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Functional Homology between the Yeast Regulatory Proteins GAL4 and LAC9: *LAC9*-Mediated Transcriptional Activation in *Kluyveromyces lactis* Involves Protein Binding to a Regulatory Sequence Homologous to the GAL4 Protein-Binding Site

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As shown previously, the β-galactosidase gene of *Kluyveromyces lactis* is transcriptionally regulated via an upstream activation site (UAS_L) which contains a sequence homologous to the GAL4 protein-binding site in *Saccharomyces cerevisiae* (M. Ruzzi, K. D. Breunig, A. G. Ficca, and C. P. Hollenberg, Mol. Cell. Biol. 7:991–997, 1987). Here we demonstrate that the region of homology specifically binds a *K. lactis* regulatory protein. The binding activity was detectable in protein extracts from wild-type cells enriched for DNA-binding proteins by heparin affinity chromatography. These extracts could be used directly for DNase I and exonuclease III protection experiments. A *lac9* deletion strain, which fails to induce the β-galactosidase gene, did not contain the binding factor. The homology of LAC9 protein with GAL4 (J. M. Salmeron and S. A. Johnston, Nucleic Acids Res. 14:7767–7781, 1986) strongly suggests that LAC9 protein binds directly to UAS_L and plays a role similar to that of GAL4 in regulating transcription.

In most well-characterized cases, yeast transcriptional regulation of protein-encoding genes has been shown to be mediated by *cis*-acting elements called upstream activation sites (UAS) (reviewed in references 16 and 37). They are located at variable distances 5′ to the structural gene and are required to activate transcription in response to particular physiological conditions. UAS elements are independent functional units which are interchangeable between genes (2, 17, 19); they can impose a specific mode of regulation on a given gene when placed upstream of its TATA box. Genetic evidence obtained from a number of systems suggests, and in some cases it has been shown directly (15, 20, 31), that a regulatory protein binds to these elements and that this binding is a prerequisite for transcriptional activation (5, 18, 23).

We have recently reported on the regulation of the β-galactosidase gene (*LAC4*) from *Kluyveromyces lactis*, a lactose-fermenting yeast (8). *LAC4* transcription is activated by lactose or galactose, and no glucose repression has been observed in the strain we use (8). In contrast to the procaryotic β-galactosidase gene *lacZ* of *Escherichia coli*, *LAC4* transcription is positively regulated (8) in a way typical for yeast genes: a UAS, (UAS_L) is located ca. 400 base pairs (bp) 5′ to the start of translation. This sequence is both necessary and sufficient to confer *LAC4*-specific regulation (34).

At least two regulatory genes affect *LAC4* regulation in *trans*, as defined by mutations which result in constitutive (*lac10*) (11) or noninducible (*lac9*) (36) expression. In these mutants, expression of genes of the galactose pathway, *GAL1*, *GAL10*, and *GAL7* (36), and of the lactose permease gene *LAC12* (10) is similarly affected, suggesting that these genes are coregulated with *LAC4*.

Recently the regulatory gene *LAC9* was cloned (35, 38) and shown to complement a *gal4* mutation of *Saccharomyces cerevisiae* (35), and vice versa, the GAL4 protein complements a *lac9* mutation (32). Although the amino acid sequences of the two proteins show little homology, some functional regions, one of which is the DNA-binding domain of GAL4, seem to be conserved (35, 38).

We have detected a DNA sequence homologous to the GAL4 protein-binding site (UAS_G) (4, 15) in the 5′ half of UAS_L (the so-called segment A) (34). This region is defined by a deletion that results in a drastic reduction of *LAC4* expression under inducing conditions but is not by itself sufficient to confer lactose regulation. For UAS_L function, an additional segment, which shows no obvious homology to segment A, is also required (34).

The complementation of *gal4* by *LAC9* in *S. cerevisiae* and the sequence homology of UAS_L and UAS_G suggest that the mode of regulation of *MEL* and *GAL* genes on the one hand and *LAC* and *GAL* genes on the other hand is similar in *S. cerevisiae* and *K. lactis*.

In this communication we present binding studies with *K. lactis* proteins to the *LAC4* regulatory region and demonstrate that a protein binds to UAS_L. Since no binding is observed in a *lac9* deletion mutant, we suggest that the DNA-binding protein is the product of the regulatory gene *LAC9*.

MATERIALS AND METHODS

Yeast strains. CBS2360 is a wild-type *K. lactis* strain. SD11 is a *trp1* derivative of CBS2360 (9), and SD11.U2 is a *ura3* mutant of SD11. W600B α *leu ade1 ade2* has been described (7), and JA6 α *trp ura ade* was obtained by crossing SD11.U2 and W600B. DL9 *lac9 trp1 ade* was derived from JA6 by gene displacement at the *LAC9* locus as described below.

Preparation of *K. lactis* protein extracts. *K. lactis* CBS2360 (wild type) was used to prepare yeast cell extracts. Cells were grown in rich medium under inducing (YEP plus 2% galactose) or noninducing (YEP plus 2% glucose) conditions to an optical density at 600 nm of 1.5. Cells were harvested and washed twice with cold H_2O and once with buffer A [200

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mM Tris hydrochloride, pH 8, 300 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 7 mM β-mercaptoethanol, 10% glycerol]. Usually extracts were prepared from about 20 g of cells which had been suspended in 25 ml of buffer A containing 1 mM phenylmethylsulfonyl fluoride and broken with 0.5 volume of glass beads (0.45 cm diameter) in a Braun homogenizer. The procedure to enrich for binding activities essentially followed the protocol of Arcangioli and Lescure (1). The cell extract was cleared by centrifugation for 10 min in a JA20 rotor (Beckman) at 12,000 × *g* and subsequently in an SW40 rotor at 100,000 × *g* for 1 h at 4°C. The supernatant (S100) with an average protein concentration of 15 mg/ml was diluted to 50 mM $(NH_4)_2SO_4$ with buffer B (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.8], 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 7 mM β-mercaptoethanol, 10% glycerol) and applied to a heparin column (Heparin Ultrogel A4R; LKB) of 2.5 cm diameter and 5 cm height at a flow rate of 50 ml/h. The column was washed with buffer B containing 50 mM $(NH_4)_2SO_4$, and the bound material, which was about 45% of the total protein, was eluted with a salt gradient [50 to 700 mM $(NH_4)_2SO_4$ in buffer B] into 40 fractions of 2 ml each with an average protein concentration of 3 to 5 mg/ml. Column fractions were used directly for retardation gels or dialyzed against buffer B with 100 mM NaCl for DNase I or exonuclease III (ExoIII) protection experiments. Binding activity was stable for at least 2 months at −70°C when portions were frozen in dry ice-acetone.

Source and preparation of fragments for binding studies. DNA fragments used for binding assays were isolated from plasmid pSH4 or its derivatives. pSH4 is a subclone of the 5' end of *LAC4*. The 2.1-kbp *Eco*RI-*Hin*dIII fragment (6) was inserted in the polylinker region of pUR250 (U. Rüther, Ph.D. thesis, University of Cologne, 1983), and a *Sal*I linker (CGTCGACG; Boehringer Mannheim) was added after filling in the *Eco*RI site (position −669 of the *LAC4* gene [6]). Derivatives containing fragments from position −669 to various positions downstream were obtained by Bal31 deletion of *Cla*I-restricted pSH4 and linker addition prior to ligation (34). p292 and p381 contain an *Eco*RI linker at position −292 and −381, respectively.

The UAS_L fragment commonly used for binding studies was, e.g., a 455-bp *Sal*I-*Hin*fI fragment from pSH4 covering nucleotides −674 to −221. The plasmid was cleaved with *Sal*I 5' end-labeled with [γ-^{32}P]ATP, cleaved with *Hin*fI, and isolated by elution from polyacrylamide gels (25). The specific activity was about 1.5×10^6 cpm/pmol (5×10^6 cpm/μg of fragment). A labeled *Nde*I-*Hin*fI fragment (−530 to −221) was prepared in the same way.

*Eco*RI fragment f292 (−669 to −292) was isolated from p292, 5'-end labeled, and cleaved with *Rsa*I (−533) or *Taq*I (−328). Reisolation of the large subfragments gave fragments labeled at the lower or upper strand, respectively.

A *Sal*I-*Taq*I fragment (−675 to −328) which covers UAS_L (26) isolated from pSH4 and a 90-bp *Mbo*II-*Eco*RI fragment covering segment A of UAS_L from p381 were isolated as unlabeled restriction fragments and used for competition experiments. The nonspecific fragment used was an *Eco*RI-*Sca*I fragment from pBR322 (position 3846 to 4361). These fragments were quantified on polyacrylamide gels stained with silver (28) by comparing serial dilutions with a DNA standard of the same size, giving an accuracy within a factor of 2.

Retardation gels. Binding tests were carried out in buffer B containing 100 mM NaCl, 5 mM $MgCl_2$, 0.2 to 1 ng of labeled fragment (1×10^3 to 5×10^3 cpm), 25 ng of poly(dI-dC) (Pharmacia), and about 1 μg of protein in a total volume of 15 μl. Samples were incubated for 10 min at 30°C and immediately loaded on 6% polyacrylamide gels. Gels were run for 3 h at 120 V at room temperature in TBE buffer (90 mM Tris, 90 mM H_3BO_3, 2.4 mM EDTA), dried, and autoradiographed.

DNase I protection experiments. Assay volume was 50 μl, containing 10 ng of labeled fragment, 2.5 μg of poly(dI-dC), and about 200 μg of protein in binding buffer (buffer B with 100 mM NaCl and 5 mM $MgCl_2$) and 5 mM $CaCl_2$. After preincubation for 7 min at 30°C and for 5 min at room temperature, DNase I (Worthington) was added to a final concentration of 0.3 μg/ml. The enzyme was freshly diluted 1:100 from a 500-μg/ml stock solution. The reaction was stopped after 30 s by the addition of 100 μl of stop solution, containing 0.6 M LiCl, 25 mM EDTA, and tRNA (250 μg/ml). DNA was purified by three extractions with phenol-chloroform (1:1) and ethanol precipitated. Samples were dissolved and loaded on 6 or 8% sequencing gels as described (25).

ExoIII protection experiments. ExoIII digestion was performed in binding buffer (buffer B plus 100 mM NaCl and 5 mM $MgCl_2$) in a 60-μl assay volume with 2.5 μg of poly(dI-dC), 15 ng of labeled fragment, and 130 μg of protein. ExoIII (P-L Biochemicals) (80 U) was added after 5 min of preincubation at 30°C. A control without protein was treated in parallel. Reactions were stopped after different times by the addition of DNase I stop solution, and samples were purified and analyzed as described for the DNase I protection experiments.

Construction of a *lac9* deletion strain. We replaced the 5' end of the chromosomal *LAC9* gene with the *URA3* gene of *S. cerevisiae* by the one-step gene displacement method of Rothstein (33).

In detail, plasmid pDL9 was constructed from the cloned *LAC9* gene of pJ431 (35) (generously provided by J. M. Salmeron and S. A. Johnston). A 0.8-kbp *Eco*RI-*Hin*dIII fragment located upstream of the *LAC9* gene was subcloned into pBR322. A *Hin*dIII fragment carrying the *URA3* gene of *S. cerevisiae* and the 3' end of the *LAC9* gene was inserted into the unique *Hin*dIII site of the resulting plasmid. This fragment was obtained by cleavage of pJ431 with *Asp*718 and *Sma*I, filling in, and ligation, thereby deleting all *K. lactis* sequences 5' to position +679 of the *LAC9* coding region and linking the 3' portion of the gene to the *URA3* gene.

As a result, in pDL9, sequences from −150 to +679 of *LAC9* are replaced by 1.1 kbp of *URA3* sequences and the *S. cerevisiae* gene is flanked on both sides by *K. lactis* sequences.

To allow homologous recombination with the *K. lactis* chromosome, pDL9 was cleaved within these flanking sequences with *Hpa*I and *Eco*RI and transformed into *K. lactis* JA6 (*ura trp ade*) by the method described by Klebe et al. (24). Of 150 Ura^+ transformants, 6 were Lac^-. In all six the *URA3* gene was integrated at the *LAC9* locus and the *LAC9* gene was replaced by the deleted version of pDL9, as verified by Southern analysis (not shown). The resulting strain was named DL9.

RESULTS

A *K. lactis* protein forms a specific complex with a UAS_L restriction fragment. Having defined the UAS of the *LAC4* gene (UAS_L [34]), we were interested in proteins interacting with this region. A very sensitive method to detect DNA-binding proteins in crude cell extracts is a gel electrophoresis

assay (12, 14) in which DNA-protein complexes are separated from free DNA by retardation due to bound proteins. We used a restriction fragment covering the 5′ noncoding region from position −674 to −221 (designated the UAS_L fragment) to perform such binding studies. This fragment covers UAS_L, which we have mapped between positions −435 and −326 and which has been shown to be sufficient to confer lactose regulation to a heterologous promoter (34). Incubation of this fragment with crude extracts from *K. lactis* cells resulted in different retarded bands which were difficult to resolve (data not shown).

We tried to enrich for DNA-binding proteins by passing the crude cell extracts over a heparin column and eluting the adsorbing material with a 50 to 700 mM salt gradient into 40 fractions as described in Materials and Methods. The retardation of the UAS_L fragment after incubation with the various fractions is shown in Fig. 1. At least three complexes, named C_1, C_2, and C_3, were formed (Fig. 1A). Treatment with proteinase K completely abolished complex formation (data not shown).

To determine whether any of these complexes was relevant to regulation of the *LAC4* gene, we compared extracts from induced and noninduced *K. lactis* cells; the same or very similar complexes were formed with both protein extracts (Fig. 1B).

We therefore tried to distinguish between nonspecific DNA-binding proteins and specific UAS_L-binding factors by competition with nonlabeled restriction fragments. For this purpose, we combined the heparin column fractions into different pools. Figure 1C shows the results of a competition experiment with a pool of fractions 15 to 22, which contained proteins forming complexes C_1 and C_2. When increasing amounts of a *SalI-TaqI* fragment (−674 to −328) which carries all sequences required for UAS_L function (lanes a to d) were added, clear competition for complex C_2 formation was observed. The addition of an equimolar amount of unlabeled DNA (lane b) resulted in a reduction of complex C_2. A 90-bp restriction fragment (−468 to −381; see Materials and Methods) which contained only the 5′ half (segment A) of UAS_L, also competed but clearly less efficiently (lanes e to h), whereas with a nonspecific DNA fragment (lanes i to l), a weak effect was seen only at a 25-fold excess (lane l).

We conclude that complex C_2 contains a protein that specifically binds to UAS_L. At least one binding site was located on the 90-bp fragment (−468 to −381), which covers the region of homology with UAS_G, but others seemed to be present on the larger UAS_L fragment (−674 to −328), as evidenced by better competition with this fragment.

UAS_L is protected from DNase I digestion. The DNA footprinting technique of Galas and Schmitz (13) allows localization of the binding site of a given protein in vitro by protecting this region against DNase I digestion. We used this approach to test the fractions from the heparin column for specific binding activity. Fractions were combined into five different pools according to their potential for complex formation. The end-labeled UAS_L fragment (−674 to −221) was incubated with about 200 μg of protein from each of the pools and subsequently treated with DNase I to allow limited digests as described in Materials and Methods (Fig. 2, lanes a to f). There was a protected region in lane d with the protein from pool III (fractions 15 to 22). As shown in Fig. 1A, these protein fractions mainly resulted in complex C_2 formation.

The footprint mapped to segment A of UAS_L (hatched box in the schematic representation) the regulatory element with sequence homology to the GAL4-binding site. A more

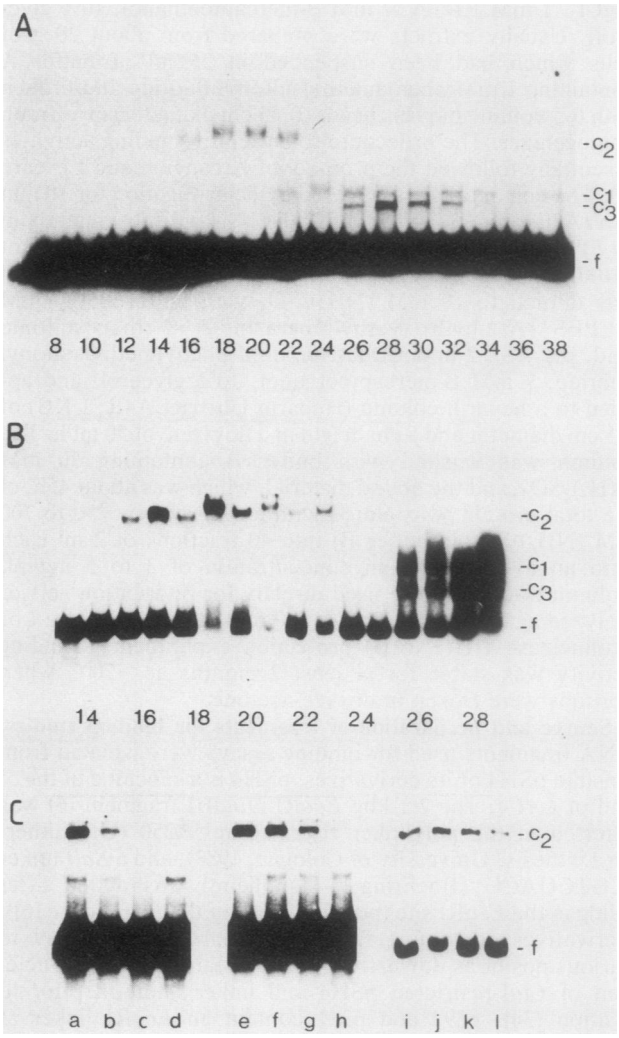

FIG. 1. Retardation of DNA-protein complexes in polyacrylamide gels. An end-labeled DNA fragment carrying UAS_L (−674 to −221) (3,000 cpm per lane; 2 fmol) was incubated with different column fractions of *K. lactis* cell protein in the presence of 25 ng of poly(dI-dC) competitor DNA. The protein fractions were eluted from a heparin column with a salt gradient into 40 fractions, each containing about 3 to 5 mg of protein per ml. About 1 μg of the indicated fraction was incubated with the labeled fragment for 10 min at 30°C and immediately loaded onto a 6% polyacrylamide gel. After 3 h of electrophoresis at 120 V in TBE buffer, gels were dried and autoradiographed. (A) Elution profile of protein from *K. lactis* cells grown in galactose (induced). The different complexes formed by the indicated protein fractions resulting in retardation of the free DNA (f) are designated C_1, C_2, and C_3. (B) Comparison of protein fractions from induced and noninduced cells. Equivalent heparin column fractions from extracts of noninduced and induced cells (left and right lanes of the indicated fractions, respectively) were run side by side. (C) Competition experiment with unlabeled restriction fragments. In lanes a to d, the *SalI-TaqI* (−674 to −328) UAS_L fragment was added, in lanes e to h an *MboII-EcoRI* (−468 to −381; see Materials and Methods) UAS_L segment A fragment, and in lanes i to l an *EcoRI-ScaI* fragment from pBR322 (position 3846 to 4361) were added. The first lane for each set serves as a control without competitor DNA; the second lanes contain equimolar amounts of labeled and unlabeled DNA; the third lanes contain a fivefold molar excess; and the fourth lanes contain a 25-fold molar excess of competitor DNA.

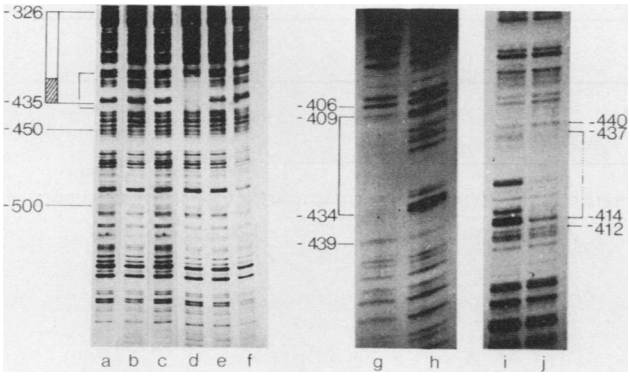

FIG. 2. DNase I digestion of a UAS_L fragment in the presence of *K. lactis* cell protein fractions. ^{32}P-labeled DNA fragments (5×10^6 cpm/μg, 50,000 cpm per assay) were preincubated with about 200 μg of protein from induced *K. lactis* cells, subsequently treated with DNase I (0.3 μg/ml), and, after purification, separated on DNA sequencing gels as described in Materials and Methods. Lanes: a to g, *Sal*I-*Hin*fI (−674 to −221) fragment labeled at the *Sal*I site incubated with various protein fractions: no protein (a) or fractions 1 to 9 (b), 10 to 14 (c), 15 to 22 (d), 23 to 32 (e), and 33 to 40 (f); lanes g to j, the pool III protein (15 to 22) was again bound to the *Sal*I-*Hin*fI fragment, now labeled at the *Hin*fI site (lanes i and j), or to an *Nde*I-*Hin*fI fragment (−530 to −221) labeled at the *Nde*I site (lanes g and h). Lanes h and i are control lanes without protein. The region protected from DNase I cleavage is indicated by a bracket. The position of the cleavage sites, as deduced from a sequencing ladder (not shown), is given as the distance from the ATG initiation codon of the *LAC4* gene. The boxed area is the UAS_L (position −435 to −326) as defined previously (34). The hatched box indicates the DNA sequence homologous to the GAL4-binding site (−434 to −418).

precise localization of the protein-binding site was obtained by labeling a restriction site (*Nde*I, position −530) closer to the protected region. Lane g shows the footprint on the *Nde*I-*Hin*fI fragment. The 5′ border mapped between positions −439 and −434, and the 3′ border mapped between −409 and −406. There was some ambiguity due to the lack of cleavage sites in free DNA.

On the opposite strand, as shown with the *Sal*I-*Hin*fI fragment (−674 to −221) labeled at the *Hin*fI site (lanes i and j), the footprint was visible but less pronounced. Between positions −412 and −440, three regions spaced at helix repeat intervals (−437 to −436, −427 to −424, and −417 to −414) were strongly protected, alternating with cleavage sites accessible for DNase I in the presence of the bound protein. The 17-bp *GAL4* homology was located in the center of the protein-binding site (cf. Fig. 4).

No additional DNase I-protected region was observed in the 3′ half (segment B) of UAS_L, which is also required for UAS_L function (34). However, a protein binding with lower affinity might easily remain undetected in this assay. We therefore complemented the DNase I protection experiments by ExoIII digestion of the DNA-protein complexes (39). This technique is based on the blocking of ExoIII digestion by a protein bound to the DNA, resulting in a positive signal in an otherwise empty lane, so that even very unstable complexes are eventually detectable as a weak band.

An *Rsa*I-*Eco*RI fragment (−533 to −292) and an *Eco*RI-*Taq*I fragment (−668 to −328) labeled at the lower or upper strand, respectively, were preincubated with protein of pool III and then treated with ExoIII for various times. No additional binding sites upstream or downstream of the DNase I-protected region were observed (Fig. 3). ExoIII stops at position −437 in the upper strand and at position −418 in the bottom strand. Again the 17 bp of GAL-homologous sequences (−434 to −418) were fully protected by the DNA-binding protein, whereas in the control lanes without protein (lanes b to d and i to k) the DNA was further degraded. There was a slight inhibition of ExoIII in digesting sequences 3′ to the binding site compared with the samples without protein (lane e versus b), but a certain slowing down of the enzyme was observed in all protein-containing samples. At the 5′ end the same border was observed with ExoIII as with DNase I, whereas at the 3′ end the protected region was slightly bigger in the DNase I footprint. These data are schematically summarized in Fig. 4 together with the results of our deletion analysis (8). We have no indication that segment B, the second element required for UAS function, binds to the same protein as segment A.

UAS_L-binding activity is also present in noninduced cells. To test whether *LAC4* regulation might be mediated by the presence or absence of active UAS_L-binding factor, we used protein fractions from glucose-grown cells for DNase I protection experiments. This led to the same, although slightly weaker, footprint as that obtained with protein from induced cells (Fig. 5, lanes b and c).

The data clearly show that a UAS_L-binding factor was present in extracts of noninduced cells and bound in vitro to the same DNA sequences as the activity from induced cells.

No UAS_L binding is detectable in a *lac9* deletion mutant. It has recently been shown that the *K. lactis* regulatory protein LAC9 can complement an *S. cerevisiae gal4* mutation (35).

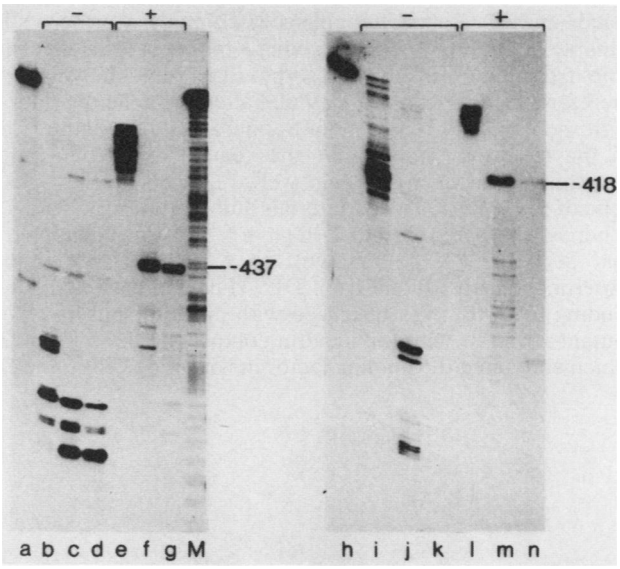

FIG. 3. ExoIII digestion of the UAS_L fragment in the presence and absence of protein. An *Rsa*I-*Eco*RI fragment (−530 to −292) (lanes a to g) and an *Eco*RI-*Taq*I fragment (−668 to −328) (lanes h to n) both 5′-end labeled at the *Eco*RI sites were digested with 40 U of ExoIII for various times in the presence (+) or absence (−) of protein (fractions 15 to 22 [pool III] from the heparin column). Samples were taken after 2.5, 5, and 7.5 min in lanes b to d, e to g, i to k, and l to n. Lanes a and h show untreated fragments. DNA was purified and subjected to electrophoresis on 8% sequencing gels. The molecular weight of the bands was deduced from a purine sequencing ladder of the same fragment (shown only for the *Rsa*I fragment, lane M). ExoIII blocking due to bound protein is indicated, giving the distance from the ATG initiation codon of *LAC4* (6).

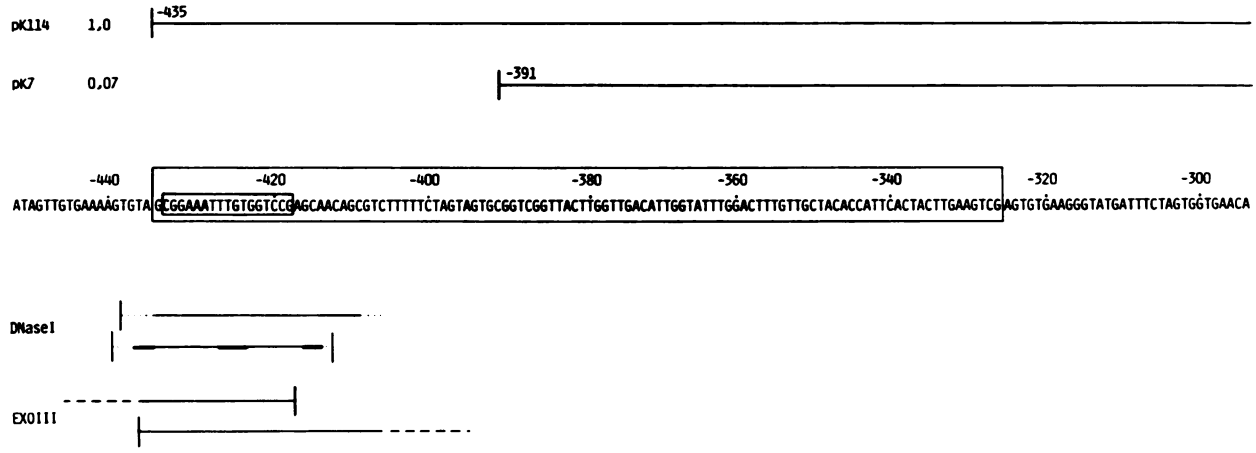

FIG. 4. Summary of DNase protection data. The DNA sequence located 5′ to the *LAC4* structural gene (6) gives the distance from the ATG initiation codon. The boxed area is the UAS_L. The internal box shows the 17-bp region homologous to the GAL4 protein-binding site of *S. cerevisiae* (34). Ratios of induced gene expression with respect to the wild type of two relevant 5′ deletion mutants are given above. The lines indicate the sequences retained within these *LAC4* plasmids (for details, compare reference 8). Below the sequence, the data presented in this work are summarized. The region protected by protein binding in the coding strand (sequence shown) and noncoding strand is indicated by the upper and lower lines, respectively, for DNase I and ExoIII. In the noncoding strand, strong DNase I protection is indicated by the thicker line; between these areas, some bands can be cleaved by the enzyme in the presence of bound protein (cf. Fig. 3, lane j).

Because of the homology of its N-terminal end to the GAL4 DNA-binding domain (23, 35, 38), this protein might bind to a similar sequence as the GAL4 protein and therefore is a likely candidate for the UAS_L-binding protein.

We analyzed *lac9* mutants for the presence of the binding activity. A noninducible *lac9* mutant obtained by mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (A. Walker-Jonah and M. Ruzzi, unpublished) contains a protein still capable of binding to UAS_L, giving a footprint indistinguishable from that of the wild type (data not shown). We therefore tried to create a *lac9* null allele by gene disruption with the cloned *LAC9* gene of Salmeron and Johnston (35). In the resulting strain DL9, the sequences encoding the N-terminal end of the protein were replaced by the *URA3* gene of *S. cerevisiae* (see Materials and Methods for details). Whereas the parent strain JA6 gave a footprint identical to that of CBS2360 (not shown), in a DNase I protection experiment with protein from DL9 (Fig. 5, lanes d to f) no binding to UAS_L was observed with protein from this *lac9* mutant strain or with the heparin column fractions 15 to 22, which contained the binding factor in wild-type cells (lane e),

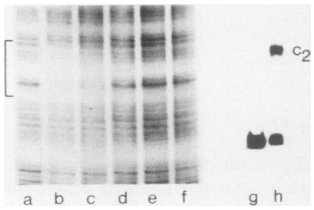

FIG. 5. Comparison of protein binding with extracts from induced, noninduced, and mutant *K. lactis* cells. Lanes a and b represent the DNase I digest of the *Sal*I-*Hin*fI fragment (−674 to −221) without protein (lane a) and with pool III protein from induced *K. lactis* CBS2360 as described in the legend to Fig. 2. In lane c, the same protein extract was made from uninduced cells. In lanes d to f, protein was isolated from the *K. lactis lac9* deletion mutant DL9. Pools II, III, and IV of the heparin column (see Fig. 2) were tested in lanes d, e, and f, respectively. Lanes g and h represent a gel retardation assay of DL9 and wild-type pool III protein (see the legend to Fig. 1).

or with those eluting at lower (lane d) or higher (lane f) salt concentration from the affinity column. In lane g a retardation gel shows that complex C_2 (lane h; cf. Fig. 1A) was not formed with the mutant protein.

These data demonstrate that the UAS_L-binding activity depends on the presence of the LAC9 protein. Although we cannot exclude an indirect effect of LAC9, its structural and functional homology with GAL4 (35) and the homology of UAS_L with the GAL4-binding site strongly suggest that the regulatory protein LAC9 itself binds to the regulatory region of the *LAC4* gene.

DISCUSSION

We have chosen the β-galactosidase gene from the yeast *K. lactis* as a model system to study gene regulation. An interesting aspect of this gene stems from the homology between its activator, the product of *LAC9* (32, 35, 38), and an extensively studied *S. cerevisiae* transcriptional activator, the *GAL4* protein.

Here we demonstrate that from a protein extract enriched for DNA-binding proteins by heparin affinity chromatography, a factor binds specifically to a *cis*-regulatory element located 400 bp 5′ to the *LAC4* structural gene. We could use a heparin column fraction obtained from wild-type *K. lactis* cell extracts directly for DNase I protection experiments, whereas in vitro footprinting data with *S. cerevisiae* regulatory proteins were mostly obtained by overproducing the binding proteins in either *E. coli* or yeast cells (3, 15, 23), by in vitro synthesis of the protein (20), or by purification of DNA-protein complexes from retardation gels (1). In this particular case no such enrichment was necessary.

We are confident that the observed binding reflects a specific protein-DNA interaction for the following reasons. (i) The affinity for a 25-bp sequence greatly exceeded that for 425 bp of flanking sequences, resulting in a clear footprint in DNase I protection experiments. (ii) The binding site coincided with an element which, by deletion analysis, has been shown to be essential for transcriptional activation in response to the inducer (34). (iii) The sequence of the binding site was homologous to the binding site of the GAL4

regulatory protein in *S. cerevisiae* (34). (iv) The binding protein was absent in a regulatory mutant.

The size of the protected region strikingly resembles that of the GAL4 protein (23): it extends a few base pairs to both sides of the 17-bp consensus sequence (Fig. 4). The GAL4 protein has been shown to bind to the same sequences in vivo as in vitro (15, 23), and in analogy we presently assume that this is also true for the protein binding to *LAC4*.

In the intergenic region of the *GAL1* and *GAL10* genes, a primary binding site for the GAL4 protein (site II in reference 23) is flanked by weaker binding sites on both sides. In the *LAC4* upstream region we detected one binding site only. However, working with rather crude fractions limits the possibility of further increasing the concentration of the binding protein. As shown in Fig. 1C, the bigger UAS_L restriction fragment competed more efficiently for complex C_2 formation than the smaller one, which indicates that additional binding sites may be present upstream or downstream of the A site (segment A of UAS_L). This would be consistent with our deletion data, which have shown that the A site is not sufficient to mediate *LAC4*-specific regulation (34).

Our data show that in vitro the binding site is occupied when protein is isolated from noninduced cells. Either the binding factor is synthesized constitutively, or a different factor, e.g., a negative regulatory protein, binds to the same site. Again by analogy to GAL4 (27, 30), we favor the first possibility. It has been suggested (26, 27, 30) that regulation of the *GAL* genes is achieved by interaction of the product of the negative regulatory gene *GAL80* with GAL4 protein. As mentioned in reference 15, GAL4 protein seems to bind to UAS_G in vivo when cells are grown under derepressed but uninduced conditions, whereas after growth in glucose no binding is detectable (15). Since DNA binding and transcriptional activation are separable functions in GAL4, it can probably remain bound to the DNA in a complex with GAL80 protein. The *K. lactis* strain we used is not glucose repressed, and growth in glucose resembles that in derepressed uninduced conditions of *S. cerevisiae*. Probably the specific induction acts on top of a bound primary regulatory protein. Our retardation gels did not show any difference in the DNA-protein complexes between extracts from induced and uninduced cells (Fig. 1B). However, a negative regulatory factor for the LAC genes might have been lost or inactivated during our enrichment procedure for the DNA-binding protein.

The UAS_L-binding protein seems to be contained within the low-mobility complex C_2 since (i) the fractions giving the footprint (Fig. 2 and unpublished data) all showed complex C_2 formation and (ii) the *lac9* mutant lacking the binding factor did not form C_2 on retardation gels (Fig. 5).

Both the facts that the binding site is a regulatory sequence and that the binding activity is lacking in a regulatory mutant demonstrate the relevance of this DNA-protein complex for regulation of the *LAC4* gene. The data do not rule out an indirect effect of the *LAC9* gene product on the binding activity. However, the structural and functional homology of this protein with GAL4 strongly suggests that LAC9 protein itself binds to UAS_L.

In a *lac9* mutant obtained by chemical mutagenesis (A. Walker-Jonah and M. Ruzzi, unpublished), the protein can bind to the DNA even though there is no induction of *LAC4* expression. Although the DNA-binding and activation functions are located in separate domains in yeast regulatory proteins (21, 23), it seems difficult to obtain mutations in the activation function. Recently Johnston and Dover (22) undertook a genetic analysis of *GAL4*. Mutants selected in vivo to lack function of the protein either have missense mutations in the DNA-binding domain that abolish binding activity or have nonsense or frameshift mutations in the carboxy-terminal 95% of the protein. We therefore expect the *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced *lac9* mutant to be of the latter class.

We could not test whether any *LAC9*-derived peptide was synthesized in the *lac9* deletion strain which we constructed. However, since sequences upstream of position +679 have been removed, the proposed DNA-binding domain (35, 38) is totally missing.

From our deletion analysis (34) we know that the presence of the presumptive LAC9-binding site in segment A of UAS_L is not sufficient to activate transcription of the *LAC4* wild-type strain. Either additional essential LAC9-binding sites in segment B were not detectable under our assay conditions or, more likely, additional factors are involved in *LAC4* regulation. In this respect the *LAC* regulon would deviate from the *GAL* genes of *S. cerevisiae*, in which the binding of GAL4 is sufficient to activate transcription.

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