Mol Gen Genet (1992) 233:97–105

MGG
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Glucose transport in the yeast *Kluyveromyces lactis*

II. Transcriptional regulation of the glucose transporter gene *RAG1*

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Received October 15, 1991 / January 14, 1992

Summary. The *RAG1* gene encodes a membrane protein involved in the low-affinity glucose/fructose transport system of the yeast *Kluyveromyces lactis*. Analysis of steady-state mRNA levels analysis and quantitation of expression by β-galactosidase from *RAG1-lacZ* fusions assays revealed that the *RAG1* gene was poorly expressed in cells grown under gluconeogenesis conditions, but was induced more than ten-fold when they were grown on various sugars. These sugars included glucose, fructose, mannose, sucrose, raffinose, as well as galactose. Nucleotide sequence and deletion analysis of the 5′ flanking region of the *RAG1* gene showed that an essential *cis*-acting element required for induced transcription of the *RAG1* gene resided between −615 and −750 from the coding sequence. This region contained a 22 bp purine stretch, and a pair of 11 bp direct repeat sequences. The 11 bp repeats harbor a CCAAT motif, a consensus sequence for binding of the yeast and mammalian HAP2/3/4-type protein complex. The transcription of the *RAG1* gene was dramatically affected by three unlinked mutations, *rag4*, *rag5* and *rag8*. We discuss the possible roles of *RAG4*, *RAG5* and *RAG8* gene products in the expression of the *RAG1* gene, as well as the importance of the inducible *RAG1* gene in the fermentative growth of *K. lactis*.

Key words: *Kluyveromyces lactis* – Transcriptional regulation – Promoter – Sugar transport

Introduction

The ability to take up glucose is a fundamental property of cells. Transport of glucose in yeast, as in most mammalian cell types, occurs via carrier-mediated facilitated diffusion (Bisson and Fraenkel 1983a; Cirillo 1962; Lang and Cirillo 1987). In the cells of *Saccharomyces cerevisiae*, the uptake of glucose appears to be mediated by at least two types of transport, distinguishable as high-affinity (K_m about 2 mM) and low-affinity (K_m about 20 mM) systems (Bisson and Fraenkel 1983b; 1984). Two glucose/fructose transporter genes, *SNF3* (Bisson et al. 1987) and *HXT2* (Kruckeberg and Bisson 1990), have been isolated. They encode an integral membrane protein sharing homology with the facilitated diffusion glucose transporter of erythrocytes (Celenza et al. 1988) and the large family of other sugar transporters (Baldwin and Henderson 1989; Cheng and Michels 1989; Szkutnicka et al. 1989). Genetic and biochemical analysis suggest that both *SNF3* and *HXT2* are required for wild-type levels of high-affinity glucose transport (Bisson et al. 1987; Kruckeberg and Bisson 1990). However, to date the nature of the components involved in the low-affinity transport system is not well understood.

In the cells of *S. cerevisiae*, the low-affinity glucose uptake appears to be constitutively expressed, whereas the high-affinity system is repressed at high extemal glucose concentration (Bisson and Fraenkel 1984).

In contrast to *S. cerevisiae*, *Kluyveromyces lactis* is a predominantly aerobic yeast which exhibits a distinct regulation pattern for glucose uptake. In 1976, Royt and Macquillan reported the existence of an inducible glucose transport system in *K. lactis* (Royt and MacQuillan 1976). This was confirmed by our recent studies (Wésolowski-Louvel et al. 1992) in which we found that the low-affinity system of *K. lactis* was induced by glucose. We also showed that this yeast, like many other yeast species, possessed at least two types of uptake mechanism for glucose, one high K_m uptake (K_m = 20–50 mM) and one low K_m uptake (K_m = 1 mM); the latter being constitutively expressed.

The *RAG1* gene of *K. lactis* codes for a low-affinity glucose transporter component (Goffrini et al. 1990; Wésolowski-Louvel et al. 1992). It is a protein of 567 amino acids with the 12 transmembrane hydrophobic domains common to many sugar carriers. Disruption of the *RAG1* gene resulted in the loss of the inducible low-

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affinity glucose transport system, leading to impaired growth in high glucose concentrations when respiration was inhibited by antimycin A (the Rag$^-$ phenotype; Goffrini et al. 1989).

In this report, we show that the induction of the *RAG1* gene is mediated by a *cis*-acting element identified in its 5′ flanking region, and is under the control of at least three *trans*-acting genes, *RAG4, 5* and *8*.

Materials and methods

Strains and media. Yeast strains used in this study are shown in Table 1. Strain MW179–1D is a *lac4* strain constructed for the β-galactosidase fusion experiments by crossing the D6 and MW121–11A strains. *lac4* is a mutation in the structural gene of the β-galactosidase enzyme (Sheetz and Dickson 1981). This strain also contains the *uraA* mutation used for transformant selection. PM6–7A and MW109–8C are two Rag$^+$ strains which have been mutagenized for the isolation of Rag$^-$ mutants (manuscript in preparation).

The complete media used for yeast cell growth consisted of 1% Difco yeast extract (Y), 1% Difco bactopeptone (P), and a carbon source as specified. YPG medium contained Y, P and 2% glucose as carbon source. YPGL contained Y, P and 2% glycerol plus 2% lactate as carbon source. The Rag phenotype was tested on YPGAA agar containing Y, P, 5% glucose and 5 mM antimycin A (Goffrini et al. 1989). Minimal medium contained 0.67% Difco yeast nitrogen base without amino acids, auxotrophic complements as required and a carbon source (2%).

Plasmids. The plasmids used in this work are shown schematically in Fig. 1.

1. pXW3. pXW3 is from the laboratory of Y.Y. Li (Fudan University of Shanghai). It contains the *URA3* gene of *S. cerevisiae,* used for transformant selection by complementation of the *uraA* mutation of *K. lactis*. The presence of the S11 fragment from the *Kluyveromyces* plasmid pKD1 (Chen et al. 1986) enables the plasmid to replicate in *K. lactis.* A polylinker with unique restriction sites (*Sal*I, *Bam*HI and *Hind*III) is present immediately upstream of the promoterless bacterial *lacZ* gene, allowing the construction of *RAG1-lacZ* fusions.
2. pBS–RAG1. pBS–RAG1 was constructed by insertion of a 1.9 kb *Asu*II-*Sal*I fragment of the *RAG1* sequence into the *Cla*I and *Sal*I sites of pBS(KS+). This *RAG1* sequence spanned nucleotides −1810 to +55 with respect to the first ATG codon of the coding sequence. The resulting plasmid, pBS–RAG1, was used for generating ordered deletions of the 5′ flanking region of the *RAG1* gene.
3. 5′ Deletions of the *RAG1* promoter and fusion to the *lacZ* gene. 5′ Deletions of the *RAG1* promoter were generated according to the method described by Hong (1982). pBS–RAG1 plasmid DNA was first partially digested with DNAase I in the presence of Mn^{2+}, giving rise to a mixture of linear double-stranded molecules. After cleavage at the *Sma*I site and filling-in the DNAase I-generated termini with Klenow enzyme, DNA was self-ligated with T4 DNA ligase, leading to a mixture of circular molecules with the *RAG1* promoter sequence fixed at one end (*Sal*I site, Fig. 1) and progressively shortened at the other. Remaining wild-type molecules in the ligation mixture were linearized by a second digestion with *Eco*RI before transforming *Escherichia coli* strain JM101. Recombinant plasmids having 5′→3′ deletions in the *RAG1* promoter region were then screened. The endpoints of each deletion were determined by DNA sequencing using a synthetic primer complementary to

Table 1. Yeast strains used in this work

Strain	Relevant genotype	Source or reference
Kluyveromyces lactis		
2359/152	*a metA1* Rag$^+$	Wésolowski et al. (1982)
D6	α *lac4*-8 *leu2 uraA* Rag$^-$ (pKD1$^+$)	M. Bolotin-Fukuhara
MW121-11A	*a metA1 uraA* Ade$^-$ *trp1* Rag$^+$ (pKD1$^+$)	This study
MW179-1D	α *lac4*-8 *uraA leu2 metA1* Ade$^-$ *trpA1* Rag$^+$ (pKD1$^+$)	This study
PM6-13A	α *uraA trpA1* Ade$^-$ *rag1*-1 (pKD1$^+$)	Wésolowski-Louvel et al. (1992)
PM6-7A	*a uraA* Ade$^-$ Rag$^+$ (pKD1$^+$)	This study
PM6-7A/VV32	*a uraA* Ade$^-$ *rag4*-1 (pKD1$^+$)	This study
PM6-7A/VV41	*a uraA* Ade$^-$ *rag5*-1 (pKD1$^+$)	This study
MW123-4C	*a ade1^{-1} lysA1 trpA1 uraA rag4*-5 (pKD1$^+$)	This study
MW 109-8C	α *lysA1 trpA1* Rag$^+$ (pKD1$^+$)	This study
MW109-8C/FA42	α *lysA1 trpA1 rag8*-2 (pKD1$^+$)	This study
MW 159-6C	α *lysA1 uraA rag8*-2 (pKD1$^+$)	This study
Saccharomyces cerevisiae		
BWG1-7A	*MATa leu2*-3 *leu2*-112 *his4*-519 *ade1*-100 *ura3*-52	Guarente and Mason (1983)
JO1-1a	*MATa leu2*-3 *leu2*-112 *his4*-519 *ade1*-100 *ura3*-52 *hap2* null	Olesen and Guarente (1990)

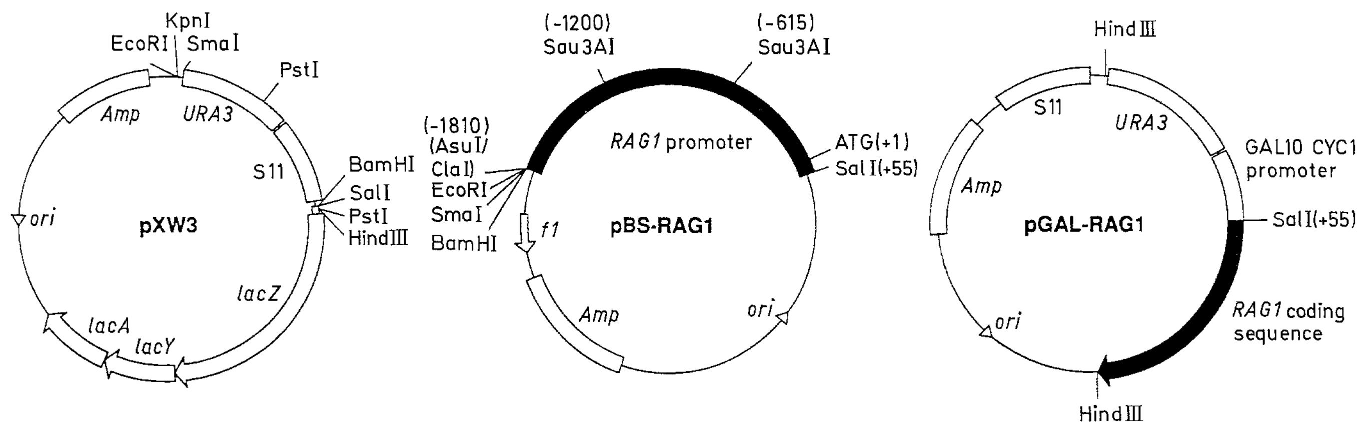

Fig. 1. Structure of the plasmids used. See Materials and methods for details of construction

the plasmid sequence upstream of the *Bam*HI site.

A *Bam*HI site immediately upstream of the disrupted *Sma*I site, which makes the junction with the endpoints of the 5′ deletions, was used to isolate *Bam*HI-*Sal*I fragments carrying *RAG1* 5′ deletions. A series of such *Bam*HI-*Sal*I fragments was then inserted into the *Bam*HI and *Sal*I sites of pXW3. This created a set of plasmids with an in-frame fusion of the first 18 N-terminal codons of the *RAG1* gene to the *lacZ* gene; each fusion having a different 5′ endpoint in the *RAG1* promoter sequence.

4. Internal deletions. A series of deletions internal to the 5′ flanking region was constructed by isolating a *Sau*3AI fragment from positions −615 to −1200 of the *RAG1* promoter, and cloning it into the *Bam*HI site situated at the endpoints of the 5′ deletions in the plasmids pD69, pD48, pD42, pD54 and pD310 (see Fig. 6). The resulting plasmids were named pDI69/1, pDI48/1, pDI42/1, pDI54/1 and pDI310/1, respectively. The deletion of the *Xho*I block in pDXX was made by *Xho*I digestion and self-ligation of the plasmid pD0.

5. Construction of the *GAL10–CYC1–RAG1* fusion. pGAL–RAG1 contains a fusion of the *S. cerevisiae GAL10–CYC1* promoter (Guarente et al. 1982) with the *RAG1* coding sequence deleted of its first 18 N-terminal codons. This was achieved by cloning a 2.0 kb *Sal*I-*Hin*dII fragment containing the truncated *RAG1* coding sequence into the *Sal*I and *Hin*dII sites of the polylinker of plasmid pE–GAL1 (Chen 1987). This created an in-frame fusion of the translational start codon of the *CYC1* sequence to the *RAG1* coding sequence.

Yeast transformation and β-galactosidase assay. K. lactis was transformed by the method of Meilhoc et al. (1990) using an electroporator (Jouan, TRX GHT 1287). Yeast transformants were selected on plates containing YNB minus uracil. Random transformants were used as soon as they were large enough to serve as inocula. Transformants were never subcultured or stored because, for unknown reasons, old cultures gave considerable variation in β-galactosidase levels. Cells were grown to an A_{600} of 2–4 in 2 ml of minimal medium containing the appropriate carbon source. Cells from 1 ml of culture were pelleted by centrifugation, resuspended in 1 ml of Z buffer (Miller 1972), then 30 μl of toluene was added. The mixtures was then vigorously vortexed for 15 s and incubated at 28° C for 1 h with shaking. β-Galactosidase assays were carried out as described by Miller (1972). The enzyme activity per ml of culture was normalized to 1 A_{600} unit.

mRNA analysis: Northern blot and primer extension. RNAs were extracted from cells grown in 50 ml complete media containing various carbon sources to an A_{600} of 2–4 (Carlson and Botstein 1982). For Northern blot analysis, total RNAs were electrophoresed through a 1.2% agarose gel containing formaldehyde, then transferred to a nitrocellulose membrane before hybridizing with radioactive probes according to Maniatis et al. (1982).

Primer extension experiments were performed as previously described (Chen et al. 1991).

Results

Nucleotide sequence of the RAG1 *upstream region*

To investigate the *cis*-acting elements involved in the regulation of the *RAG1* gene, we first analyzed the nucleotide sequence of the region upstream from the *RAG1* coding sequence. This was achieved by sequencing a 1.9 kb *Kpn*I-*Sal*I fragment from the plasmid pGW4A (Wésolowski-Louvel et al. 1992). Figure 2 shows the nucleotide sequence from positions +55 (*Sal*I site) to −1834. We can note the following features:

1. Two TATA-like motifs are present at positions −33 and − 150.
2. Three 9 bp perfect (repeats a), and four 14 bp near-perfect (repeats b, containing *Xho*I sites) direct repeats, are found between −1091 and −1219.
3. Between −788 and −863 around the *BSp*EI site, there are three 12 bp repeated sequences (repeats c), one of which is in the reverse orientation.
4. A 15 bp near-perfect dyad symmetry occurs between −762 and −776 (repeats d).
5. Two other distinct 11 bp perfect direct repeats (repeats e, with sequence 5′-ATACCACCAAT-3′) occur be-

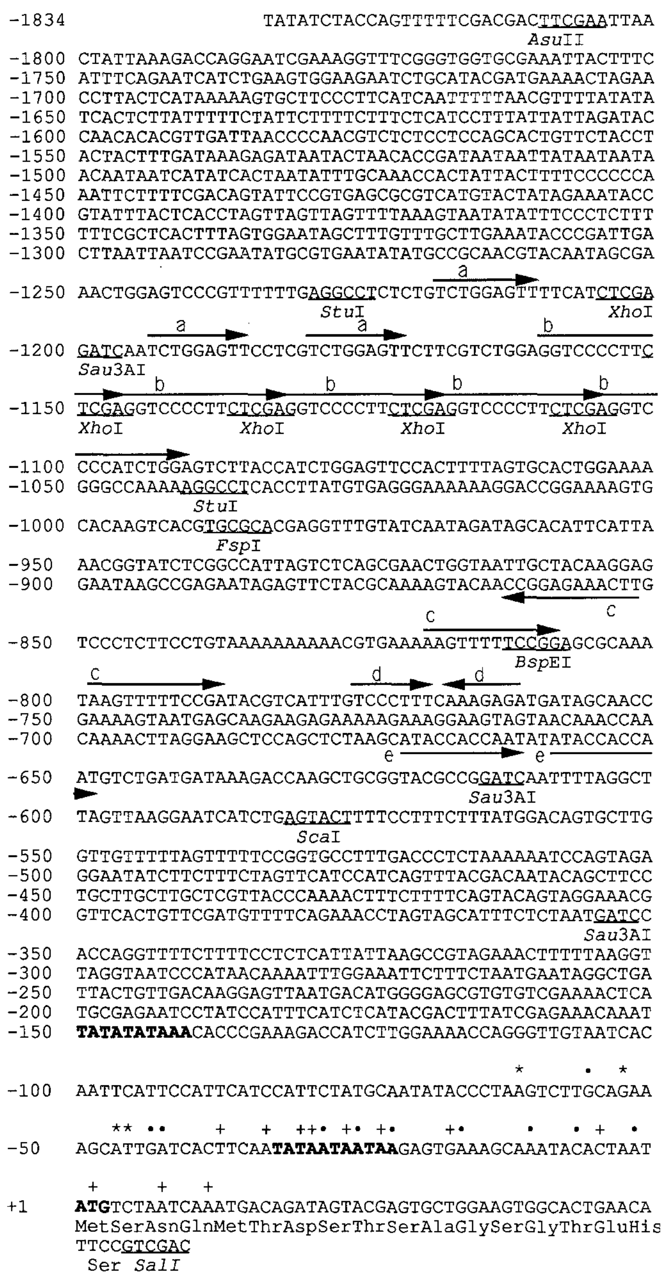

Fig. 2. Nucleotide sequence of the *RAG1* 5′ flanking region (EMBL accession number: X53752). The sequencing of the *RAG1* coding region has been described previously (Goffrini et al. 1990). The nucleotide sequence is numbered from the putative ATG translational start codon. TATA sequences and the putative translation start codon are marked by *bold* letters. 5′ ends of mRNA determined by primer extension are marked by *, • and +, representing strong, moderate and weak mRNA signals, respectively. The repeat sequences are shown by *lettered horizontal arrows*

tween − 649 and − 672. These sequences are reminiscent of the pentanucleotide sequence CCAAT motif, which is the binding site of the HAP2/3/4-type protein complex in *S. cerevisiae* (Olesen et al. 1987; Olesen and Guarente 1990) and in mammalian cells (Chodosh et al. 1988a, b).

Nucleotide sequence comparison also revealed that the sequence at positions − 982 to − 995, 5′-GTCAC-GTGCGCACG-3′, was very similar (one base in excess) to the consensus binding site for the yeast protein ABF/GF1, which is RTCRYNYNNNACG (Dorsman et al. 1989). ABF/GF1 has been shown to bind to the consensus sequence in the upstream regions of the glucose-induced genes of *S. cerevisiae* encoding the glycolytic enzymes phosphoglycerate kinase and pyruvate kinase (Chambers et al. 1990).

RAG1 *has multiple transcription initiation sites*

To examine the transcription initiation pattern of the *RAG1* gene, the 5′ ends of mRNAs were mapped by primer extension. The source of *RAG1* mRNA was the Rag$^+$ *K. lactis* strain 2359/152 (Table 1) grown in YPG. The primer was a synthetic 20mer oligonucleotide complementary to the coding sequence + 17 to + 36. Figure 3 illustrates the 5′ ends of *RAG1* mRNAs. It was found that *RAG1* transcripts have heterogeneous 5′ ends (up to 25), suggesting the presence of multiple sites for transcription initiation. With the RNA samples prepared from cells grown on glycerol, we observed an almost complete absence of cDNAs (data not shown). This indicated that *RAG1* transcription was not induced in the glycerol medium (see later), showing also that the oligonucleotide primer was specific for *RAG1* mRNA. The 5′ ends of the *RAG1* transcripts mapped over a 75 bp region, from positions + 12 to − 62 relative to the first nucleotide at position + 1 of the protein-coding DNA sequence. It is to be noted that the *RAG1* sequence possesses two in-frame ATG codons, the second starting at nucleotide + 13. Therefore, the transcription of the *RAG1* gene seems to involve multiple start sites. However, we cannot rule out the possibility of 5′ degradation of mRNAs or arrests of the reverse transcriptase. It is noteworthy that no RNA start sites are found upstream of the most distal TATA-like element at position − 150.

Transcription of the RAG1 *gene is induced by various sugars*

We have previously shown that transcription of the *RAG1* gene is observed not only on glucose and fructose, but also on other sugars. Before identifying the regulatory sequences of the *RAG1* gene, we fisrt examined which substrates could modify the expression of the *RAG1* gene. For this purpose, we constructed a gene fusion system in which the first 18 codons of the *RAG1* gene were fused to the *lacZ* gene of *E. coli*. This fusion, carried by the plasmid pD0, was preceded by 1810 nucleotides of the *RAG1* upstream sequence. pD0 was constructed by cloning the 1.9 kb *Bam*HI-*Sal*I fragment from pBS–RAG1 into the *Bam*HI and *Sal*I sites of the multicopy plasmid pXW3 (Fig. 1). pD0 was then introduced into the *lac4*, Rag$^+$ recipient strain MW179–1D (Table 1).

β-Galactosidase levels in these transformants were determined under a variety of growth conditions. The results are illustrated in Fig. 4. It was found that on the non-fermentable substrate glycerol plus lactate, only a very low level of expression could be detected. In con-

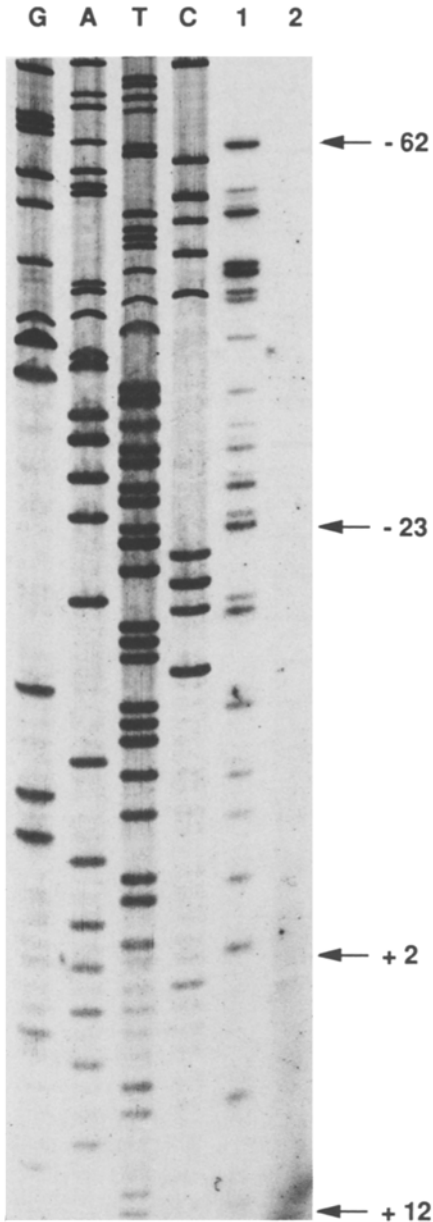

Fig. 3. Primer extension mapping of the *RAG1* mRNA termini. An oligonucleotide (5′-TCCAGCACTCGTACTATCTG-3′) complementary to nucleotides +17 to +36 of the coding sequence of the *RAG1* gene was labelled with T4 polynucleotide kinase and γ-[^{32}P]ATP. Labelled oligonucleotide (5 ng) and 4 mg poly(A)$^{+}$-enriched RNA were hybridized for 1 h at 42° C. Extension reactions were done with 6 units of AMV reverse transcriptase (USB) for 30 min at 37° C. The cDNA products (lane 1) were electrophoresed through an 8% polyacrylamide-urea sequencing gel. Lane 2, the reaction without RNA. Lanes G, A, T and C contain the products of Sanger sequencing reactions made on a pTZ18R clone of *RAG1* and the same primer. Numbers on the right indicate the positions of the nucleotides with respect to the putative start of the *RAG1* coding sequence (see Fig. 2)

trast, the *lacZ* expression was induced about tenfold by various sugars examined, including glucose, fructose, mannose, sucrose, raffinose, as well as galactose. In the case of glucose, maximal induction could be seen when the glucose concentration exceeded 2%. The level of

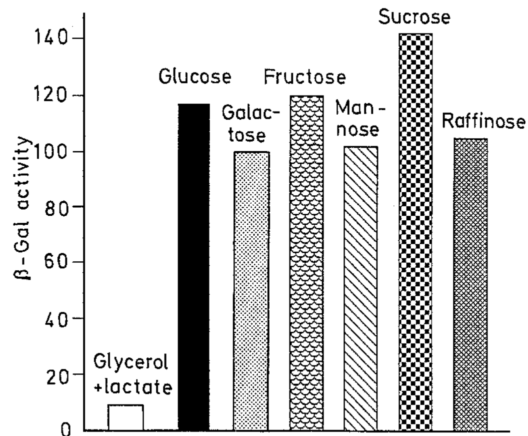

Fig. 4. Relative β-galactosidase activities from the cells transformed with the *RAG1-lacZ* fusion. Cells were grown to exponential phase in minimal medium containing the indicated sugar at 2%. β-Galactosidase activity was determined (Miller 1972), and is expressed as described in Materials and methods

expression did not vary significantly between exponentially growing and stationary phase cells. Glycerol and lactate-grown cells had a low constitutive level of *RAG1* expression. Once transferred to glucose media, the expression of *RAG1* was induced slowly in these cells (about a twofold increase after 4 h).

To test whether the increased β-galactosidase levels reflected transcriptional regulation, we examined the level of *RAG1* mRNA in standard Rag^{+} strains grown on various substrates. In Fig. 5, Northern blot analyses are shown for two Rag^{+} strains, 2359/152 and PM6–7A (see Table 1). They were grown on either glycerol plus lactate, glucose, or galactose. The results obtained are in agreement with those obtained by the β-galactosidase assay, that is, *RAG1* expression is induced by all of the fermentative substrates tested. The results thus confirmed that the *RAG1* gene was transcriptionally regulated and that the regulation occurred via some upstream elements, probably of the upstream activation sequence (UAS) type. Since both Rag^{+} strains used in these experiments were Lac^{+}, we also tested the effect of lactose on the induction of *RAG1*. Surprisingly, we found that the effect of lactose on *RAG1* mRNA level was highly strain-dependent (Fig. 5). In strain 2359/152, lactose acted like glucose and galactose, while in strain PM6–7A, the mRNA level of *RAG1* was as low as that found on glycerol plus lactate. The genetic basis of this variation has not been studied further.

Mapping of the cis*-acting regulatory sequences within the 5′ flanking region of* RAG1

To locate the *cis*-acting sequences which regulate the expression of the *RAG1* structural gene, we constructed a series of deletions in the 5′ flanking region of the *RAG1* gene and analyzed their effect on the expression of the fused *lacZ* gene. The plasmids with the sequentially deleted *RAG1* promoter were transformed into the *lac4* host strain MW179–1D. Since the *RAG1* gene was poorly

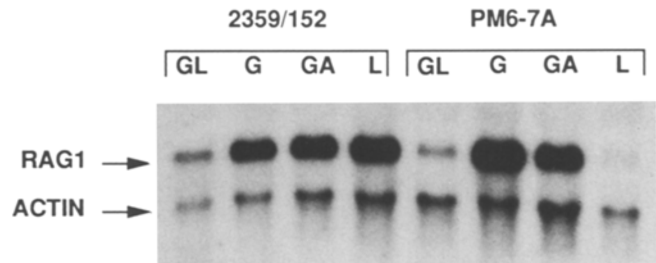

Fig. 5. Northern blot hybridization showing *RAG1* mRNA levels in Rag$^+$ strains 2359/152 and PM6–7A, grown on various carbon sources. Total RNA (10 μg) was electrophoresed, blotted to nitrocellulose membranes and hybridized with a probe made of an internal segment of the *RAG1* gene. A probe of an actin gene (plasmid pKL7, from J.J. Rossi) was used in parallel as a quantitative reference. Both probes were labelled by nick-translation using α-[^{32}P]dCTP. GL, 2% glycerol and 2% lactate; G, 2% glucose; GA, 2% galactose; L, 2% lactose

expressed in cells grown on glycerol and induced by many fermentable substrates, the transformants were grown on glucose or glycerol plus lactate. In each case the levels of β-galactosidase were assayed (Fig. 6). Plasmid pD0, which has no deletion, was used as a control.

5′ Deletions extending from −1810 to −1059 had little effect on the expression of the *RAG1-lacZ* fusion when the cells were grown either on glucose or on glycerol plus lactate. The internal deletion of pDXX in which the 14 and 9 bp repeat sequences (repeats b and a, see Fig. 2) have been removed did not affect the expression.

Deletions up to position −839 reduced the expression in glucose-grown cells by approximately twofold. In this case (pD57), one of the 12 bp repeat sequences (repeats c) and the putative consensus sequence for binding of the ABF/GF1 protein in *S. cerevisiae* have been removed. Dorsman et al. (1989) have shown that a GF1-like factor exists in *K. lactis* and that it binds to the *S. cerevisiae* target sequence, but with much lower affinity. Whether the putative consensus sequence present in the *RAG1* promoter is the target site of such a factor remains to be clarified.

Several deletions ending between positions −839 to −750 did not lead to a further decrease of expression. Deleting up to −621, however, resulted in about a 20-fold decrease in the expression on glucose medium. It was deduced, therefore, that essential sequences for the induced transcription had a 5′ border around position −750. Cells grown on glycerol plus lactate medium gave a low but detectable level of activity, even for deletions up to −186, indicating the existence of a basal level of expression of the *RAG1* gene which was independent of the upstream sequences.

Finally, the deletion ending at −146 activity below the level of detection, both on glucose and on glycerol plus lactate medium. This indicated that the putative TATA box located at −150 was necessary for basal expression. Therefore, sequences sufficient for a basal promoter activity seemed to lie downstream from nucleotide −186.

Several intemal deletions (pDI series) were construct-

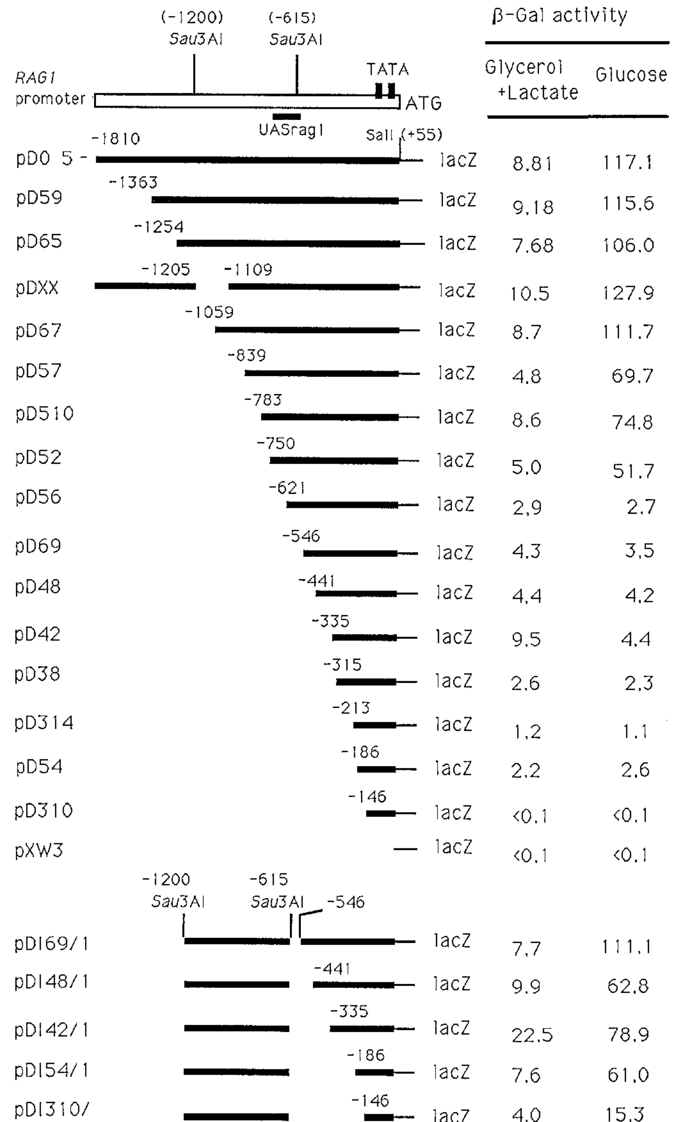

Fig. 6. Progressive deletions of the 5′ flanking region of *RAG1* and their effects on *lacZ* expression. All deletion endpoints are numbered as shown in Fig. 2 and verified by sequencing. β-Galactosidase levels are expressed as described in Materials and methods. Cells were grown in minimal media containing 2% glucose or 2% glycerol plus 2% lactate

ed to map the 3′ border of upstream elements. Deletions with endpoints between −615 and −546 gave the same level of expression as the entire promoter. Deletions ending between −615 and −186 gave rise to about a twofold decrease in activity. Deletions ending at −146, which disrupted the putative TATA box at −150, caused another fourfold reduction in expression (for unknown reasons, the expression level from pDI42/1 on glycerol plus lactate was relatively high). These experiments suggested that sequences between −615 and −186 are not critical for *RAG1* expression. β-Galactosidase expression of each of these deletions has also been measured in galactose- or raffinose-grown cells. In both cases the results were very similar to those obtained with glucose. We thus concluded that a *cis*-acting essential element, located between −615 and −750, is required for the induction by sugars.

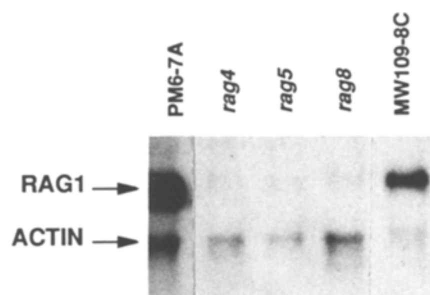

Fig. 7. Levels of *RAG1* transcripts in the Rag⁻ mutants. Northern blot analyses were performed as in Fig. 5. All the strains were grown on YPG medium (2% glucose). PM6–7A and MW109–8C are the Rag⁺ parental strains. *rag4, rag5* and *rag8* mutant strains are PM6–7A/VV32, PM6–7A/VV41 and MW109–8C/FA42, respectively (see Table 1)

The products of RAG4, RAG5 *and* RAG8 *genes affect the transcription of* RAG1

The Rag⁻ phenotype (inability to grow on glucose in the presence of antimycin A, see Wésolowski-Louvel et al. 1992) can be obtained by mutation of many genes including those encoding enzymes for glycolysis (Goffrini et al. 1991; Wésolowski-Louvel et al. 1988). Any such mutation is a potential candidate for the components that control *RAG1* expression. Indeed, we have isolated many Rag⁻ mutants (to be published). Among these mutations, three were found to affect transcription of the *RAG1* gene. They are called *rag4, 5* and *8;* all belong to different complementation groups.

Figure 7 shows the results of Northern blot analysis. Total RNAs were prepared from glucose-grown cells. In *rag4, rag5* and *rag8* strains, mRNA levels of *RAG1* were significantly reduced with respect to their wild-type parents (PM6–7A for *rag4* and *rag5,* and MW109–8C for *rag8* mutants). Analysis of other alleles (two for *rag4* and *rag5,* one for *rag8*) resulted in the same pattern (data not shown). These findings suggest that the products of *RAG4, 5* and *8* genes play an essential role in the expression of the *RAG1* gene.

The RAG1 *gene fused to a heterologous promoter complements* rag4 *and* rag8 *but not* rag5 *mutations*

It appeared most likely that the defect in *RAG1* expression is responsible for the Rag⁻ phenotype of the three mutant strains, *rag4, rag5* and *rag8.* We thus supposed that the three genes either directly control *RAG1* expression via its 5′ upstream sequences, or play a role in the transmission of signals induced by glucose (or other sugars) and thus indirectly affect the transcription of *RAG1.* To test such possibilities, we replaced the native upstream sequence of *RAG1* by the heterologous promoter, *GAL10–CYC1* of *S. cerevisiae* (Guarente et al. 1982). The multicopy plasmid, pGAL–RAG1, bearing the *GAL10–CYC1–RAG1* fusion, was introduced into *rag4, rag5* and *rag8* mutants. As a control, a *rag1* mutant was transformed in parallel. The Rag phenotype of the transformed cells was tested on YPGAA medium (see Materials and methods). The results are shown in Fig. 8.

It has been shown that in *K. lactis,* expression from the *GAL10–CYC1* promoter is regulated by the lactose/galactose regulon and that there is constitutive basal level of expression on glucose (Chen, 1987). Indeed, as expected, the *GAL10–CYC1–RAG1* fusion restored the Rag⁺ phenotype in a *rag1* mutant on YPGAA medium. Interestingly, it also conferred the Rag⁺ phenotype to *rag4* and *rag8,* though *rag4* and *rag8* transformants grew more slowly than *rag1* transformants. However, complementation was not observed in the case of the *rag5* mutant. In addition, a multicopy plasmid bearing *RAG1* with its native promoter did not show complementation in any of the three mutants (data not shown). These results suggested that *RAG4* and *RAG8,* but not *RAG5,* are probably involved in controlling *RAG1* expression via its upstream sequences.

Discussion

Early biochemical studies on the oxidative yeast *K. lactis* revealed an inducible component of the glucose transport system (Royt and McQuillan 1976). We demonstrated

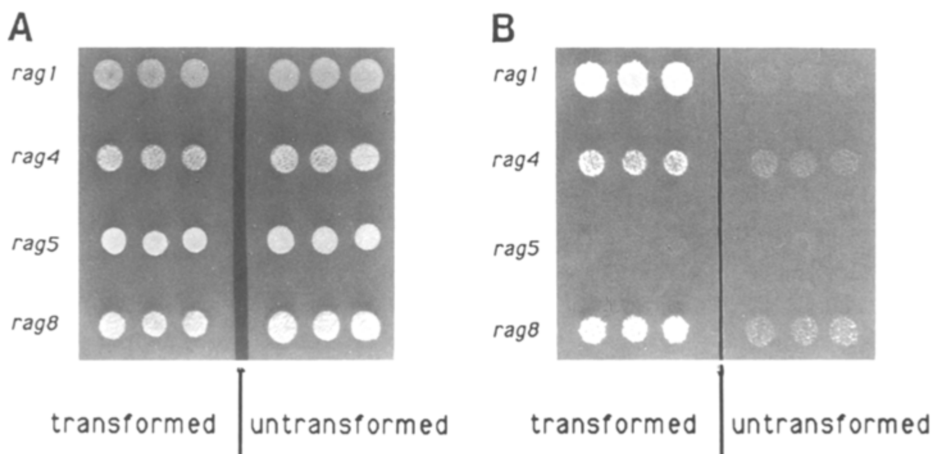

Fig. 8A, B. Complementation of the Rag⁻ phenotype of *rag4, rag5* and *rag8* mutants by transformation with the *GAL10–CYC1–RAG1* fusion plasmid, pGAL–RAG1. Recipient strains were MW123–4C *(rag4),* PM6–7A/VV41 *(rag5)* and MW159–6C *(rag8).* Ura⁺ transformants that grew on minimal medium (**A**) were replica-plated on YPGAA agar (**B**). The Rag⁺ phenotype was observed after incubation at 28° C for 24 h

that this inducible component is a low-affinity transport system (Wésolowski-Louvel et al. 1992). The inducibility of the low-affinity glucose transport system in *K. lactis* is interesting in that most yeasts investigated so far have been reported to be constitutive in this respect (Does and Bisson 1989; Postma and van den Broek 1990; van Urk et al. 1989), independent of external glucose concentration. However, the regulation of high-affinity transport seems to vary in different species. It has been suggested that the control of glucose uptake is associated with the fermentative ability of each yeast species (Does and Bisson 1989). In *S. cerevisiae,* a predominantly fermentative species, the high-affinity transport is repressed at high glucose concentration and derepressed upon glucose limitation (Bisson and Fraenkel 1984). In contrast, studies on several oxidative yeasts (Does and Bisson 1989) do not seem to show repression/derepression of high-affinity glucose uptake. In *K. lactis* also, the high-affinity system appears to be constitutive, while the low-affinity system is induced by glucose. An inducible transport system has also been described in the case of galactose/lactose transport in *K. lactis* (Dickson and Barr 1983).

The expression of the low-affinity transport in *K. lactis* is controlled, at least in part, by the regulated expression of the *RAG1* gene. Mutation or disruption at the *RAG1* locus abolishes the low-affinity transport system, and leads to a respiration-dependent growth on high glucose (Rag$^-$ phenotype). A gene fusion system indicated that maximal expression of the *RAG1* gene was reached when the glucose concentration exceeded 2% (data not shown). This can be correlated with the fact that a high glucose concentration is required to distinguish the Rag$^+$ from the Rag$^-$ phenotype. Therefore it is likely that the inducibility of this low-affinity transport system is required for fermentative growth in *K. lactis.*

Transcription of the *RAG1* gene is induced not only by its substrates, glucose and fructose, but also by other sugars. These sugars include galactose and lactose, which are transported by a distinct carrier (Dickson and Barr 1983). This would support the idea that there is a specific intracellular metabolite(s) which is the common signal(s) generated from metabolism of fermentative carbon sources. However, active glycolysis does not seem to be required for induction of the *RAG1* gene. Indeed, a *rag2* mutant, which is defective for phosphoglucose isomerase and does not produce ethanol (Goffrini et al. 1991; Wésolowski-Louvel et al. 1988), did not show decreased mRNA levels of *RAG1* (data not shown). Royt (1981) has shown that the non-phosphorylatable glucose analog 6-deoxyglucose can serve as a gratuitous inducer for a glucose transport system in *K. lactis.* Therefore, the signal(s) for *RAG1* induction must be generated before the production of phosphorylated sugars. Several difficult questions remain to be answered: (1) what is the primary trigger for induction of *RAG1*?; (2) which is the signalling pathway?; and (3) are there any common points between the triggering of glucose transport and other glucose-induced signals, for example the cAMP signal, or glucose-induced expression of glycolytic genes?

The 1810 bp 5′ non-coding region of the *RAG1* gene shows several typical sequence motifs, among which are found two TATA-like sequences, at positions −150 and −33. Primer extension experiments indicated the presence of a family of *RAG1* messenger RNAs whose 5′ ends mapped within a 75 bp region. The fact that no start sites are found upstream of the TATA sequence at position −150 suggests that this TATA is required for mRNA starts. This is emphasized by the finding that a partial deletion of this TATA sequence (in plasmid pD310) dramatically reduced the expression of the *RAG1* gene in the fusion experiments. We do not know whether the second potential TATA element (at position −33) is required for transcription.

We have identified an UAS-like sequence between positions −615 and −750. We conclude that this sequence is an essential positive regulatory element of the *RAG1* promoter, though other elements upstream or downstrem from it might be required for full activation. From nucleotide sequence analysis, we distinguished two structural motifs within this region. First, a continuous stretch of 22 purines occurs between −715 and −736, which might be a nucleosome-free region. Secondly, we found a pair of 11 bp direct repeats between −649 and −672. The repeats contain a CCAAT motif which might be related to the binding site for the HAP2/3/4 protein complex of *S. cerevisiae* (Olesen et al. 1987; 1990) and its homolog in mammalian cells (Chodosh et al. 1988a, b). This protein complex has been known to activate the transcription of genes involved in mitochondrial respiratory functions, including *CYC1* (Forsberg and Guarente 1989; Olesen et al. 1987), *HEM1* (Keng and Guarente 1987), *COX4* and *CYT1* (Schneider 1989).

Gel retardation assays showed that the CCAAT-containing sequences in the UAS-like region were able to bind unidentified protein factors from crude extracts from both *S. cerevisiae* and *K. lactis* (data not shown), whatever the carbon source of the culture from which proteins were extracted. In addition, the bound protein factors did not seem to be the HAP2/3/4-type complex. Whether the protein(s) that binds to that CCAAT sequence is in fact involved in *RAG1* regulation still remains to be demonstrated.

The transcription of the *RAG1* gene was dramatically reduced in *rag4, rag5* and *rag8* mutants. The effect of these mutations on *RAG1* transcription is mediated by the upstream region of the *RAG1* gene. Indeed, expression of the *RAG1* gene from a heterologous promoter can bypass, at least partially, *rag4* and *rag8,* but not *rag5* mutations. What are the roles of their products in the modulation of *RAG1* expression? We consider it probable that at least one of the functions of the *RAG4* and *RAG8* genes in fermentative growth is to activate the transcription of *RAG1.* They may act on *RAG1* expression either by direct interaction with the promoter region or, indirectly, in the transmission of glucose-induced signals. The *RAG5* gene, which is clearly involved in the transcription of *RAG1,* should have a more general function controlling other genes related to fermentation. This is presently under investigation.

Acknowledgements. We thank Dr. J.J. Rossi for the kind gift of plasmid pKL7 containing the actin gene, Dr. M. Bolotin-Fukuhara

for strain D6, Prof. Y.Y. Li for plasmid pXW3, and Dr. L. Guarente for *S. cerevisiae* strains. We also thank B. Séraphin for advice on the primer extension experiments and helpful discussions. This work was supported by a grant from the European Communities (BAP 0026F). X.J.C. has received a fellowship from the H. de Rothschild Foundation.

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Communicated by C.P. Hollenberg