids, radioactively labeled with ^{32}P, and used to probe the CV13-35 yeast genomic library (35). The Southern crossblotting kit (Du Pont, NEN Research Products, Boston, Mass.) was used to generate a restriction map for each of the inserts (7.3 kbp was the average size) isolated from the CV13-35 library.

Grunstein and Hogness screening. Grunstein and Hogness screening (21) of the CV13-35 yeast genomic library was done by established procedures. Probes were labeled with α-^{32}P-labeled deoxynucleoside triphosphates by the random primer method of Feinberg and Vogelstein (17).

DNA sequencing. DNA was sequenced by the dideoxy

TABLE 1. Assays of reporter gene products in DC45 transformants

Strain	ADH enzyme activity[a]	ADH phenotype[b]	β-Galactosidase enzyme activity[c]
DC45	–	–	–
pPC0	–	–	–
pPC0-A7	1,199	+	–
pPC0-A7I	11	–	185.1
pPC0-A23	1,608	+	6.1
pPC0-A23I	587	+	336.9
pPC0-A32	315	+	6.6
pPC0-A32I	260	+	91.0
pPC0-A45	1,017	+	0.4
pPC0-A45I	814	+	366.9
pPC0-L44	1,594	+	–
pPC0-L44I	509	+	20.8

[a] The mean of determinations for four independent transformants. Units of ADH are expressed as micromoles of NAD reduced per minute (49) per optical density unit at 600 nm of each culture. –, Activity of less than 1 U.

[b] The ADH$^+$ phenotype (+) is defined as the capacity for growth in the presence of the respiratory inhibitor antimycin A.

[c] The mean of determinations for four independent transformants. Units of β-galactosidase are expressed as nanomoles of *o*-nitrophenol produced per minute per optical density unit at 600 nm (24, 41). –, Activity of less than 0.1 U.

chain termination method of Sanger et al. (42); the modifications of Chen and Seeburg (11) were used. The primer (complementary to bases −13 to −32 in the *ADH1* gene [3]) was synthesized by International Biotechnologies, Inc., New Haven, Conn.

RNA isolation and analysis. Cells were grown and harvested as for enzyme assays. Total RNA was isolated as described by Gallis et al. (19), by using yeast cells instead of polysomes as the starting material. Poly(A)$^+$ RNA was selected by chromatography on oligo(dT)-cellulose (Collaborative Research, Inc., Bedford, Mass.) as described by Aviv and Leder (2). S1 nuclease analyses of mRNA were done (under conditions of DNA excess) by the method of Favaloro et al. (16).

RESULTS

Use of silent reporter genes to detect putative yeast promoters. *adh1* and *lac4* reporter gene expression is not detectable in ADH$^-$ yeast cells that harbor the promoter-cloning vector pPC0 (Table 1). The in vivo ADH and β-galactosidase assays, as well as the genetic selection for ADH, were used to detect the subset of yeast transformants that harbored positive pPC0 library members (Fig. 2); positive members presumably contained a promoter inserted at the *Xho*I site. An equal proportion (14%) of positive transformants was detected by the in vivo ADH assay and the genetic selection for ADH activity (Fig. 2).

We isolated five of the *Xho*I fragments (A7, A23, A32, A45, and L44; size range, 155 to 543 bp) on the basis of activation of the *ADH1* gene; these fragments were associated with a dramatic increase either in the level of ADH alone (A7, A45, and L44) or in the levels of both ADH and β-galactosidase (A23 and A32) in transformed cells (Table 1). Similarly, when fragments were selected on the basis of activation of the *LAC4* gene, they were associated with a dramatic increase either in β-galactosidase activity alone or in both β-galactosidase and ADH activity (data not shown).

Evidence that the cloned fragments contain bona fide yeast promoters. To rule out the possibility that a promoter element had been created accidentally at the insert/vector

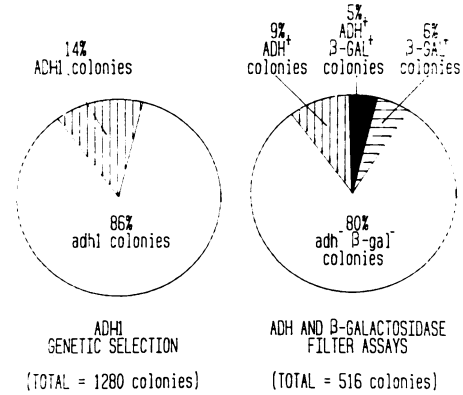

FIG. 2. In vivo assays for ADH and β-galactosidase expression. LEU2$^+$ yeast colonies, obtained after transformation of DC45 with the pPC0 library, were transferred directly to (left) YPD agar (plus antimycin A), for *ADH1* genetic selection, or (right) nitrocellulose filters, which were then assayed for ADH and β-galactosidase as described in Materials and Methods.

junctions, we recloned each fragment at the *Xho*I site of pPC0 in its inverted, or I, orientation. The association between each fragment and the restoration of reporter gene expression was retained after inversion (Table 1). Surprisingly, inversion of the A45 and L44 fragments, which in their original orientation activated only the *ADH1* gene, resulted in activation of both reporter genes. In contrast, the A7 fragment appeared to be a truly unidirectional promoter: only *ADH1* expression was restored in pPC0-A7, and only *LAC4* expression was restored in pPC0-A7I (Table 1). However, the failure of pPC0-A7, pPC0-A45, and pPC0-L44 to activate expression of both reporter genes was due to the location of RNA start sites downstream from the *LAC4* initiation codon; similarly, *ADH1* RNA start sites in pPC0-A7I were located downstream from the *ADH1* initiation codon (see below).

Other possible sources of artifacts in transformants were the high copy number and episomal environment of pPC0 library members. Therefore, integrants were generated for pPC0, all five positive library members, and each I counterpart. Intergrant strains were identical to transformants, except that the plasmid (minus the 2μm sequences) was in a chromosomal environment. For all but one integrant strain (the one that contained pPC0-A23), plasmid sequences were present in a single copy (data not shown). The results of ADH and β-galactosidase assays of the integrants are shown in Table 2. Reporter gene expression persisted after integration of positive pPC0 library members, albeit at lower levels with respect to the corresponding transformants (compare Tables 1 and 2). The lower levels in integrant strains reflected the low copy number: strains containing plasmids integrated at high copy number produced enzyme levels comparable to those in transformants (data not shown).

Artifacts could also have resulted from the presence of vector sequences in the vicinity of each putative yeast promoter. For example, a latent promoter element in pPC0 could be activated by an insert that, in its original location, is not normally associated with promoter activity. To test for promoter activity in the absence of vector sequences, we looked for RNA start sites in the vicinity of each insert in its original genomic environment. For all five randomly cloned fragments, at least one genomic poly(A)$^+$ RNA 5′ end was identified by S1 nuclease analysis (Fig. 3A; summarized in

TABLE 2. Assays of reporter gene products in DC45 single-copy integrants

Strain	ADH enzyme activity[a]	ADH phenotype[b]	β-Galactosidase enzyme activity[c]
DC45	–	–	–
pPC0	–	–	–
pPC0-A7	150	+	–
pPC0-A7I	–	–	3.0
pPC0-A23[d]	585	+	–
pPC0-A23I	64	+	10.4
pPC0-A32	23	+	–
pPC0-A32I	10	+	2.0
pPC0-A45	152	+	–
pPC0-A45I	41	+	11.7
pPC0-L44	198	+	–
pPC0-L44I	18	+	–

[a] The mean of four determinations. For units and symbols, see Table 1, footnote *a*.
[b] See Table 1, footnote *b*.
[c] The mean of four determinations. For units and symbols, see Table 1, footnote *c*.
[d] Contains approximately three integrated copies of this plasmid (data not shown).

Fig. 3B and C). For two of the five fragments, A32 (Fig. 3A, lanes 5 and 6) and L44 (lanes 9 and 10), nearby genomic RNA start sites were identified on both strands.

S1 nuclease mapping of RNA start sites in pPC0 derivatives. The reporter gene transcripts produced by each transformant and corresponding integrant were compared by S1 nuclease analysis (Fig. 4). For all positive library members, each RNA start site in the transformant had a counterpart in the corresponding integrant. Transcriptional activation of *adh1* (Fig. 4A) and *lac4* (Fig. 4B) by all of the randomly cloned yeast promoters, independent of their orientation in the *Xho*I site of pPC0, was bidirectional in both integrants and transformants. The failure of several integrants and transformants to produce both reporter enzymes (Tables 1 and 2) was caused by the location of RNA start sites downstream from the *ADH1* or *LAC4* initiation codon (Fig. 5; shown only for the original orientation of each insert).

DNA sequence analysis of promoter-containing fragments. We have determined the DNA sequence of the five fragments. As would be expected of promoter regions, none of these randomly cloned fragments were spanned by open reading frames. With two exceptions, TATA elements were found within 40 to 100 bp upstream from each RNA start site

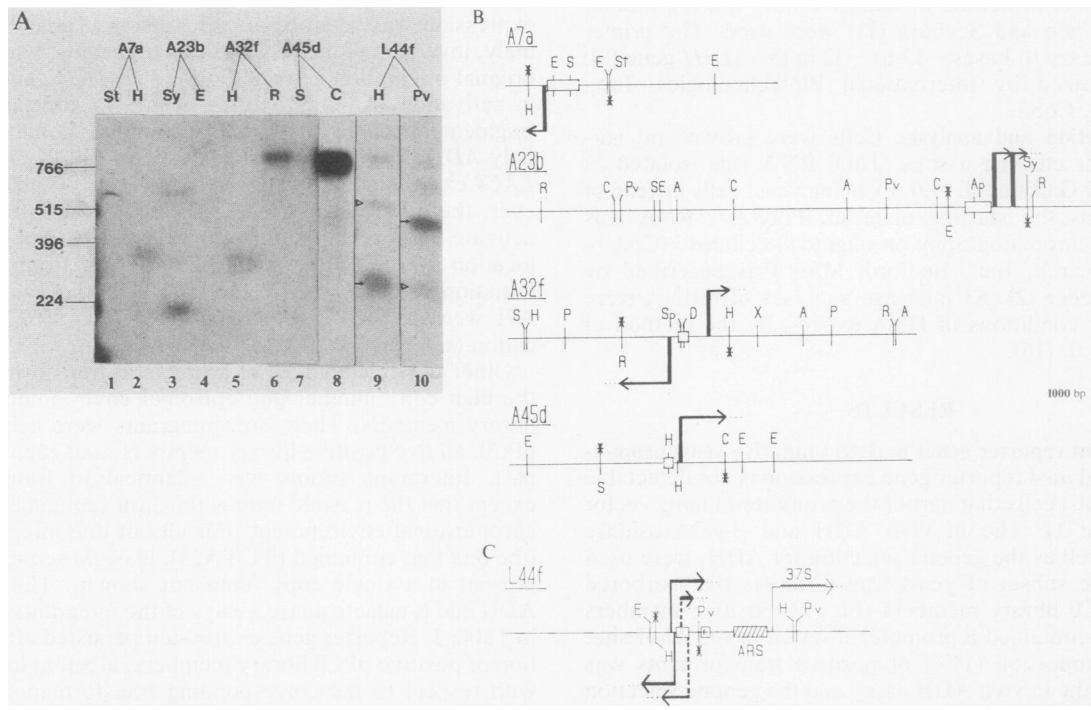

FIG. 3. Search for RNA polymerase II transcriptional start sites near randomly isolated promoters (S1 nuclease analysis of genomic RNA). (A) Poly(A)$^+$ RNA (10 μg per reaction), isolated from LL20 cells grown in YPD, was hybridized to strand-specific probes (prepared after 5′ labeling the indicated site with T4 polynucleotide kinase and [γ-^{32}P]ATP). A7a, A23b, A32f, A45d, and L44f refer to the CV13-35 library inserts that were isolated as a result of their homology to the A7, A23, A32, A45, and L44 *Xho*I fragments, respectively (see Materials and Methods). Two sites (one for each strand) were labeled in each CV13-35 library insert (panel B) as follows: *Stu*I (St) and *Hin*dIII (H) for A7; *Sty*I (Sy) and *Eco*RI (E) for A23b; *Eco*RV (R) and *Hin*dIII (H) for A32f; *Sal*I (S) and *Cla*I (C) for A45d; *Hin*dIII (H) and *Pvu*II (Pv) for L44f. The S1 nuclease-treated products were subjected to electrophoresis in alkaline 2% agarose gels. Symbols: →, major L44-associated RNA; △, minor L44-associated RNA. The molecular sizes of marker fragments are shown in bases. (B) Restriction maps of inserts from the CV13-35 library. Asterisks mark the sites labeled strand specifically for S1 nuclease analysis (panel A); the asterisks above each line indicate labeling of the top strand, and the ones below each line indicate labeling of the bottom, or opposite, strand. Symbols: ⌐→, RNA start sites (mapped in panel A); ▭, randomly isolated promoter fragments. Abbreviations (apart from those defined in panel A): A, *Ava*I; Ap, *Apa*LI; D, *Dra*I; P, *Pst*I; Sp, *Ssp*I; X, *Xba*I. (C) Diagram of the rDNA spacer region of *S. cerevisiae* (44). The enhancer of rRNA transcription is located between the *Eco*RI and *Hin*dIII sites (15). The L44 insert (▭) and the major (⌐→) and minor (↩) L44-associated genomic transcripts (mapped in panel A) are included, as are the 5S transcript (⇐), the major 5′ end of the 37S rRNA precursor (↷), and the autonomous replicating sequence (ARS; ▨). Asterisks and restriction sites are as described for panel B.

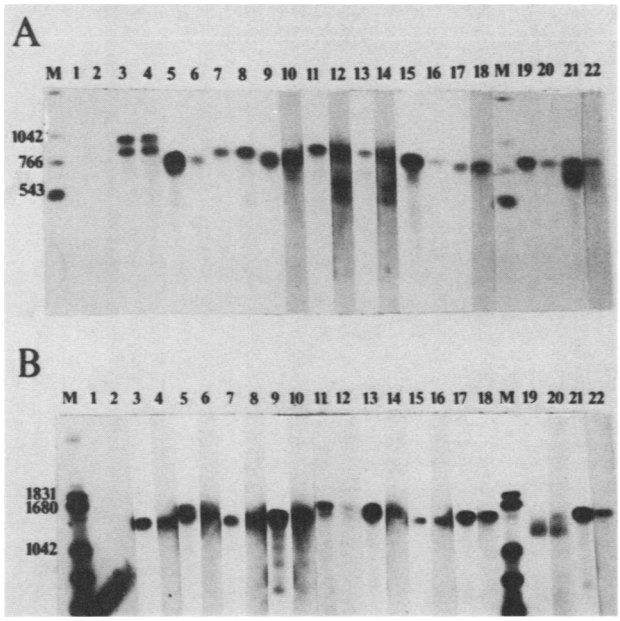

FIG. 4. S1 nuclease analysis of RNA initiation sites in pPC0 derivatives. Total RNA (15 μg per reaction for transformants; 75 μg per reaction for integrants) from cells grown in minimal medium was hybridized to homologous, single strand-specific probes. The S1 nuclease-treated products were subjected to electrophoresis in an alkaline 1.5% agarose gel. (A) Mapping of *ADH1* transcripts. Probes were prepared after 5′ labeling the *Pvu*II site of each plasmid (Fig. 1). (B) Mapping of *LAC4* transcripts. Probes were prepared after 5′ labeling the *Bam*HI site of each plasmid (Fig. 1). Lanes: M, molecular size markers (sizes given in bases); 1 and 2, pPC0; 3 and 4, pPC0-A7; 5 and 6, pPC0-A7I; 7 and 8, pPC0-A23; 9 and 10, pPC0-A23I; 11 and 12, pPC0-A32; 13 and 14, pPC0-A32I; 15 and 16, pPC0-A45; 17 and 18, pPC0-A45I; 19 and 20, pPC0-L44; 21 and 22, pPC0-L44I. Odd-numbered lanes, transformants; even-numbered lanes, integrants.

(Fig. 5). The exceptions are on the bottom strand in A7 (222 bp downstream from the nearest match) and on the top strand in A23 (171 bp downstream from the nearest match). Poly(dA-dT) homopolymer tracts were identified in three fragments (A7, A23, and A32 [Fig. 5]). The role, if any, of these randomly cloned TATA or homopolymer sequences in restoring expression of the reporter genes remains to be determined.

Identification of a regulatory switch in the rDNA spacer region. When the DNA sequence of each promoter was compared with the Genbank data base (5), only one (L44) was found to match a known sequence. The 221-bp L44 fragment (Fig. 5) is 96% identical to a segment of 1 of the approximately 100 copies of the rDNA spacer region of *S. cerevisiae* (44). The L44 fragment mapped to a region just downstream from the major RNA polymerase I enhancer (Fig. 3C) (15). S1 nuclease mapping of genomic RNA indicated that activation of bidirectional transcription by RNA polymerase II occurred in the vicinity of the L44 fragment. The two major L44-associated RNAs (Fig. 3A, lanes 9 and 10, arrows), which are transcribed divergently (Fig. 3C, bent solid arrows), have opposite regulatory responses: when ethanol was used in place of glucose as a carbon source, the level of one of the transcripts decreased (Fig. 6A, compare lanes 1G and 1E), whereas the level of the other transcript increased (Fig. 6A, compare lanes 2G and 2E). This regulatory switch is summarized in Fig. 6B. On the basis of an analysis of mitochondrially regulated RNA by subtractive hybridization, Parikh et al. (37) have also identified a transcript (ρ19) that maps near the L44 region of the ribosomal spacer (Fig. 6B); the relative levels of ρ19 in glucose-grown versus ethanol-grown cells were not determined.

DISCUSSION

A promoter-cloning approach can be used to isolate promoters from yeast genomes. We have ruled out the accidental creation of a promoter at an insert/vector junction, since disruption of the junctions by inversion of the inserts failed to deactivate transcription. By comparing transformants with corresponding single-copy integrants, we have also ruled out high copy number or episomal location as contributors to transcriptional activation. However, we have not eliminated the possibility that a latent promoter element in pPC0 was activated in the positive isolates from the promoter library. Nevertheless, we consider this to be unlikely for three reasons: (i) the start sites of reporter gene transcripts varied among plasmids containing positive inserts (Fig. 5); (ii) the pattern of transcriptional activation, which in several pPC0 derivatives was distinct with respect to the two reporter genes, was roughly reversed on inversion of each insert (for example, in Fig. 4A and B, compare lanes 19 and 20 with lanes 21 and 22); (iii) all of the randomly cloned fragments were adjacent to at least one genomic poly(A)$^+$ RNA start site (assuming that the 5′ ends identified in Fig. 3 were transcription initiation sites and not splice sites). The simplest explanation for both (i) and (ii) is that promoter elements were in a different location within each insert. The conclusion that these fragments contain bona fide promoters in a genomic context is consistent with the fact that none of the fragments were spanned by open reading frames.

Several of the randomly cloned fragments contained poly(dA-dT) homopolymer tracts. Poly(dA-dT) stretches of at least 8 bp are found in 80% of known yeast DNA sequences and, with one exception, are located in noncoding regions (48). The frequent identification of poly(dA-dT) tracts within randomly cloned yeast promoters is consistent with the hypothesis that these elements play an essential role in a prevalent mechanism of transcriptional activation in *S. cerevisiae* (48).

When two reporter genes were used to identify promoters, one-fifth of all transformants were positive. An interpretation of these results is made difficult by the influence of additional factors. For example, approximately two different plasmid species (on average) are harbored by each yeast transformant (G. Santangelo, unpublished data); this increases the proportion of positive transformants. Concomitantly, this proportion is decreased artifactually by false-negatives; occasionally the spacing of a promoter within the intergenic region of pPC0 precludes the production of functional reporter gene transcripts (Tables 1 and 2; Fig. 5). We therefore cannot use our results to estimate the frequency with which promoters occur in the *S. cerevisiae* genome. Nevertheless, it should be possible to identify classes of coordinately regulated promoters (and genes) by screening the pPC0 library.

Constitutive transcriptional activation in *S. cerevisiae* may be bidirectional. None of the promoters analyzed in pPC0 activated reporter gene transcription in an exclusively unidirectional fashion. It is possible that divergent transcription in pPC0 derivatives was artifactual, since a similar phenomenon was not observed for three of the five randomly cloned promoters in their original genomic location (Fig. 3). The

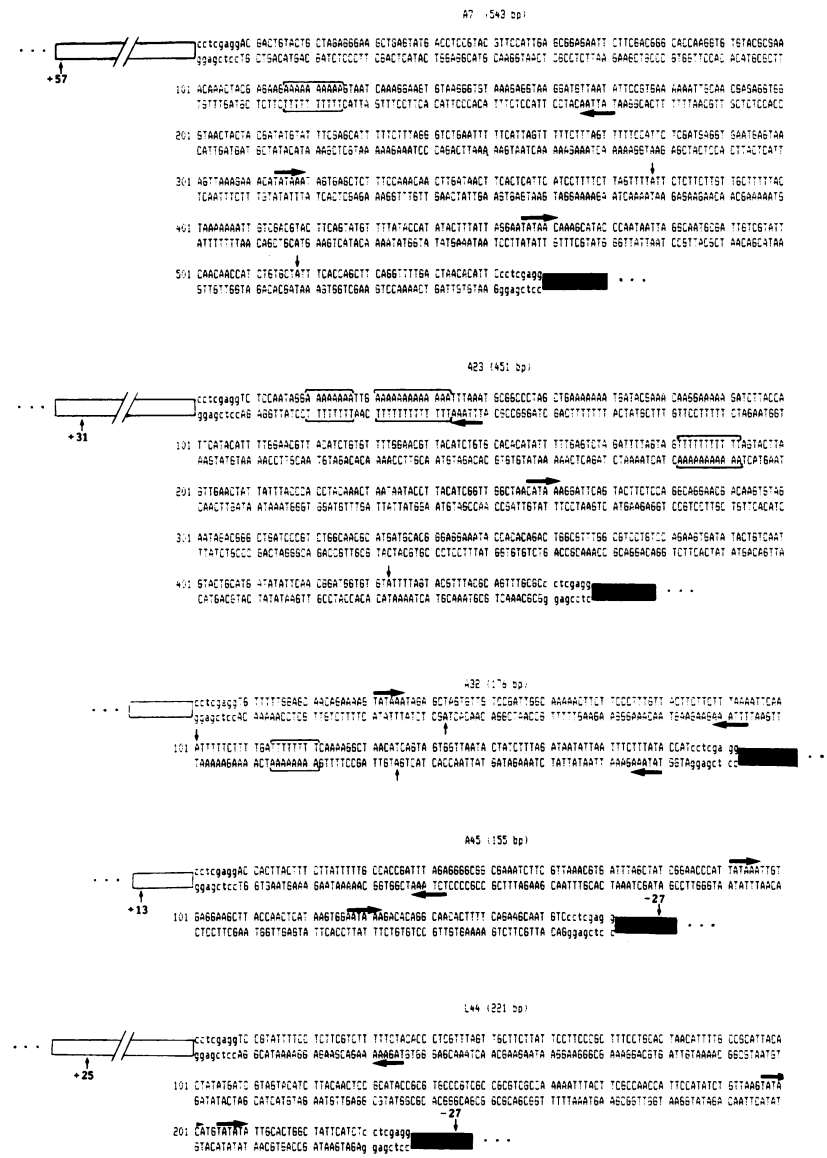

FIG. 5. DNA sequence of randomly cloned yeast promoters. The top (*ADH1* coding) strand of each sequence (in its originally isolated orientation) is 5′ to 3′. The bottom (*LAC4* coding) strand is 3′ to 5′, complementary to the top strand. The *ADH1* initiation codon is 36 bp from the rightmost *Xho*I site; the *LAC4* initiation codon (bottom strand) is 15 bp from the leftmost *Xho*I site. RNA start sites were determined from Fig. 4 and from S1 nuclease analyses with shorter probes that allowed better resolution (data not shown). For plasmids that contained start sites outside the insert, only the site nearest the insert/vector junction is shown. The numbers assigned to such sites are given (in bases) relative to the first base in the respective initiation codon (designated +1). Symbols: ▭, *LAC4* sequences; ▬, *ADH1* sequences; lower-case letters, *Xho*I site (insert/vector junction); brackets, poly(dA-dT) tracts (only those 8 bp or longer); ↑, location (±15 bp) of each *adh1* RNA start site (above the sequence) or *lac4* RNA start site (below the sequence); →, upstream TATA elements nearest each RNA start site, based on a five-of-six or six-of-six match with the sequence 5′-TATAAA-3′ (12, 34). There are no matches within the relevant segments of *ADH1* (bases −36 to +36 [3]) or *LAC4* (bases −15 to +57 [10]) DNA.

analysis of integrants has eliminated two possible sources of artifacts in transformants: high copy number and episomal environment. Although we cannot rule out a vector sequence as a cause of artifactual bidirectionality, such a mechanism would have to involve an element that was latent in pPC0 yet was capable of being activated by a variety of promoter-containing fragments (independent of their orientation). There are simpler explanations for the failure to detect divergent transcription in the original genomic context of every promoter-containing fragment. For example, the labeled site chosen for S1 protection experiments may not have been located within the transcription unit. Also, it is possible that genomic downstream processing signals (e.g., those required for polyadenylation) are absent from the strands for which RNA start sites were not detected. If so, bidirectionality would become detectable only after the downstream signals of the reporter genes in pPC0 were provided.

Two types of upstream elements have been identified in *S. cerevisiae*: poly(dA-dT) tracts and UASs. Poly(dA-dT) tracts activate transcription bidirectionally (48); they may act as entry sites by disfavoring nucleosome formation in vivo, as they do in vitro (29, 39). UASs also function bidirectionally: the *CYC1* UASs (22), UAS_G (47), and

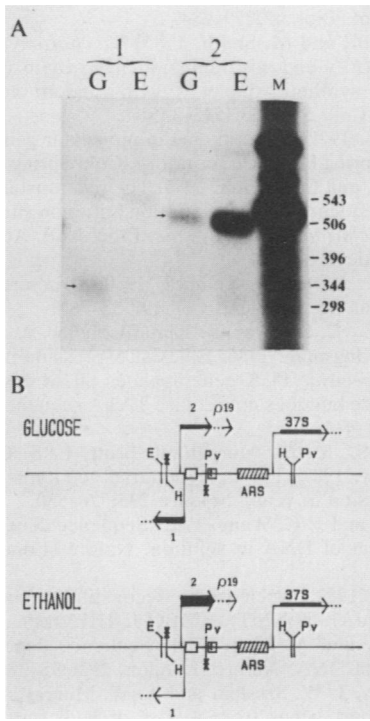

FIG. 6. Regulatory switch in the rDNA spacer region. (A) S1 nuclease analysis of L44-associated transcripts. Poly(A)$^+$ RNA (20 μg per reaction), isolated from cells grown on YP8D (G) or YPE (E), was hybridized to strand-specific probes (prepared after 5′ labeling the *Hin*dIII site [1] or the *Pvu*II site [2] with T4 polynucleotide kinase and [γ-^{32}P]ATP). S1 nuclease-resistant products were subjected to electrophoresis in an alkaline 2% agarose gel. A similar analysis with an actin-specific probe was done as a control; actin mRNA levels were identical in YP8D-grown and YPE-grown cells (data not shown). Arrows identify the major L44-associated transcripts (also see Fig. 3A, lanes 9 and 10, arrows). The molecular sizes of marker fragments (M) are shown in bases. (B) Schematic representation of the regulatory response to the carbon source. Restriction sites and landmarks are as described in Fig. 3C. The thickness of arrows 1 and 2 reflects approximate RNA levels, as determined in panel A (lanes 1 and 2, respectively). ρ19 was identified elsewhere (37) by cDNA subtractive hybridization; the arrow indicates its approximate origin (between the 5S gene and the ARS); the ρ19 start site has not been mapped.

UAS_{PGK} (36), among others, are known to activate transcription in both orientations. The TATA element (consensus TATAAA) is the only unidirectional promoter element yet identified; the opposite orientation (TTTATA) does not work (34). Therefore, if TATA elements are not absolutely required, transcriptional activation should be intrinsically bidirectional. Two apparent examples of transcriptional activation in the absence of a TATA element were found here; two other examples are associated with the *PGK* (36) and *ADH1* promoters (G. Santangelo and J. Tornow, manuscript in preparation).Therefore, bidirectionality may indeed be a property of constitutive promoter function in *S. cerevisiae*. The relatively frequent occurrence of divergently arrayed gene pairs in the *S. cerevisiae* genome might reflect an evolutionary adaptation to this aspect of transcriptional activation.

A regulatory switch in the *S. cerevisiae* rDNA spacer responds to changes in the carbon source. The L44 promoter fragment mapped near the RNA start sites of two regulated divergent transcripts whose responses to different carbon sources (fermentable versus nonfermentable) were inversely related (Fig. 6). This situation is reminiscent of the *CYC1-tr2* divergently transcribed mRNAs: *CYC1* mRNAs predominate in aerobically grown cells, whereas the *tr2* transcript predominates under anaerobic conditions (30). One of two mechanisms is likely to account for the regulation of L44-associated RNAs: differential mRNA stability or differential efficiency in the frequency of transcription initiation. Experiments that distinguish between these possibilities are in progress.

The ρ19 transcript (37) also maps to the rDNA spacer region (occasionally referred to, inappropriately, as "nontranscribed"). It is unclear whether the ρ19 transcript and the L44-associated transcript 2 are identical; they are both transcribed from the same strand (Fig. 6B). At least two distinct regulatory controls seem to occur: the L44-associated transcript 2 is expressed at high levels in ethanol-grown cells, whereas ρ19 is in abundance in ρ^- cells (which are incapable of metabolizing ethanol). The regulatory controls that act on transcripts mapping to the rDNA spacer region (including the rRNA precursor) may therefore be quite complex; further experiments are required to elucidate the underlying mechanism(s). Since this region seems to be a hot spot for transcription initiation (containing start sites for synthesis by RNA polymerases I, II, and III), an understanding of this regulation may reveal some important and possibly interlocking controls of rRNA synthesis, mitochondrial function, and cellular growth rate.

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