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Identification of Upstream Activator Sequences That Regulate Induction of the β-Galactosidase Gene in *Kluyveromyces lactis*

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Transcription of the *Kluyveromyces lactis* β-galactosidase gene, *LAC4*, is inducible by galactose and lactose. We examined the effects of deletion mutations within the *LAC4* promoter on the expression of β-galactosidase activity. The results of these experiments indicate that at least two upstream activator sequences (UAS) mediate maximum induction by galactose. These UAS sequence elements are homologous to UAS that regulate induction of the melibiose-galactose regulon of *Saccharomyces cerevisiae*. We also show that a synthetic copy of one of the *K. lactis* UAS restores the inducibility of a deleted, noninducible *LAC4* promoter. Since the uninduced or basal level of *LAC4* expression was increased in several promoter deletion strains and in deletion strains carrying one or two synthetic UAS, we examined the contribution of the LAC9 positive regulatory protein to this effect. The LAC9 protein is thought to bind to UAS and activate transcription of *LAC4* (L. V. Wray, M. M. Witte, R. C. Dickson, and M. I. Riley, Mol. Cell. Biol. 7:1111–1121, 1987). Our results demonstrate that LAC9 protein plays a role in setting the uninduced level of gene expression, but other factors also participate. For example, in a *lac9* background a *LAC4* promoter deletion mutant with two copies of a synthetic 17-base-pair UAS yields a sevenfold higher level of uninduced *LAC4* expression than the same strain with one UAS. These and other data indicate that the basal level of gene expression is strongly influenced by the base sequence of the promoter.

When the yeast *Kluyveromyces lactis* is grown in medium containing lactose or galactose, the expression of several genes required for the catabolism of these sugars is induced. Among these are the genes for the Leloir pathway enzymes galactokinase (EC 2.7.1.6), galactose-1-phosphate uridylyltransferase (EC 2.7.7.10), and UDP-glucose-4-epimerase (EC 5.1.3.2), encoded by *GAL1*, *GAL7*, and *GAL10*, respectively, and *LAC4* and *LAC12*, which code for a β-galactosidase (EC 3.2.1.23) and a lactose permease, respectively (21, 26, 29). β-Galactosidase activity is inducible up to 100-fold over a moderate uninduced level; this activity and those of the *GAL1*, *GAL7*, and *GAL10* genes have been shown to be regulated at the transcriptional level (18; T. Webster, Ph.D. Thesis, University of Kentucky, 1986). The expression of these genes is dependent upon the presence of a *trans*-acting gene, *LAC9*. *LAC9* is thought to code for a positive regulatory factor, since *lac9* strains are incapable of inducing expression of β-galactosidase and the Leloir pathway enzymes (25, 31). Another locus, *LAC10*, is presumed to encode a negative regulatory factor. This hypothesis is based on the observation that strains harboring mutations mapping at this locus are constitutive for the expression of lactose and galactose catabolic enzymes (10).

A related regulatory system which has been studied in detail is the melibiose-galactose regulon of *Saccharomyces cerevisiae*. Induction of the Leloir enzymes in this yeast is dependent upon the presence of a *trans*-acting, positive regulatory factor, the GAL4 protein (11, 16). This protein has been shown to bind specifically to 17-base-pair (bp) sequences upstream of the transcription initiation sites in the *GAL1*, *GAL10*, and *GAL7* promoters (2, 14). The presence of these galactose-specific upstream activating sequences (UAS_G) is necessary for induction of gene expression. Recent results from this laboratory showed that the *GAL4* gene of *S. cerevisiae* can complement a *lac9* mutant of *K. lactis* (22). Similarly, the *LAC9* gene of *K. lactis* can complement a *gal4* mutant strain of *S. cerevisiae* (31). These results suggest that the LAC9 and GAL4 proteins activate transcription by binding to similar UAS_G or sequences related to UAS_G.

To further understand how the *trans*-acting LAC9 protein regulates the expression of the lactose-galactose regulon of *K. lactis* and to determine whether UAS_G-related sequences are present in *K. lactis*, we examined the organization and function of nucleotides on the 5′ side of *LAC4*. Our results demonstrate that the promoter for this gene is larger than previously thought (6), that a synthetic UAS_G restores inducibility of the gene, and that there are probably at least two and perhaps more UAS_G that regulate expression of this gene.

MATERIALS AND METHODS

Bacteria, yeast, plasmids, and media. *Escherichia coli* DG75 (*hsdI leu-6 ara-14 galK2 xyl-5 mtl-1 rpsL20 thi-1 supE44 lacΔZ39* λ⁻) was used for routine bacterial transformations and plasmid propagations; *E. coli* MC1066 (*leu trp pyrF*::Tn*5 lacX74 galKU ΔstrA*) was used for construction of plasmids carrying the *S. cerevisiae URA3* gene. *K. lactis* strains Y1140 (wild type [25]) and 7B520 (*his2-2 trp1 ura3-1*, Riley and Dickson, unpublished data) were the Lac⁺ strains used in this study; *K. lactis* MS425 (α *lac4-8 ade1-1* [26]) was used as a nonreverting *lac4* mutant. This mutation is not a large deletion in *LAC4*, but its exact nature is unknown. Plasmid pKR1B is an 8-kilobase (kb) pBR322-based vector carrying the kanamycin resistance gene of Tn*903* and a 2.4-kb *K. lactis* autonomous replication sequence (*ARS1B* [29]); pKR1B-*LAC4*-1 is pKR1B containing a 12-kb *Xho*I fragment of *K. lactis* DNA inserted at the *Sal*II site. The

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insert carries the *K. lactis LAC4* and *LAC12* genes (28). Plasmid pKR1B-*LAC4*ΔXmaIII was derived from pKR1B-*LAC4*-1 by removal of a 7-kb *Xma*III fragment located on the 5′ side of *LAC4* (see Fig. 2).

Bacteria were grown on LB medium or plates. For selection of transformants LB plates were supplemented with 25 μg of kanamycin per ml, 50 μg of ampicillin per ml, or 20 μg of tetracycline per ml. To screen colonies for β-galactosidase activity, LB plates were supplemented with 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Yeast media and culture conditions were described previously (21). For the experiment in Table 1 the cells were grown overnight at 30°C to saturation in double-strength yeast nitrogen base (Difco Laboratories) containing 40 μg of both adenine and uracil per ml and 1% glucose. Cells were diluted into fresh medium, and grown from an A_{600} of 0.5 to 3.0, and extracts were prepared (21).

Oligonucleotides. Complementary oligonucleotides corresponding to the UASI sequence (Fig. 1) were synthesized so that insertion into a *Sal*I or *Xho*I site would generate only one end which could be recleaved by *Sal*I or *Xho*I; the structure of the duplex is:

5′-TCGAGCGGAAATTTGTGGTCCG
CGCCTTTAAACACCAGGCAGCT-5′

In addition, the duplex contains an *Ava*II site (underlined), which facilitates identification of plasmid constructs carrying inserts. A sequencing primer, 5′-CTCCTAAGAAACACA AGCCTC, complementary to the coding strand of the *LAC4* promoter at positions −148 to −168 was used to determine the nucleotide sequence of plasmids carrying inserts.

Construction of *Bal* 31 deletion mutations. Plasmid pKR1B-*LAC4*-1 was cleaved at its unique *Sst*II site and then treated with *Bal* 31 (0.14 U/μg of DNA) for various times. *Sal*I linkers (8 bp) were added (50- to 100-fold molar excess of ends), and the mixtures were ligated, digested with *Sal*I, and religated under conditions which promote circularization. Ligation mixtures were used to transform *E. coli* DG75. Transforming plasmids were isolated by alkaline lysis (1) and analyzed by restriction digestion to map the endpoints of deletions. The *LAC4*-proximal endpoints of putative deletion plasmids were determined by Maxam and Gilbert sequencing (19).

Construction of *Sal*I linker insertions. Plasmid pKR1B-*LAC4*-ΔXmaIII was partially digested with *Hin*cII or *Xmn*I under conditions which yielded a high percentage of singly cut, full-length linear plasmid molecules. An 8-bp *Sal*I linker, at a 50- to 100-fold molar excess of ends, was ligated to the partially cleaved vector overnight at 16°C. Ligation mixtures were electrophoresed on a 0.8% agarose gel until the full-length linear plasmid molecules were well separated from other forms. Full-length linear plasmids were collected by electroelution and purified by passage over an Elutip-D minicolumn (Schleicher and Scheull Co., Keene, N.H.). Purified DNAs were digested with *Sal*I, ligated under conditions which promote circularization, and used to transform *E. coli* DG75. *Sal*I linker insertions were located by restriction analysis. Linker insertion plasmids were designated pKR1B-*LAC4*::S195, ::S376, ::S519, and ::S1065 corresponding to the insertion sites −195, −376, −519, and −1065, respectively (Fig. 1).

Construction of *LAC4* promoters with internal deletions. Plasmids containing *Sal*I linker insertions in the *LAC4* promoter (pKR1B-*LAC4*::S195, ::S376, ::S519, and ::S1065) were digested with *Sal*I and *Xho*I to yield fragments

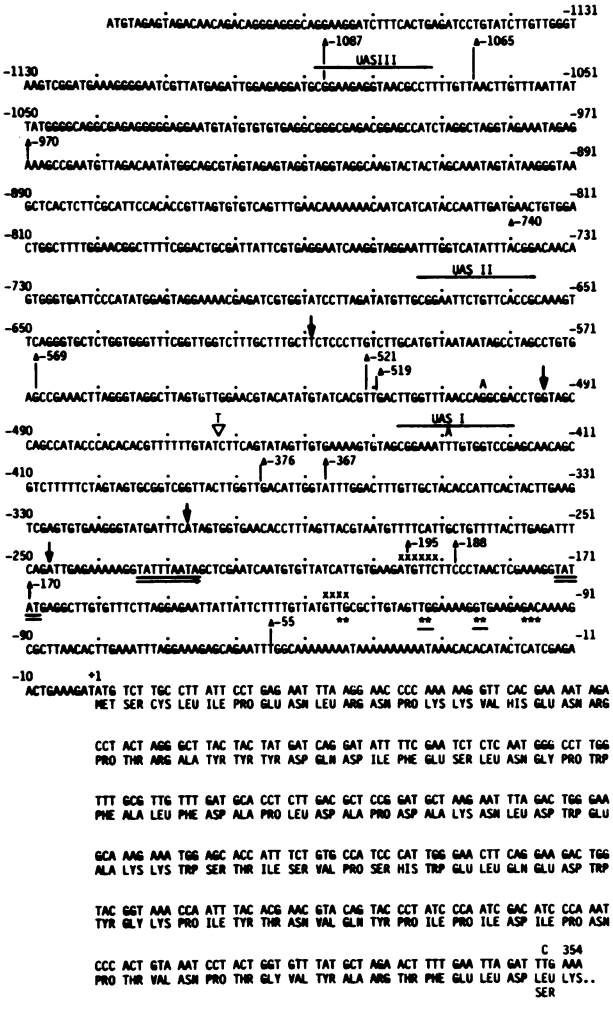

FIG. 1. Sequence of the *LAC4* promoter region. Symbols: ▲, sites of 8-bp *Sal*I linker insertions; △, endpoints of *Bal* 31-generated deletions. Putative TATAAA boxes are indicated by double underlines. *K. lactis* transcription start sites are indicated by asterisks below the sequence; the major sites (nucleotides −104/−105 and −112/−113) are underlined. *E. coli* transcription starts are indicated (×) above the sequence. The DNA sequence was determined as described in the legend to Figure 2. Differences between this sequence and a previously published sequence (4) are as follows. Arrows above the sequence indicate nucleotides which are insertions relative to the previously published sequence, and these are (i) T between nucleotides −608 and −610, (ii) G between nucleotides −494 and −496, (iii) A between nucleotides −307 and −308, and (iv) A between nucleotides −246 and −248. The inverted triangle between nucleotides −462 and −463 indicates a deletion of a T in our sequence relative to the sequence previously published (4). Nucleotides written above the sequence (−504, −429, +350) indicate differences, with the base above the sequence being that from the previous report.

of 2.5 and 12 kb. The *Xho*I site is within the kanamycin resistance gene (Fig. 2). The fragments were purified after electrophoresis in agarose gels. For the construction of pKR1B-*LAC4*Δ1 (pKR1B-*LAC4*ΔXmaIII with a deletion of the *LAC4* promoter between nucleotides −1065 and −519), the 2.5-kb fragment of pKR1B-*LAC4*::S1065 was ligated with the 12-kb fragment of pKR1B-*LAC4*::S519. pKR1B-*LAC4*Δ2 (deleted from nucleotides −1065 to −376), -Δ3 (−1065 to −196), -Δ4 (−519 to −377), -Δ5 (−519 to −196),

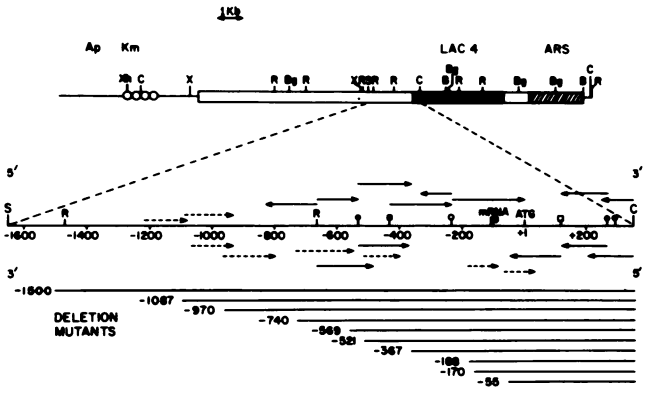

FIG. 2. Restriction map of pKR1B-*LAC4*-1, strategy for determining the nucleotide sequence of the *LAC4* promoter, and 5'-deletion mutations. The structure of pKR1B-*LAC4*-1 (28) is shown in the top of the figure. Symbols: ——, pBR322 sequences including the ampicillin resistance gene (Ap); OOOO, kanamycin resistance gene (Km) of Tn*903*; ▭, *K. lactis* DNA including (▬) *LAC4* and (▨) *ARS1B*. An expanded segment of the vector is shown in the middle of the figure. The strategy for determining the nucleotide sequence of this region is shown by arrows; those pointing in the 5'-to-3' direction were labeled with ^{32}P at their 5' ends, and those pointing in the 3'-to-5' direction were labeled with ^{32}P at their 3' ends (19). Fragments shown by solid arrows were labeled at the indicated restriction site, and those shown by broken arrows were labeled at the *Sal*I linker of deletion mutations. The transcription initiation codon, ATG, and the two major transcripts of *LAC4* are indicated. The endpoint of each deletion mutation closest to *LAC4* is given as a negative number relative to the ATG. The other endpoints (nucleotide) was determined by restriction mapping to be −1700 for Δ−1500; −2000 for Δ−1087; −3200 for Δ−970; −3900 for Δ−740; −3100 for Δ−561; and −3000 for Δ−521, Δ−188, and Δ−55. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; R, *Eco*RI; P, *Pvu*I; S, *Sst*II; X, *Xma*III; Xh, *Xho*I. Symbols: ○, *Alu*I sites; ●, RsaI sites; □, *Hae*III sites; ■, *Ava*II sites.

and -Δ6 (−376 to −196) were all constructed by using a strategy similar to that for Δ1. Plasmids, KR1B-*LAC4*Δ7 (deleted from nucleotides −1065 to −970) and pKR1B-*LAC4*Δ8 (−1065 to −740) were constructed by ligating the 2.5-kb fragment from pKR1B-*LAC4*::S1065 with the 12-kb fragments of pKR1B-*LAC4*Δ−970 and pKR1B-*LAC4*Δ−740, respectively. The structure of these deletion plasmids was verified by restriction analysis.

Construction of chromosomal deletions. A *LAC4* chromosomal promoter deletion strain carrying a synthetic UASI was constructed as follows. Plasmid KR1B-*LAC4*Δ2 was digested with *Sal*I and treated with calf intestine alkaline phosphatase. A 20- to 50-fold molar excess of synthetic UASI duplex, phosphorylated with T4 polynucleotide kinase, was ligated to the vector for 4 to 16 h at room temperature. The ligation mixture was used to transform DG75. Plasmids carrying UAS inserts were identified by digestion with *Ava*II, and the number of UAS and their orientation were determined by the Sanger sequencing procedure with double-stranded plasmid DNA (5).

Each plasmid was used to transplace the *LAC4* promoter by the procedure of Rudolph et al. (23). Plasmid DNA (20 μg) digested with *Sst*II and *Bgl*II and uncut pKARS2 (5 μg) (7) were used to transform strain 7B520 *LAC4*Δ3::*URA3* (see below) to Trp^+, a phenotype conferred by pKARS2. Transformants were pooled, suspended in sterile water, and plated onto minimal agar plates containing 200 μg of 5-fluoroorotic acid per ml. Colonies resistant to 5-fluoroorotic acid were streak purified onto YPD plates, and then replica-plated to SD plates with and without uracil (23). Ura^- colonies were analyzed by Southern blots to confirm that the *URA3* gene had been transplaced from the *LAC4* promoter by the *Sst*II-*Bgl*II DNA fragment of pKR1B-*LAC4*Δ21, pKR1B-*LAC4*Δ214, or pKR1B-*LAC4*Δ215 (see Fig. 5).

A deletion-disruption of the*LAC4* promoter was constructed as follows. A 1.1-kb *Sal*I fragment carrying the *S. cerevisiae URA3* gene was inserted into the *Sal*I site of pKR1B-*LAC4*Δ3 to give pKR1B-*LAC4*Δ3::*URA3*. The wild-type *LAC4* promoter was transplaced (23) by transforming *K. lactis* 7B520 with *Sst*II-*Bam*HI-digested pKR1B-*LAC4*Δ3::*URA3*. Ura^+ transformants were selected, and the structure of the putative promoter transplacement was confirmed by a Southern blot (data not shown). Enzyme assays were done on two to four separate isolates of each deletion strain to verify that a consensus phenotype was being examined.

Strains carrying a *lac9* deletion mutation were constructed by gene transplacement (23). The *LAC9*-containing plasmid RS2 (31) was digested with *Bgl*II and *Bst*EII and then mung bean nuclease to remove protruding ends. The *Bgl*II site is 41 bases in front of the ATG initiation codon, and the *Bst*EII site is 131 bases after the termination codon. A 1.1-kb *Sma*I fragment carrying the *S. cerevisiae URA3* gene was inserted to give *plac9*Δ1::*URA3*. The wild-type *LAC9* gene was transplaced by transforming strains 7B520 Δ1, Δ2, Δ4, Δ6, and Δ21 with *Eco*RI-digested *plac9*Δ1::*URA*. Ura^+ transformants were selected, and the transplacement was verified by Southern blotting (data not shown).

Selection of plasmid integrants. G418-resistant transformants carrying autonomously replicating plasmids were grown for approximately 100 generations in nonselective (YPD) medium. Dilutions of these outgrowths were plated onto YPD agar containing 50 μg of G418 per ml (G50 plates) to select for strains carrying a copy of the plasmid integrated into a chromosome. After 3 days of growth at 30°C, G418-resistant colonies were streak purified onto YPD agar lacking G418. After the streaks had grown, they were again replicated to G50 plates. If the original G418-resistant isolate was an integrant, 100% of the colonies within the streak grew vigorously within 1 to 2 days. However, if the transformant carried only an autonomous plasmid, most of the single colonies within the streak failed to grow on G50 plates.

Miscellaneous procedures. Procedures for the preparation of cell extracts for enzyme assays have been described (21). *K. lactis* was transformed by the method of Sreekrishna et al. (29), except that polyethylene glycol 4000 (BDH chemicals, Poole, England) was used at 60% instead of 40%. Southern blots were done essentially as described by Southern (27) with ^{32}P-labeled probes (13). Poly(A)$^+$ RNA was prepared from *K. lactis* by chromatography on an oligo(dT)-cellulose column by the procedure of Desrosiers et al. (8). The 5' termini of *LAC4* mRNAs were mapped by the method of Sures et al. (30).

RESULTS

Multiple transcripts made from *LAC4*. Before constructing deletions in the promoter of LAC4 we determined where transcription initiated. A 78-bp *Hae*II-*Taq*I DNA fragment spanning nucleotides −14 to −92 (Fig. 1) was labeled with ^{32}P at its 5' ends and used to prime transcription of poly(A)$^+$ RNA isolated from lactose-induced cells. The sizes of the reverse transcripts and their 5' end points were determined by comparison with a Maxam and Gilbert sequence ladder of a known DNA sequence. The data (Fig. 3) show two strong

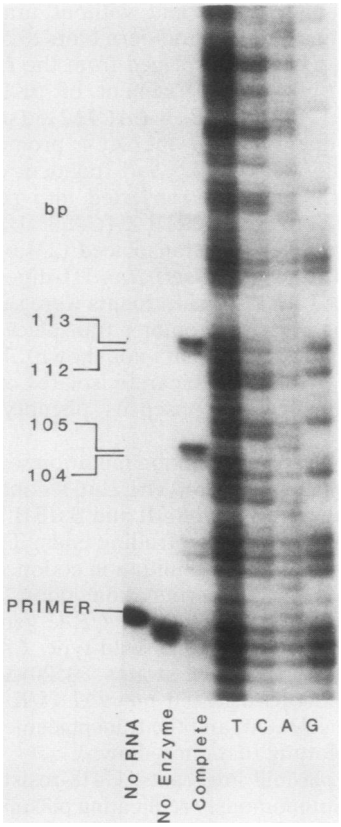

FIG. 3. Mapping 5′ termini of mRNAs synthesized in vivo from *LAC4*. The size of reverse transcripts, synthesized as described in the text and Materials and Methods, was determined by electrophoresis on an 8% polyacrylamide gel in the presence of 7 M urea followed by autoradiography of the gel. Lanes: No RNA, ^{32}P-labeled 78-bp DNA primer after incubation with reverse transcriptase in the absence of RNA; No Enzyme, complete transcription reaction without reverse transcriptase; Complete, reverse transcription products made in a complete reaction containing 50 μg of RNA from *K. lactis* induced with lactose; T, C, A, and G, nucleotide sequences of a ^{32}P-labeled DNA fragment sequenced by the method of Maxam and Gilbert (19).

bands with 5′ endpoints at nucleotides −105 and −113 along with minor 5′ endpoints at nucleotides −96, −97, −98, −104, and −112. In other experiments minor transcripts were also noted at nucleotides −124 and −125. To rule out the possibility that transcripts might arise from the region between nucleotides −96 and −15, we used a primer defined by the *Taq*I sites at nucleotides −15 and +95. The transcripts obtained with this primer have endpoints in the regions near nucleotides −98, −105, and −113 (data not shown) and are thus similar to those obtained with the 78-bp *Hae*II-*Taq*I primer. Results similar to these were obtained with the S1 nuclease mapping procedure of Weaver and Weissman (data not shown). The above results are in close agreement with an earlier report by Breunig et al. (4), in which these authors mapped the major *LAC4* initiation sites at nucleotides −105 and −115.

One mechanism for regulating induction of *LAC4* might involve switching of transcription initiation sites. This possibility was examined by comparing the 5′ endpoints of poly(A)$^+$ RNA from induced and uninduced cells. The patterns of reverse transcripts from these two samples were identical (data not shown), indicating that induction of *LAC4* did not proceed by changing transcript initiation sites.

Since *LAC4* is also expressed in *E. coli* DG75 (9) we were interested in determining where transcription initiated in this organism. The data (not shown) placed a major 5′ transcript endpoint around nucleotides −190 to −196 upstream of the *LAC4* initiation codon, with a minor endpoint around nucleotides −124 to −127. The 5′ endpoints of *LAC4* transcripts are clearly different in *K. lactis* and *E. coli*.

Effect of 5′ upstream promoter deletions on the expression of *LAC4*. To identify regions of the *LAC4* promoter necessary for induction by galactose, we constructed plasmids containing deletion mutations within the 5′-flanking sequence by using exonuclease *Bal* 31 as described in Materials and Methods. The deletion endpoint closest to *LAC4* was determined by nucleotide sequencing and is shown in Fig. 1 and 2 as a negative number [e.g., Δ–55; the plasmid carrying this deletion is called pKR1B-*LAC4*Δ–55, and strain MS425 carrying an integrated copy of the plasmid is referred to as MS425(Δ–55)].

To determine the effects of deletion mutations on the expression of β-galactosidase activity, deletion-carrying plasmids were integrated into a chromosome of a strain of *K. lactis* that has a nonreverting mutation in the *LAC4* coding region (strain MS425). Southern blots were used to verify plasmid integration, to determine the number of copies of plasmid that integrated, and to determine the site of integration. Plasmid pKR1B-*LAC4* and derivatives of it should be able to integrate into a chromosome by homologous recombination at either *LAC4* or *ARS1B* (examples of Southern blots [27] are shown in Fig. 4).

The level of β-galactosidase activity in promoter deletion strains is shown in Table 1. Deletions Δ–1500 and Δ–1087 have no effect on β-galactosidase activity as compared with the control strain, integrated pKR1B-*LAC4*-1. However, deletions extending to nucleotide −970 and further toward *LAC4* all have diminished levels of β-galactosidase activity under both inducing and noninducing conditions. Inducibility gradually decreased as the endpoint of the deletion approached nucleotide −521, beyond which inducibility was lost altogether. These results indicate that a region of the promoter between nucleotides −521 and −367 is necessary for induction. They also suggest that the level of expression of *LAC4* in both the uninduced and induced state is influenced by sequences upstream of nucleotide −970.

Interpretation of the data obtained with integrated deletion plasmids may be complicated by the presence of the normal *LAC4* promoter, by the presence of vector sequences, and by the different DNA sequence that is present in each deletion plasmid upstream of the deleted region. To avoid some of these potential problems and to further localize functional regions of the *LAC4* promoter, we constructed strains carrying chromosomal deletions within the promoter.

Effect of internal promoter deletions on *LAC4* expression. Figure 5 shows the chromosome structure of *LAC4* promoters carrying internal deletions. When the wild-type *LAC4* promoter of strain 7B520 was replaced by a deletion spanning nucleotides −196 through −1065 (Δ3, Fig. 5), the uninduced level of β-galactosidase was reduced to a level about 50% of that of the wild type. This is probably not significant, since we often observe up to a twofold variation in uninduced enzyme activities. The major effect of deletion 3 is to nearly abolish inducibility, as evidenced by the low β-galactosidase activity of the Δ3 strain grown in the presence of galactose. Thus, deletion 3 removes sequences necessary for induction. The Δ6 strain has β-galactosidase

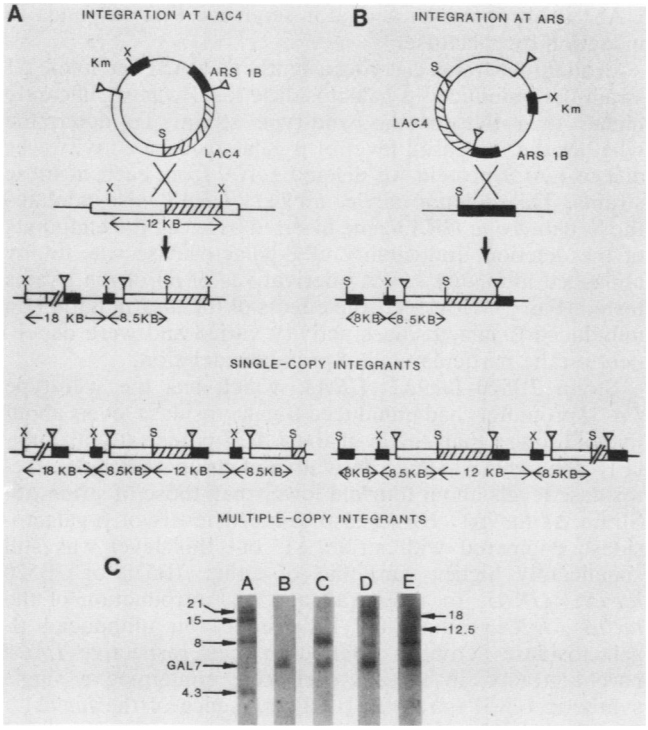

FIG. 4. Southern blot analysis of *LAC4* promoter deletion plasmids integrated into *K. lactis* chromosomes. Southern blot analysis was used to determine the site and number of *LAC4* promoter deletion plasmids that had integrated into a chromosome (see Table 1). Total DNA was isolated from putative plasmid integrant strains and digested with *Sal*I and *Xho*I. The probe was ^{32}P-labeled (13) BS2Δ*ARS*, which is identical to pKR1B except that it is deleted for *ARS* sequences and also carries a 3.2-kb *Bam*HI-*Sal*I insert containing the *K. lactis GAL7* gene. When total cellular DNA is digested with *Xho*I and *Sal*I, the *GAL7* gene migrates as a unique, 6-kb fragment (panel C, lane B). Panel A shows the structure of plasmids integrated at *LAC4* (▨) and the expected hybridizable bands. Integration of a single plasmid at *LAC4* generates an 18-kb band corresponding to the chromosomal flanking sequence and an 8.5-kb plasmid band. Integration of a single plasmid at *ARS1B* (panel B, ▬) generates an 8-kb flanking band and an 8.5-kb plasmid band, and these comigrate (panel C, lane C). Integration of multiple plasmids at either site will generate a new 12-kb band, and the intensity of the 8.5-kb band will increase proportionally with the number of copies integrated. In multiple-copy integrants, the intensity of the 8.5-kb band relative to the 6-kb *GAL7* band, as determined by densitometric scanning, was used to estimate copy number. (C) Lanes: A, molecular size markers pKR1B-*LAC4*-1, pKR1B-*LAC4*ΔXmaIII, pKR1B, and pBR322; B, *Sal*I-*Xho*I digest of MS425 DNA; C, *Sal*I-*Xho*I digest of MS425(Δ−367) (one copy, *ARS1B*); D, *Sal*I-*Xho*I digest of MS425(Δ−970) (one copy, *LAC4*); E, *Sal*I-*Xho*I digest of MS425(Δ−188) (two copies, *LAC4*). Restriction sites: X, *Xho*I; S, *Sal*I. Symbol: ▽, *Sal*I-*Xho*I junction.

activity which is as inducible as that of 7B520, but both the induced and uninduced β-galactosidase activities are reduced to about 15% of the wild-type level.

These data indicate that the region between nucleotides −196 and −376 is not necessary for induction but is necessary for maximal expression of *LAC4*. Strain Δ5 has an uninduced β-galactosidase activity similar to Δ6; however, the inducibility of β-galactosidase activity is reduced in this strain. These data suggest that the region between nucleotides −376 and −519 is required for maximal induction. This prediction was verified with strain Δ4, in which the inducibility of β-galactosidase activity was reduced to 13-fold. Strain Δ2, in which nucleotides −377 through −1065 are deleted, showed a further loss of inducibility (reduced to 5-fold) as compared with Δ4 (13-fold), indicating that sequences within the region of nucleotides −520 to −1065 are also necessary for maximal induction. To test this possibility, we measured the β-galactosidase activities in a strain containing a deletion of these nucleotides (Δ1) and observed that β-galactosidase inducibility was reduced to eightfold. Surprisingly, both the uninduced and induced β-galactosidase activities were elevated compared with the wild type, although the fold inducibility was reduced. Possible explanations for this effect are presented in the Discussion. The next upstream deletion, Δ8, in which nucleotides −1065 through −740 are deleted, had uninduced β-galactosidase activity slightly higher than wild-type and induced levels essentially identical to those of strain 7B520. Similarly, deletion of sequences between nucleotides −970 and −1065 had little effect on *LAC4* expression. Taken together, the data from these experiments indicate that the *LAC4* promoter contains at least two types of elements. One, located between nucleotides −195 and −376, is necessary for maximal expression of *LAC4* under both induced and uninduced conditions. The other elements, located between nucleotides −377 and −519 and nucleotides −520 and −740 are necessary for maximal induction.

TABLE 1. Effect of 5′ upstream promoter deletion mutations on the expression of *LAC4*[a]

Yeast strain (deletion plasmid)	Copies	β-Galactosidase activity	
		Uninduced	Induced
MS425(Δ-1500)	1[b]	84 ± 65	2,541 ± 684
MS425(Δ-1087)	1[b]	157 ± 96	3,787 ± 238
MS425(Δ-970)	1[b]	6	864 ± 324
MS425(Δ-740)	1[b]	1.5 ± 0.2	438 ± 54
MS425(Δ-569)	1[c]	1.7 ± 0.5	178 ± 15
MS425(Δ-521)	2[b]	1.0 ± 0.6	107 ± 37
MS425(Δ-367)	1[c]	0.6 ± 0.5	2.0 ± 0.6
MS425(Δ-188)	2[b]	3.5 ± 2.7	3.6 ± 0.3
MS425(Δ-170)	2[b]	5.7 ± 1.5	6.5 ± 1.6
MS425(Δ-55)	1[c]	0.1 ± 0.02	0.5
MS425(pKR1B-*LAC4*-1)	1[b]	190 ± 112	2,806 ± 1,137
Y1140(pKR1B)	1[c]	37 ± 18	2,443 ± 1,485

[a] For these experiments each *LAC4* promoter deletion plasmid shown in Fig. 2 was integrated into a chromosome of *K. lactis* MS425 (*lac4-8*). The specific activity of β-galactosidase, expressed as nanomoles of substrate hydrolyzed per milligram of protein, was measured in cell extracts (21). Cells were grown as described in Materials and Methods. Induced samples were supplemented with 1% galactose during all stages of growth. Enzyme values with a standard deviation were determined on three or more separate cell extracts. Values without a standard deviation were determined once or twice. Plasmid pKR1B-*LAC4*-1 has the wild-type promoter and is a positive control for these experiments. Another positive control is Y1140(pKR1B) (29). Deletion -367 has 70 bp of DNA of unknown origin between nucleotides -367 and -297.

[b] Number of copies of plasmid integrated at *LAC4*.

[c] Number of copies of plasmid integrated at *ARS1B*.

Activation of *LAC4* gene expression by a synthetic UAS. Because of the similarities which exist between the regulation of galactose metabolism in *S. cerevisiae* and *K. lactis*, we searched the *LAC4* promoter for sequences homologous to UAS_G of several *S. cerevisiae* genes comprising the melibiose-galactose regulon. The only constraints that we placed on potential *K. lactis* UAS were that they be at least 50% homologous to a *S. cerevisiae* GAL UAS and that they be of the form 5′-CGG(N_{11})CCG, since these 6 bp are nearly 100% conserved among all of the identified *S. cerevisiae*

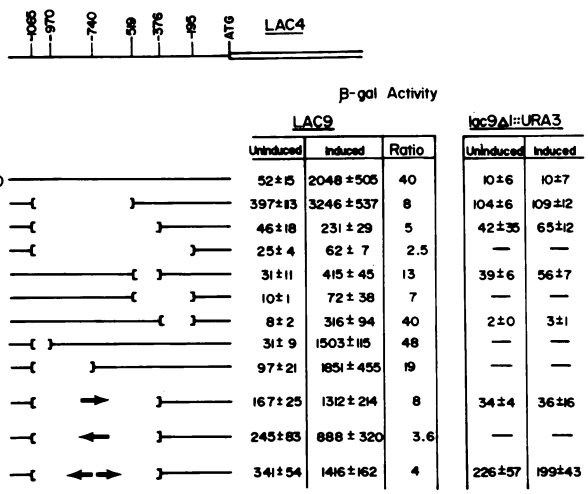

FIG. 5. Effect of internal promoter deletions on the expression of *LAC4*. The DNA sequence upstream of the *LAC4* coding sequence is diagrammed at the top of the figure with deletion endpoints indicated by negative numbers. The wild-type *LAC4* promoter of strain 7B520 was deleted by gene transplacement as described in Materials and Methods. The strain resulting from the transplacement of the *LAC4* promoter by that of pKR1B-*LAC4*Δ1 is designated Δ1, and the other strains are designated similarly. Deleted sequences are indicated by the space between the brackets in the lower part of figure. β-Galactosidase activity was measured in a strain carrying *LAC9* and in an isogenic mutant strain in which LAC9 was deleted by gene transplacement and replaced by *lac9*Δ1::*URA3*. Cells were grown and β-galactosidase activities were determined as described in Materials and Methods. Uninduced refers to cultures grown in minimal medium containing 1% glucose as the carbon source; induced refers to cultures grown in minimal medium containing 1% glucose and 1% galactose as an inducer. All activities are given as the mean of three or more separate determinations with standard deviations as indicated. Ratio refers to the ratio of induced β-galactosidase activity to uninduced β-galactosidase activity. Each arrow in Δ21, Δ215, and Δ214 represents a single synthetic UASI sequence. The direction of the arrow indicates the orientation of UASI, where the arrow pointing to the right is in the native orientation.

GAL UAS (3). In addition, the penultimate G · C pair at either end of these sequences has been implicated to form close contacts with the GAL4 protein, since methylation of either guanine in these pairs is prevented by GAL4 binding (14). Two such sequences were identified (Fig. 1); UASI, between nucleotides −420 and −436, and UASII, between nucleotides −657 and −673. To determine whether UASI could function in vivo, we inserted a synthetic copy of it into the *Sal*I site of pKR1B-*LAC4*Δ2. Three types of constructs were identified by DNA sequencing (Fig. 5).

Each of these plasmid constructs was used to transplace the *LAC4* promoter of strain 7B520 *LAC4*Δ3::*URA3*. The β-galactosidase activities obtained with the resultant strains are shown in Fig. 5. With a single UASI in the native orientation (strain Δ21), the induced level of β-galactosidase activity is restored to 64% of that of the wild type. Strain Δ215, which contains a single copy of UASI in the reverse orientation, has induced levels of β-galactosidase similar to those of strain Δ21. Strain Δ214, containing two copies of the synthetic UASI, has induced β-galactosidase levels identical to those of strain Δ21. Thus, introduction of a second UASI adjacent to the first has no further effect on increasing induced *LAC4* expression. These results demonstrate that UASI is an upstream activator sequence that responds to induction by galactose.

In all three strains carrying a synthetic UASI and in the Δ1 strain the uninduced β-galactosidase level was significantly higher than that of the wild-type strain. To determine whether the increased level of β-galactosidase activity was due to LAC9 protein we deleted *LAC9* from each of these strains. This deletion allele, *lac9*Δ1::*URA3*, also contains the *S. cerevisiae URA3* gene inserted between the endpoints of the deletion. Inducibility of β-galactosidase was totally abolished in *lac9*Δ1::*URA3* derivatives of all of the strains tested (Fig. 5). However, the effects of the *lac9* mutation on uninduced β-galactosidase activity varied and were dependent on the particular *LAC4* promoter deletion.

Strain 7B520 *lac9*Δ1::*URA3*, which has the wild-type *LAC4* promoter, had uninduced β-galactosidase levels about fivefold lower than those of the *LAC9* parent strain. Similarly, the *lac9*Δ1::*URA3* derivative of strain Δ6 had β-galactosidase levels about fourfold lower than those of strain Δ6. Strain Δ1 *lac9*Δ1::*URA3* also reduced levels of β-galactosidase compared with strain Δ1, but this level was still significantly higher than that of either 7B520 or 7B520 *lac9*Δ1::*URA3*. In strains Δ2 and Δ4, introduction of the *lac9*Δ1::*URA3* allele had little effect on uninduced β-galactosidase activities relative to their respective *LAC9* parent strains. In the Δ2 derivative containing a single synthetic UASI (strain Δ21), the presence of the *lac9*Δ1::*URA3* allele decreased β-galactosidase activity to a level comparable to that of Δ2 *lac9*Δ1::*URA3*. However, when a second synthetic UASI was also present (strain Δ214), inactivation of *LAC9* caused only a slight decrease in β-galactosidase activity. Taken together, these data indicate that *LAC9* and multiple UAS elements are responsible for the induction of *LAC4* expression, and that these same factors are, at least in part, necessary for the maintenance of uninduced β-galactosidase activity. In addition, comparison of the uninduced and induced enzyme activities of strains Δ2 and Δ2 *lac9*Δ1::*URA3* indicates that at least one other *LAC9*-responsive UAS sequence in addition to those at positions −420 to −436 and positions −657 to −673 must exist within the *LAC4* promoter.

DISCUSSION

The data presented here show that the *LAC4* promoter of *K. lactis* contains at least two UAS that are necessary for maximum induction of the gene by galactose and a region between nucleotides −195 and −376 that is necessary for maximum expression of the gene.

Support for the existence of UASI, nucleotides −420 to −436, and UASII, nucleotides −657 to −673 (Fig. 1), is as follows. First, a strain carrying promoter deletion 2 (Fig. 5) has normal uninduced levels of β-galactosidase activity but only induces 5-fold, whereas the wild-type strain induces 40-fold. The region deleted in this strain is large, between nucleotides −376 and −1065, and could contain multiple sequences that are required for inducibility. The strain carrying Δ4 subdivides the region into two parts, both of which contribute to induction. This conclusion follows from the inducibility of the Δ4 strain, 13-fold, which is between the inducibility of the wild type and the Δ2 strain. Further support for UASI as a transcriptional activator comes from a comparison of plasmid Δ−521, which is inducible for β-galactosidase activity (Table 1), and plasmid Δ−367, which is not inducible. This result is in agreement with a previous analysis of the *LAC4* promoter (4). Second, be-

cause of functional similarities between the regulation of genes involved in galactose catabolism in *S. cerevisiae* and *K. lactis*, we reasoned that the galactose-responsive upstream-activating sequences in both organisms should be similar. Bram et al. (3) compiled a list of GAL upstream-activating sequences from *S. cerevisiae*. Nearly all of these have a 17-bp core consisting of the sequence 5'-CGG (N_{11})CCG. A computer search of the *LAC4* sequence shown in Fig. 1 with this UAS core sequence identified UASI and UASII as shown in Fig. 1. These two UAS lie within the region which we identified by deletion analysis as being necessary for maximal induction. Finally, to determine whether UASI could function as an upstream-activating sequence we synthesized and inserted it into the *LAC4*Δ2 promoter deletion strain that has low β-galactosidase inducibility. The synthetic UASI sequence restored the induced level of β-galactosidase activity to about 60% of the wild-type level (Fig. 5; compare strain Δ21 with strain 7B520). This is similar to *S. cerevisiae*, in which a synthetic consensus, UAS_G, restores the inducibility of the *GAL1* gene to 50 to 70% of the wild-type-induced level (14). Thus, the synthetic UASI functions as an upstream-activator sequence in the context of Δ2 and it is likely to do the same in a normal sequence context.

Synthetic UASI functioned in either orientation, which is similar to the behavior of eucaryotic enhancer sequences. Two UASI oriented head to head (strain Δ214, Fig. 5) did not increase the induced level of β-galactosidase over the level produced by one UASI. However, the two UAS did increase the uninduced level of enzyme activity. We discuss this result below.

Although we have not tested the putative UASII sequence for UAS activity, we believe that it functions as a UAS in vivo because it is 71% homologous to UASI, and in a strain in which UASI is deleted (Δ4), β-galactosidase expression is still 13-fold inducible. This induction requires LAC9 protein, indicating that the promoter must still contain one or more UAS.

There is also a third potential UAS sequence upstream of *LAC4*. The sequence 5'-CGGAAGAGGTAACGCCT, located at nucleotides −1088 to −1072, is 41 and 47% homologous to UASI and UASII, respectively. In the strain containing a deletion of nucleotides −1065 through −377 of the *LAC4* promoter (Δ2, Fig. 5), β-galactosidase expression is still fivefold inducible. When *LAC9* was deleted in this strain, β-galactosidase activity was no longer inducible. These results suggest that one or more galactose-responsive elements other than UASI and UASII exist. These are probably upstream of nucleotide −1065 rather than downstream of nucleotide −376, since a strain carrying a deletion of nucleotides −376 through −195 induces 40-fold just like the wild-type parent. Whether the sequence between nucleotides −1088 and −1072 is a GAL UAS cannot be determined from the present study.

Deletion of nucleotides −196 to −376 (strain Δ6) decreased expression of *LAC4* in both uninduced and induced growth conditions but had no effect on inducibility as measured by the induction ratio of 40-fold. Deletion of *LAC9* in this strain completely abolished induction, indicating that the induction was due to LAC9 protein. Examination of the *LAC4* promoter sequence in this region revealed a sequence, 5'-TATTTAATA, centered around nucleotide −230. This sequence is similar to others which have been identified as TATAAA boxes, and it may function as such (24). TATAAA sequences are thought to be necessary for proper transcription initiation, and deletion of this sequence could account for the phenotype observed with strain Δ6. To be functional, TATAAA sequences are usually between 60 and 120 nucleotides upstream of transcription start sites in *S. cerevisiae* (15). By this criterion, the first two transcription start sites of *LAC4*, nucleotides −124 and −125 and nucleotides −112 and −113, could be determined by the nucleotide −230 sequence. However, it is possible that deletion of the region of nucleotides −196 to −376 removes other sequences important for *LAC4* transcription. We conclude that this region is not required for *LAC4* induction, but it is necessary for maximum expression under both inducing and noninducing conditions.

All of our conclusions about the location of UASs and TATA sequences are further supported by the data presented in Table 1.

Wray et al. (31) have shown that the LAC9 protein of *K. lactis* can activate transcription of the *S. cerevisiae GAL1*, *GAL7*, *GAL10*, and *MEL1* genes in a *gal4* mutant strain of *S. cerevisiae*. Riley et al. (22) have shown that *GAL4* activates transcription of the *K. lactis GAL1*, *GAL7*, *GAL10*, and *LAC4* genes in a *lac9* mutant strain of *K. lactis*. These results suggest that the LAC9 protein, like the GAL4 protein, binds to UAS sequences and activates transcription. This hypothesis is supported by the data in Fig. 5, which demonstrate that induction of *LAC4* is dependent upon a UAS and the LAC9 protein. This hypothesis should be verified by more direct experiments, such as footprinting analysis.

An interesting aspect of the present study is the elevated uninduced β-galactosidase level observed with strains Δ1, Δ21, Δ214, and Δ215 (Fig. 5). There are several mechanisms that could explain the increased level of enzyme activity. First, in strains Δ21, Δ214, Δ215, and Δ1, a sequence that is necessary for controlling the transcriptional activator function of the LAC9 protein had been deleted. Second, the deletions bring another UAS into closer proximity to UASI. These two UAS sequences than act cooperatively to facilitate transcription in the absence of inducer. A third possibility is that multiple UAS elements, properly spaced, are necessary to repress transcription mediated by the LAC9 protein. Finally, the uninduced level might have nothing to do with UAS or LAC9 protein.

We examined the role of *LAC9* in setting the uninduced level of *LAC4* gene expression by deleting *LAC9* from strains having promoter mutations (Fig. 5). In the wild type and in strains having an elevated uninduced β-galactosidase level (Δ1, Δ21, and Δ214), deletion of *LAC9* reduced the uninduced level of gene expression. So in these strains the LAC9 protein is participating in setting the uninduced level of gene expression. However, it is not the only factor, since the level of expression in strains Δ1 and Δ214 is still elevated above the wild-type level even when *LAC9* is deleted. Furthermore, the uninduced level of *LAC4* expression was not affected by *LAC9* in strain Δ2 or Δ4. These results demonstrate that factors besides LAC9 protein play a role in setting the uninduced level of *LAC4* expression. This is particularly striking when comparing strain Δ21 *lac9*Δ1::*URA3* (34 units) to strain Δ214 *lac9*Δ1::*URA3* (226 units). These two strains only differ by the presence of a synthetic UAS. The mechanism by which UAS and promoter deletions affect basal level gene expression remains to be determined.

There is some evidence that UAS function in pairs (reviewed in reference 20). Bram et al. (3) have shown that two UAS_G are necessary for complete repression of transcription of *S. cerevisiae* galactose-inducible genes in the absence of inducer. Since repression of galactose-inducible genes in *S.*

cerevisiae is dependent upon the presence of the negative regulatory protein GAL80 (12, 17), Bram et al. have suggested that the GAL80 protein interacts with the GAL4 protein, bound at two upstream activating sequences, to effect repression. If such a mechanism is used for repression in vivo, then it is likely that the number of UAS and spacing between them are important for normal function. It is possible that the deletion in strains with elevated uninduced β-galactosidase activity has altered the spacing between interactive UAS elements or has removed one element, rendering them incapable of forming the hypothetical repressor complex.

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