Use of the *KlADH4* Promoter for Ethanol-Dependent Production of Recombinant Human Serum Albumin in *Kluyveromyces lactis*

MICHELE SALIOLA,[1] CRISTINA MAZZONI,[1] NICOLA SOLIMANDO,[1] ALESSANDRA CRISÀ,[1] CLAUDIO FALCONE,[1]* GERARD JUNG,[2] AND REINHARD FLEER[2]

Department of Cell and Developmental Biology, Pasteur Institute-Cenci Bolognetti Foundation, University of Rome "La Sapienza," 00185 Rome, Italy,[1] and Biotechnology Department, Rhone-Poulenc Rorer, 94403 Vitry cedex, France[2]

KlADH4 is a gene of *Kluyveromyces lactis* encoding a mitochondrial alcohol dehydrogenase activity which is specifically induced by ethanol. The promoter of this gene was used for the expression of heterologous proteins in *K. lactis*, a very promising organism which can be used as an alternative host to *Saccharomyces cerevisiae* due to its good secretory properties. In this paper we report the ethanol-driven expression in *K. lactis* of the bacterial β-glucuronidase and of the human serum albumin (HSA) genes under the control of the *KlADH4* promoter. In particular, we studied the extracellular production of recombinant HSA (rHSA) with integrative and replicative vectors and obtained a significant increase in the amount of the protein with multicopy vectors, showing that no limitation of *KlADH4* *trans*-acting factors occurred in the cells. By deletion analysis of the promoter, we identified an element (UAS$_E$) which is sufficient for the induction of *KlADH4* by ethanol and, when inserted in the respective promoters, allows ethanol-dependent activation of other yeast genes, such as *PGK* and *LAC4*. We also analyzed the effect of medium composition on cell growth and protein secretion. A clear improvement in the production of the recombinant protein was achieved by shifting from batch cultures (0.3 g/liter) to fed-batch cultures (1 g/liter) with ethanol as the preferred carbon source.

Kluyveromyces lactis is an aerobic yeast which is able to grow on lactose as the sole carbon source. Due to this property, which is rare among yeasts, *K. lactis* has been used for the purification of the enzyme lactase (β-galactosidase) and for the production of low-lactose milk in the dairy industry (22), and the conditions used for cultivation of this organism on the large scale have been well established. In the last few years, *K. lactis* has been successfully used as an alternative host to *Saccharomyces cerevisiae* for heterologous gene expression, and good transformation systems and stable multicopy vectors are now available for the genetic manipulation of this yeast (for reviews, see references 16, 32, and 44). Expression of heterologous genes in *K. lactis* can be achieved by the use of constitutive promoters which have also been isolated in *S. cerevisiae*; such promoters are interchangeable between the two yeasts. However, *S. cerevisiae* inducible promoters are not always tightly regulated in *K. lactis* cells, suggesting the existence of different regulatory circuits on gene expression in the two organisms.

Regulated promoters have been obtained from the *K. lactis* genes lactase (*LAC4*) (18, 26, 33, 46) and acid phosphatase (*KlPHO5*) (14, 15). These genes are regulated in a very similar way to the corresponding *GAL* and *PHO5* genes of *S. cerevisiae*, which are induced at the transcriptional level in the presence of lactose or galactose and of low phosphate concentrations, respectively (40, 45).

KlADH4 is a *K. lactis* gene induced by ethanol, encoding a mitochondrial alcohol dehydrogenase (ADH) activity with interesting regulatory properties (35). Such regulation can be observed in Rag⁻ (resistance to antibiotics on glucose) strains of *K. lactis*, which are unable to grow on glucose in the presence of mitochondrial inhibitors (19). In these strains, which are impaired in sugar fermentation and are poor producers of ethanol, the addition of ethanol specifically induced *KlADH4* while the addition of glycerol and other nonfermentable carbon sources did not induce the expression of this gene. Moreover, differently from the *ADH2* gene of *S. cerevisiae* (8–10), the induction of *KlADH4* is not sensitive to glucose repression, since the gene is expressed when both glucose and ethanol are present in the culture medium (29, 34).

All these findings suggested to us the possibility of using the promoter regions of *KlADH4* for ethanol-dependent expression of heterologous genes in *K. lactis* Rag⁻ strains. Such strains can be either naturally isolated or constructed in the laboratory by disrupting genes which affect the glycolytic flux, such as *KlPGI1* (29), the gene encoding phosphoglucoisomerase, or genes directly involved in ethanol production, such as *KlPDC1*, the gene encoding pyruvate decarboxylase of *K. lactis* (3). By contrast, in Rag⁺ strains, the *KlADH4* promoter is also active in cells growing in glucose and other fermentable carbon sources because of the ethanol produced by cells through fermentation, but its transcriptional activity can be still increased (two- to threefold) by the addition of exogenous ethanol (data not shown).

In this paper, we report the isolation of the *KlADH4* promoter and its use for the regulated expression of bacterial and human genes. We also identified a small region of the promoter which is responsible for the induction of the gene in the presence of ethanol.

In *K. lactis* cells, an efficient production of heterologous proteins with enzymatic activity, such as prochymosine (46) and α-galactosidase (1), and proteins of therapeutic interest, such as interleukin-1β (4, 17), hepatitis B surface antigen (28), granulocyte colony-stimulating factor (21), and human serum

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* Corresponding author. Mailing address: Department of Cell and Developmental Biology, Pasteur Institute-Cenci Bolognetti Foundation, University of Rome "La Sapienza," Piazzale A. Moro, 00185 Rome, Italy. Phone: (39) 0649912278. Fax: (39) 0649912256. E-mail: FALCONEC@AXCASP.CASPUR.IT.

albumin (HSA) (5, 18), have been efficiently produced (for a review, see reference 44).

To optimize the production of recombinant HSA (rHSA) under the control of the *KlADH4* promoter, we focused on the medium composition and growth conditions; in preliminary attempts, we obtained good yields of rHSA in fed-batch fermentation.

MATERIALS AND METHODS

Strains. The following *K. lactis* strains were used in this work. MW98-8C (α *uraA lys arg rag1 pgi1 adh3*) is the parental strain (2). MS1 is a derivative of MW98-8C carrying an integrated copy of plasmid P4GUS into the *KlADH4* chromosomal locus (12) CMK5 (**a** *thr lys pgi1 adh3 adh1::URA3 adh2::URA3*), which expresses only *KlADH4* activity, was obtained by crossing strains that lacked single ADH activities (29). CBS 293.91 is a prototrophic natural *K. lactis* isolate (12). Y 721 is an isogenic derivative of CBS 293.91 carrying a disrupted copy of the phosphoglycerate kinase (*PGK*) gene (12). This strain, used for many generations in batch and fed-batch fermentations, showed a Rag$^+$ phenotype for the presence of the *PGK* gene on plasmid pYG156. Since the gene is essential for the growth on fermentable carbon sources, such a host-vector combination resulted in the stabilization of the expression system.

Media and culture conditions. YPD consists of (grams/liter) glucose, 20; yeast extract (Difco), 10; and Bacto Peptone (Difco), 20; supplemented, when required, with 100 mg of Geneticin G418 (Sigma) per liter. When indicated, ethanol (YPE), glycerol (YPG), or glucose plus ethanol (YPDE) at 20, 30, and 20 + 10 g/liter, respectively, were added instead of glucose.

M9YE consists of (grams/liter) carbon substrate, 20; yeast extract, 10; Na_2HPO_4, 6; KH_2PO_4, 3; NaCl, 0.5, NH_4Cl, 1; $CaCl_2 \cdot 2H_2O$, 0.015; and $MgSO_4 \cdot 7H_2O$, 0.24.

DM1 (defined medium 1) consists of (grams/liter) carbon substrate, 20; Na_2HPO_4, 4.4; KH_2PO_4, 4; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.1; sodium glutamate, 3; and ammonium acetate, 7; 1 ml of salt solution (13 mg of $FeSO_4 \cdot 7H_2O$ per ml, 9 mg of $MnSO_4 \cdot H_2O$ per ml, 1.8 mg of $CoCl_2 \cdot 6H_2O$ per ml, 18 mg of $ZnSO_4 \cdot 7H_2O$ per ml, 0.9 mg of $AlCl_3 \cdot 6H_2O$ per ml, 0.22 mg of $CuCl_2 \cdot 2H_2O$ per ml) per liter, and 1 ml of vitamin solution (13.5 mg of niacin per ml, 0.036 mg of biotin per ml, 1.8 mg of calcium pantothenate per ml, 2 mg of *m*-inositol per ml) per liter.

Shake flask cultures. Flasks (300 ml) containing 50 ml of medium were seeded with 0.5 ml of an inoculum grown to the stationary phase on the same medium. Cultures were grown for 3 days at 28°C on a rotary shaker (220 rpm) for rHSA production.

Fed-batch cultures. Fermentations were carried out in a 2-liter fermentor (SETRIC France) containing 0.8 liter of medium. The fermentor was inoculated with 40 ml of a fresh preculture grown to the stationary phase on the same medium. During fermentation, the pH was automatically controlled and adjusted between 6.5 and 7.0 with 10% (wt/vol) ammonia and the temperature was maintained at 28°C. Aeration was maintained at 75 liters/h under 0.2×10^5 Pa, and the dissolved oxygen was controlled at ~30% air saturation by the impeller speed controller and, when necessary, by enrichment of the air stream with pure O_2. All the process variables (pH, temperature, agitation, dissolved oxygen, and O_2 and CO_2 contents in the exhaust gases) were transduced on-line and interfaced by a PH3852 unit to a Hewlett-Packard 9000 computer. The compositions of the feed media were carbon source, 340 g/liter; yeast extract, 100 g/liter; ammonium acetate, 50 g/liter; or carbon source, 340 g/liter; sodium glutamate, 40 g/liter; ammonium acetate, 70 g/liter; and vitamins for complex and defined media, respectively.

General methods. Restriction enzyme digestions, plasmid engineering, and standard techniques were performed as specified by Sambrook et al. (37). *Escherichia coli* and yeast transformation was performed by electroporation with a Bio-Rad Gene-Pulser apparatus as specified by the manufacturer.

PCR amplification and cloning of *KlADH4* promoter fragments. The oligonucleotides used in PCR amplifications are listed in Table 1. PCRs were performed with the GeneAmp kit (Perkin-Elmer Cetus), using as template a 4.0-kbp genomic DNA region of *K. lactis*, which contained the *KlADH4* gene, cloned on a plasmid. The reaction mixture (100 μl) contained 0.2 mM each primer, 0.2 mM each deoxynucleoside triphosphate, 1× PCR buffer, 25 ng of DNA, 2 mM $MgCl_2$, and 2.5 U of Ampli-Taq DNA polymerase. PCR was carried out for 30 cycles of 94°C for 1 min, 52°C for 2 min, and 72°C for 3 min. After amplification, the DNA fragments were purified on agarose gels, digested with the appropriate enzyme, cloned into the pKSII plasmid (Stratagene cloning system), and transferred to *K. lactis* replicating plasmids by standard procedures.

Vector constructions. *E. coli* β-glucuronidase (GUS) and HSA expression vectors pP4-GUS, pYG132, and pYG156 (Fig. 1) were engineered as derivatives of pKD1, a natural plasmid originally isolated from *Kluyveromyces drosophilarum* (7, 13). pKD1 is structurally related to the *S. cerevisiae* plasmid 2μm and can stably replicate in *K. lactis* (2).

The details of the construction of pP4-GUS and pYG132 were described previously (12). Briefly, pP4-GUS is a vector derived from vector pSK-Kan401, which contains the pKD1 origin of replication (6). The *GUS* gene was isolated

TABLE 1. Nucleotide sequence of the oligonucleotides used in PCR amplification of different regions of the *KlADH4* promoter

	Nucleotide sequence[a]	
	−1197	−1179
1 ....(*Sal*I) ccccg/tcgac	CGGCTACTAATAGAAGTTC	
	−936	−953
2 ....(*Sal*I) cgcgg/tcgac	GGAACGACGGTACATAAG	
	−758	−741
3 ....(*Sal*I) ccccg/tcgac	CGGCGCCTGAAATTTTCC	
	−741	−758
4 ....(*Eco*RI) cgcgg/aattc	GGAAAATTTCAGGCGCCG	
	−536	−519
5 ....(*Sal*I) ccccg/tcgac	CCAGAAGTCGGGAGTTTAC	
	−519	−536
6 ....(*Hin*dIII) cgcga/agctt	GTAAACTCCGACTTCTGG	
	−758	−741
7S ....(*Sal*I) ccccg/tcgac	GTGTGTGCTGTGCCTTCC	
	−400	−383
7E ....(*Eco*RI) cgcgg/aattc	GTGTGTGCTGTGCCTTCC	
	−400	−383
8 ....(*Hin*dIII) cgcga/agctt	ATGTTGTGTTGTTGGTGG	
	−13	−30

[a] Chromosomal sequences of *K. lactis* are in capital letters.

from plasmid pBI221 (Clontech) as a *Bam*HI-*Eco*RI fragment, 2.1 kb in length, and placed under control of the *KlADH4* *Bgl*II-*Bam*HI portable promoter. The resulting expression cassette was introduced as a *Bgl*II-*Eco*RI fragment into the *Bam*HI and *Eco*RI sites of pSK-Kan401. The stability of plasmid pYG132, measured as the percentage of G418-resistant colonies, was 100% after 200 generations.

Construction of *KlADH4* promoter-HSA fusion vectors. In contrast to pP4-GUS, plasmid pYG132 contains the entire sequence of pKD1, linearized at the unique *Eco*RI site, as well as an HSA expression cassette consisting of the *KlADH4* *Sal*I-*Hin*dIII portable promoter, the prepro-HSA cDNA gene, and the *S. cerevisiae* phosphoglycerate kinase (*PGK*) terminator. pYG156 contains the entire pKD1 and the *K. lactis* *PGK* gene that can be used as a selectable marker in *pgk* mutant host strains (12). The common features of all three vectors are the presence of the *E. coli* origin of replication, allowing the plasmids to work as shuttle vectors, and the presence of the *bla* and *aph* genes conferring ampicillin and geneticin resistance to *E. coli* and yeast, respectively. In addition, pP4-GUS carries the *S. cerevisiae* *URA3* gene as an auxotrophic marker.

The vectors pYG107 and pYG108 contain the entire pKD1 sequence and the HSA cDNA fused to LAC4 and PGK promoters, respectively (18).

To construct pYG534 and pYG397, the region from −536 to −13 was obtained by PCR amplification with oligonucleotides 5 and 8, and the region from −399 to −13 was obtained with oligonucleotides 7S and 8. After *Sal*I-*Hin*dIII digestion, these fragments were inserted into plasmid pYG108 to give pYG534 and pYG397, respectively.

To construct pYG108/700M and pYG108ΔUAS, the region from −1199 to −519 was obtained by PCR amplification with oligonucleotides 1 and 6. This *Sal*I-*Hin*dIII fragment was cloned into the polylinker of pKSII plasmid. The *Hin*dIII site was eliminated by filling in with Klenow polymerase to give plasmid pKSII/700M. The 700-bp fragment was cut by a *Sal*I-*Not*I digestion and cloned into plasmid pYG108 to give plasmid pYG108/700M. In plasmid pYG108/700M, the region of the *PGK* promoter from −1499 to −402 containing the upstream activation site (UAS) was replaced by the 700-bp fragment of the *KlADH4* promoter. As a control, the pYG108 plasmid was digested with *Sal*I-*Not*I, filled in with Klenow DNA polymerase, and religated with DNA T4 ligase. The resulting plasmid was called pYG108ΔUAS.

Plasmid pYG108/2-4 contains the promoter regions from −953 to −741 (amplified with oligonucleotides 2 and 4) cloned into the *Sal*I-*Eco*RI sites of plasmid pYG108/700M.

Plasmid pCM13 carries the region of the *KlADH4* promoter spanning from −399 to −13 (amplified with oligonucleotides 7E and 8). The amplified fragment was inserted into the *Eco*RI-*Hin*dIII sites of plasmid pYG108/700M. In this way, the *PGK* promoter TATA box was replaced with the *KlADH4* TATA box.

Plasmid pCM15 was obtained by cloning the promoter fragment contained in plasmids pYG108/2-4 into the *Sal*I-*Eco*RI sites of pCM13. The promoter regions from −758 to −519 (amplified with oligonucleotides 3 and 6) were cloned in the *Sal*I-*Hin*dIII sites of the pKSII polylinker to yield plasmid pKS/3-6. After *Sal*I-*Eco*RI digestion, the promoter fragment was cloned into pCM13 plasmid to yield plasmid pCM16.

In pCM17, two of the three UAS elements contained in the *LAC4* promoter were replaced with the 200-bp fragment containing UAS$_E$.

Protein assays. Cultures (10 ml) of *K. lactis* were grown to the early stationary phase in YP medium containing different carbon sources as specified in the text.
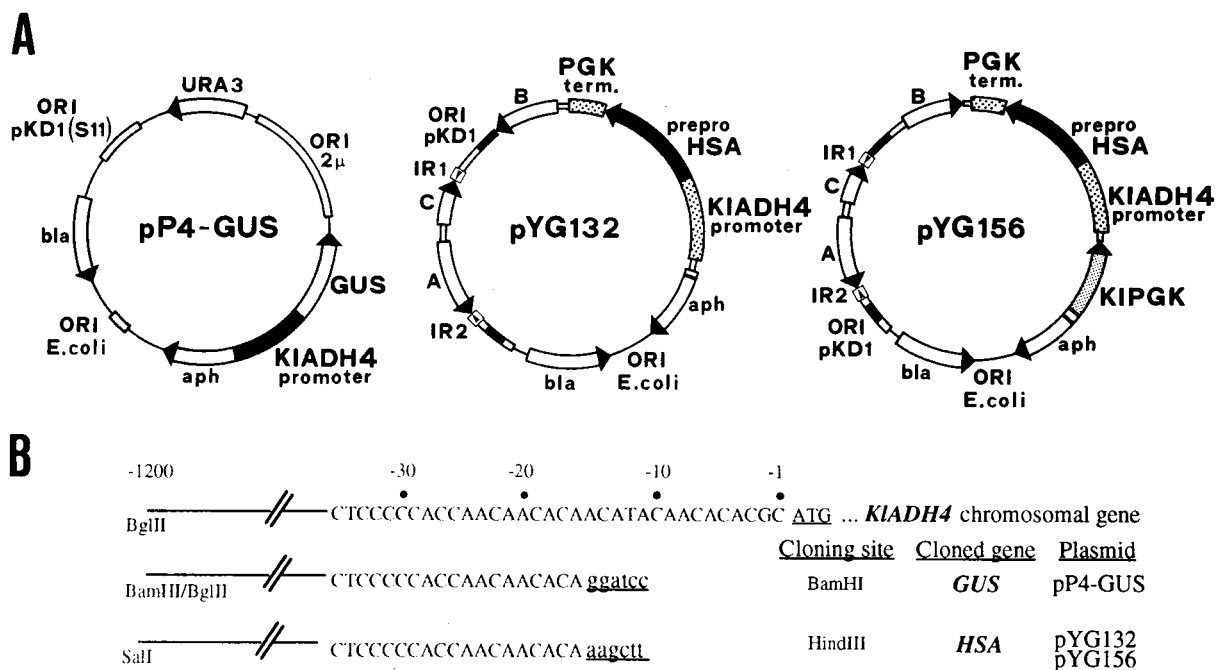
FIG. 1. (A) pKD1-derived expression vectors for *K. lactis*. pP4GUS harbours the replication origin (S11) of pKD1 and the β-glucuronidase (GUS) gene under the control of the *KlADH4* promoter. pYG132 and pYG156 contain the entire pKD1 sequence and the human serum albumin (HSA) gene under the control of the *KlADH4* promoter. A, B, and C are pKD1 genes; IR1 and IR2 indicate the inverted repeats of the plasmid. β-Lactamase (bla) and aminoglycoside phosphotransferase (aph) are the genes conferring ampicillin and geneticin (G418) resistance in bacterial and yeast cells, respectively. The uracil auxotrophic marker gene (URA3) and the promoter regions of phosphoglycerate kinase (PGK) and killer toxin (KI) are also indicated. (B) 3′ end sequences and cloning sites of the natural and engineered promoters present in vectors pP4GUS, pYG132, and pYG156.

The cells were broken with glass beads in Eppendorf tubes and centrifuged, and the supernatants were analyzed by electrophoresis on 5% nondenaturing acrylamide minigels. Gel preparation and buffers were essentially as described by Williamson et al., (47). Samples corresponding to 20 to 40 μg of proteins were run at 4°C for 60 min at 20 mA. For the detection of ADH activity, the gels were stained as described by Lutstorf and Megnet (27). GUS activity (39) was detected by soaking the gels in a staining solution containing 50 mM Na_2HPO_4 (pH 7.0) and 50 μg of 5-bromo-4-chloro-3-indolyl glucuronide (X-Glu) per ml dissolved in dimethylformamide (5 mg/ml). The gels were then incubated at 37°C until bands corresponding to the enzymatic activities became visibly stained.

Albumin detection. To detect albumin, 5×10^5 cells/ml were inoculated into YPD or YPDE medium and supernatants were tested after 3 days of fermentation. Aliquots of the supernatants were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, and the protein was revealed by Coomassie brilliant blue staining or by Western blotting. When the latter method was used, aliquots of supernatants were separated on sodium dodecyl sulfate–10% polyacrylamide gels and proteins were electroblotted onto Immobilon P membranes (Millipore) with a Bio-Rad wet blotting system. Filters were saturated by incubation for 30 min at room temperature in phosphate-buffered saline (PBS) with 5% nonfat dry milk. The blots were washed twice in PBST (PBS, 0.05% Tween 20) and incubated for 2 h with the anti-HSA monoclonal antibody (Sigma Co.A-0433) diluted 1:16,000 in PBS. After two washes with PBST, the filters were incubated for 1 h with the secondary antibody linked to the alkaline phosphatase. After two final washes, the blots were developed with the 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium system (20).

RESULTS

Cloning of the *K. lactis KlADH4* promoter. The *KlADH4* gene was isolated as an 8.4-kb *Bam*HI fragment from a *K. lactis* genomic bank by using an *S. cerevisiae ADH2*-derived probe (36). Based on the nucleotide sequence of the *KlADH4* coding region (35), we located the ATG initiation codon close to a *Hin*dIII site. Accordingly, we subcloned a 2.2-kb *Hin*dIII fragment containing the *KlADH4* upstream region and the first 14 codons of the coding region into plasmid pTZ19 (Pharmacia). This construct was used to introduce a *Bam*HI site 10 nucleotides upstream from the ATG codon by site-direct mutagen-

esis by using the double-primer procedures (37). The resulting plasmid allowed the isolation of a 1.2-kb *Bgl*II-*Bam*HI portable promoter used in the construction of the pP4-GUS expression vector (Fig. 1). A promoter variant in which the *Bgl*II and *Bam*HI sites were replaced by *Sal*I and *Hin*dIII sites, respectively, was obtained by the PCR procedure (pBluescript, mung bean nuclease). This variant was used in the engineering of albumin expression vectors pYG132 and pYG156 (Fig. 1).

Ethanol-regulated expression of bacterial GUS. We then wondered whether the *KlADH4* promoter could be successfully used for the regulated expression of heterologous genes. First we used the *E. coli GUS* gene as a reporter gene under control of the *KlADH4* upstream region (see Materials and Methods). The resulting plasmid, named pP4-GUS (Fig. 1), was used to transform *K. lactis* MW98-8C to uracil auxotrophy. This autonomously replicating sequence-containing vector, which is unstable in *K. lactis*, was used to select for chromosomal integration of the expression cassette. One of the isolated Ura[+] transformants, named MS1, expressed GUS activity over many generations. Southern analysis revealed that integration of the expression vector occurred at the chromosomal *KlADH4* locus by homologous recombination, which resulted in the duplication of the promoter region without interfering with the integrity of the *KlADH4* structural gene (data not shown). In fact, as demonstrated in Fig. 2A, both genes are functionally expressed and they are coregulated in an ethanol-dependent manner. Furthermore, these results show that all elements relevant for regulation are present on the 1.2-kb portable *KlADH4* promoter. Moreover, as shown by Northern analysis, *GUS*- and *KlADH4*-specific transcripts can be detected as soon as 30 min after induction by ethanol (Fig. 2B). For the *GUS* gene, besides the mRNA of the expected length (about 2,300 nucleotides), another, larger transcript which migrated at the level of
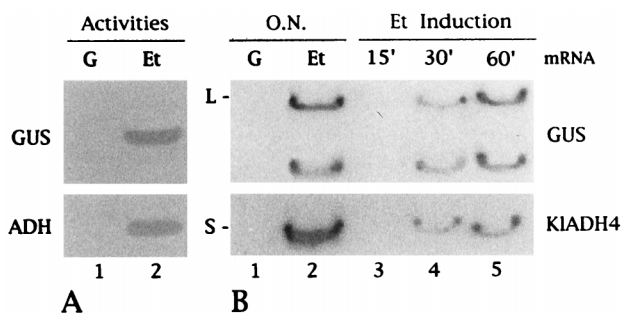
FIG. 2. Ethanol-dependent expression of *KlADH4* and *GUS* genes. (A) Strain MS1, carrying the *GUS* gene under the control of the *KlADH4* promoter integrated at the chromosomal *KlADH4* locus, was grown overnight on 20 g of glucose per liter (G, lane 1) and 20 g of ethanol per liter (Et, lane 2). The presence in cellular extracts of the ADH IV and GUS activities were revealed by specific staining on native acrylamide gels (see also Materials and Methods). (B) Transcription analysis of *KlADH4* and *GUS* genes after an overnight (O.N.) growth on glucose and ethanol (lanes 1 and 2) and 15 to 60 min after the addition of ethanol to glucose-grown cells (lanes 3 to 5). L and S indicate the migration positions of the large and small rRNAs, respectively.
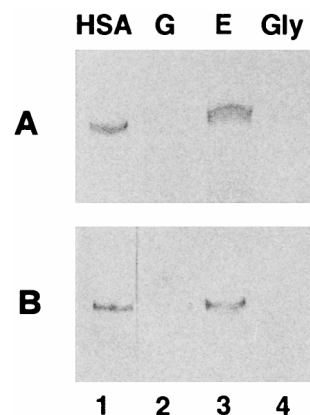

FIG. 3. pYG132-driven production of HSA with strain CMK5. Cells were grown on 20 g of glucose per liter (G), 20 g of glucose per liter plus 10 g of ethanol per liter (E), and 30 g of glycerol per liter (Gly) for three days. After SDS-polyacrylamide gel electrophoresis of supernatants HSA was revealed by Coomassie Blue staining (A) and by Western blotting with a monoclonal antibody, anti-HSA (B). Fifteen and 5 μl of supernatants were loaded in panels A and B, respectively. Commercial HSA was loaded in lane 1 as a marker.

the large rRNA was detected. This transcript, regulated in the same way as the mature mRNA, could represent a precursor molecule.

Production of HSA under control of the *KlADH4* promoter on multicopy vectors. Recently, increasing interest has been focused on the production of HSA in *K. lactis*. The HSA cDNA gene has been cloned into pKD1-derived vectors under the control of the constitutive *PGK* promoter of *S. cerevisiae* and the regulated *LAC4* promoter of *K. lactis*, and good production of the protein has been obtained under different growth conditions (batch, fed batch, and chemostat) (5, 18).

To test whether the *KlADH4* promoter could be efficiently used for the biotechnological production of a protein of industrial interest, we engineered vectors carrying the HSA cDNA gene under the control of this promoter. In the first experiment, we integrated the P*KlADH4*–prepro-HSA expression cassette into the genome of *K. lactis*. Since the resulting integrants showed very low levels of secreted HSA which could be detected only by immunological methods (data not shown), we examined the production of this protein in *K. lactis* strains transformed with the pKD1-derived multicopy vector pYG132 (Fig. 1A), which carries 1.2 kbp of the *KlADH4* promoter fused to the HSA cDNA. This plasmid was introduced into the non-fermenting strain CMK5, and after 3 days of growth in YP medium containing different carbon sources, aliquots of supernatants were analyzed for the presence of HSA by gel electrophoresis under denaturing conditions. The protein was revealed either by Coomassie blue staining or by Western blot analysis with an anti-HSA monoclonal antibody (Fig. 3A and B). In CMK5, as in other nonfermenting strains, the HSA was present only in the supernatant of cells grown in ethanol (lane 3) and not in the supernatant of glucose- or glycerol-grown cells (lanes 2 and 4).

In these experiments, HSA expression levels were at least 30 to 40 times higher than those observed with the single-copy integrant, which is in agreement with the average copy number of pKD1-derived vectors (40 to 60 per cell). Interestingly, these results indicate that even in the presence of multiple copies of the *KlADH4* promoter, there is no limitation of *trans*-acting factors for the induction of the *KlADH4* gene.

Identification of an ethanol-responsive element. To identify possible *cis*-acting elements, we introduced deletions into the *KlADH4* promoter and parts of it were also cloned upstream of

the TATA box of the heterologous *PGK* promoter of *S. cerevisiae*. All constructions were introduced into CMK5 cells, and the level of HSA produced by transformant clones was analyzed by gel electrophoresis. The results of our analysis are reported in Fig. 4. HSA production was not observed on ethanol with *KlADH4* promoter deletions upstream of position −536 (plasmids pYG534 and pYG397), suggesting that the putative activating sequences were localized within the deleted regions. On the other hand, we could observe ethanol-driven expression of the *HSA* cDNA when the region from bp −1197 to −536 was fused to the TATA box of both the *KlADH4* (pCM13) and the *PGK* (pYG108/700M) genes. For this reason, we performed a more detailed analysis of this region.

The most important results were obtained with plasmids pCM15 and pYG108/2-4, which contained a region of about 200 bp spanning from −953 to −741 fused to the TATA box of the *KlADH4* and *PGK* genes, respectively. Both plasmids allowed ethanol-dependent expression of the *HSA* cDNA. In contrast, when this 200-bp region was missing (plasmid pCM16), the regulatory property of the promoter was completely lost. No HSA production could be detected with the control vector pYG108ΔUAS, which contained only the TATA element of *PGK*.

To further confirm that the region from bp −953 to −741 contained the ethanol-responsive element(s), we substituted this fragment to the upstream activation site (UAS) region of the promoter of *LAC4*. This is the β-galactosidase-encoding gene of *K. lactis*, which is induced by lactose and is repressed in the presence of glucose (1, 26, 33, 38). The resulting hybrid *LAC4*-*KlADH4* promoter was fused to the *HSA* cDNA (plasmid pCM17), and the production of HSA on different carbon sources was monitored. As shown in Fig. 4, this promoter could drive HSA production only in ethanol-grown cells and, as expected, was no longer inducible by lactose (data not shown). In control experiments (plasmid pYG107), the *LAC4* promoter fused to the *HSA* cDNA failed to drive HSA production on glucose, glucose plus ethanol, and glycerol, whereas it allowed HSA production in lactose-grown cells (not shown).

All the above results indicate that the element(s) necessary and sufficient for the induction of *KlADH4* by ethanol is located within the 200-bp fragment spanning bp −953 to −741.
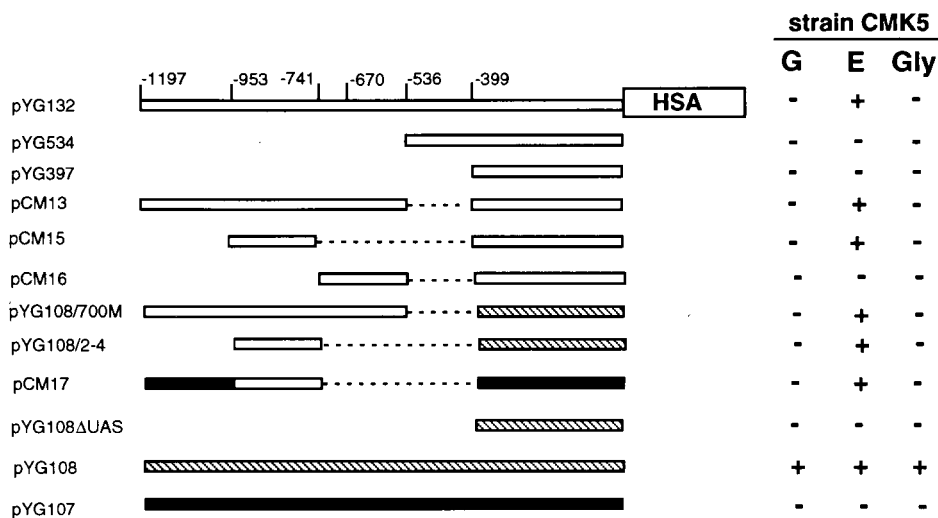
FIG. 4. HSA production with CMK5 cells carrying different regions of the *KlADH4* promoter grown on 20 g of glucose per liter (G), 20 g of glucose plus 10 g of ethanol per liter (E), and 30 g of glycerol per liter (Gly). Numbers, in approximate scale, refer to the *KlADH4* promoter. HSA in supernatants was detected on SDS-polyacrylamide gels followed by Coomassie Blue staining and quantified by comparison to known amounts of commercial HSA. All constructions were tested in three independent fermentation experiments. + indicates HSA amounts similar to those observed with the entire *KlADH4* promoter induced by ethanol; − indicates undetectable HSA levels. □, *KlADH4* promoter; ▨, *PGK* promoter; ■, *LAC4* promoter.

This element, which also proved functional when inserted in heterologous promoters, was named UAS$_E$.

p*KlADH4*-driven rHSA secretion in fed-batch fermentations. In shaken-flask cultures, the production of rHSA under inducing conditions reached 200 to 250 mg/liter in defined medium and 300 mg/liter in complex medium, which corresponded to yields of 20 to 30 mg of rHSA/g of dry biomass. These values represent 5 to 7% of the yeast total proteins and 80 to 90% of the total soluble proteins (data not shown).

To improve the rHSA yields, we performed fed-batch fermentations (0.8 liter) on different media. In these experiments, the vector used was pYG156, which carries the PGK gene for selection (see Materials and Methods) and does not confer resistance to the aminoglycoside antibiotic G418. After initial batchwise growth of strain Y 721 in defined medium (DM1) and consumption of the carbon substrate, the feed medium was added to the fermentor by using a peristaltic pump coupled to a preprogrammed time-based feeding profile, deduced from previous experiments where the feeding rate was coupled to the carbon dioxide evolution rate. The carbon substrate used was a mixture of lactose and ethanol (final concentration, 20 g/liter) in different ratios: 50/50, 12/88, and 0/100.

As shown in Fig. 5A, when 100 g of carbon sources was added, 45% of the carbon was recovered in the biomass when 100% ethanol was used, and this value increased to 53% when lactose plus ethanol (12/88) was used. On the other side, the best results in terms of rHSA production were obtained with ethanol as the only carbon substrate (Fig. 5B). In fact, under these conditions, about 800 mg of the protein per liter was produced, compared to 200 and 350 mg/liter obtained when different mixtures of ethanol and lactose were used. In conclusion, at the end of the fermentation time (72 h) with ethanol as the only carbon source, we obtained 71 g (dry-cell weight) of biomass per liter with a yield ($Y_{x/s}$) of 45%. At the same time, 620 mg of rHSA per liter was produced, with a yield ($Y_{p/x}$) of 8.7 mg of rHSA/g of biomass and a productivity of 8.7 mg of rHSA/liter · h.

Kinetics of rHSA secretion in fed-batch fermentations. The growth and production kinetics of rHSA in complex medium

(ethanol, yeast extract, and ammonium acetate) and a defined medium (ethanol, sodium glutamate, ammonium acetate, vitamins) have been compared. As shown in Fig. 6A and B, the kinetics of growth and the production levels of rHSA were very similar in the two media during the first 3 days, and we could observe an increase in the specific productivity of rHSA from 5 to 30 mg/liter · h between 24 and 72 h of fermentation. After 4 days of culture, a high biomass production occurred in both complex and defined media (70 and 80 g [dry-cell weight]/liter, respectively), with a yield of about 45% as a result of the added ethanol. In this period, the amount of rHSA produced was 1.05 g/liter in complex medium and 0.85 g/liter in defined medium, which corresponded to a productivity of 15 and 10 mg of rHSA/g of biomass, respectively. In both complex and defined media, biomass and rHSA yields were well correlated with the ethanol added during the fed-batch culture (Fig. 6C).

DISCUSSION

HSA is the most abundant protein in human plasma involved in the maintenance of a normal osmolarity and also in the transport of hydrophobic molecules. This protein has a big market requirement in that it can be used as replacement fluid during septic or traumatic shock, to compensate for blood loss, and to treat burn victims. At present, HSA is largely produced (300 tons/year) by conventional techniques involving fractionation of plasma obtained from blood donors. It would be a great advantage to be able to use genetic engineering to obtain rHSA in good yield and at lower cost, with no danger of contamination by human pathogens. For this reason, great efforts have been dedicated to the production of this protein on a large scale by genetically engineered microorganisms. For this purpose, rHSA production has been studied in *E. coli* (24, 25) *Bacillus subtilis* (38), *S. cerevisiae* (11, 23, 31, 42, 43), *Pichia pastoris* (30), and plants (41).

K. lactis is a very promising nonpathogenic organism because of its good secretory capabilities (17, 18, 44, 46), and new promoters with specific properties can be derived from this yeast for the regulated expression of heterologous proteins. In
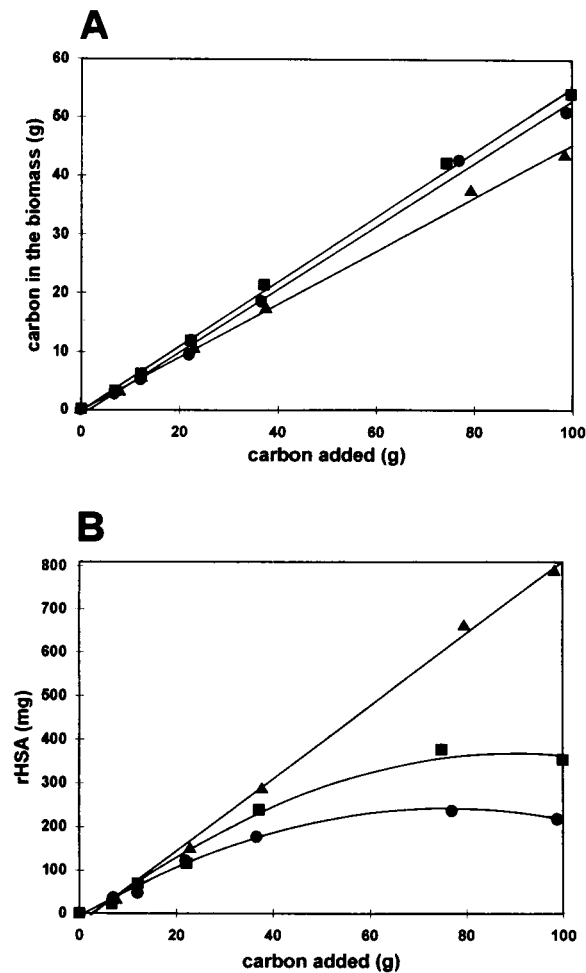
FIG. 5. Influence of carbon sources on biomass production (A) and rHSA secretion (B) in fed-batch fermentation. *K. lactis* Y721 (*pgk*, Rag$^+$), harboring plasmid pYG156, was grown in defined medium containing 20 g of ethanol per liter or mixtures of ethanol and lactose (final concentration, 20 g/liter) as the carbon source. ●, lactose-ethanol (50/50); ■, lactose-ethanol (12/88); ▲, lactose-ethanol (0/100).
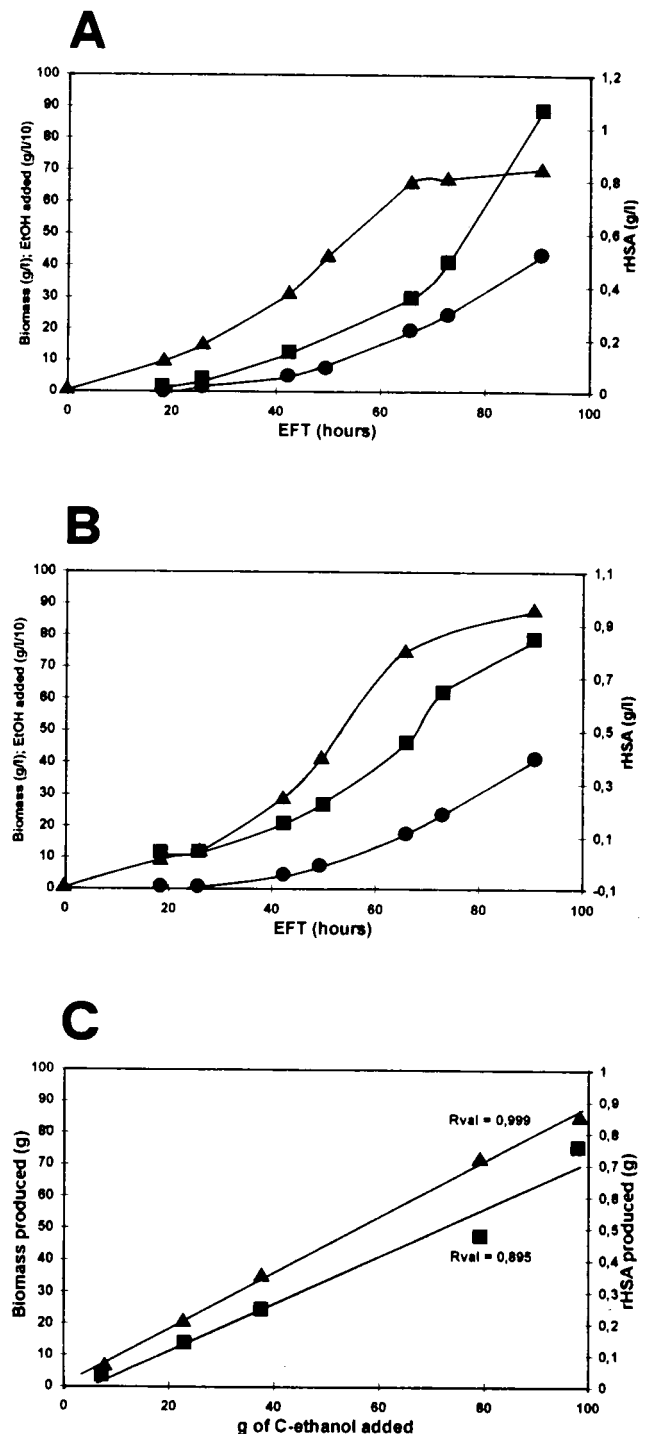

FIG. 6. Kinetics of rHSA secretion, biomass production, and ethanol consumption in fed-batch cultures of *K. lactis* Y721 (*pgk*, Rag$^+$) grown in complex medium (A) and defined medium (B and C). ●, ethanol; ■, rHSA; ▲, biomass.

fact, we isolated a new promoter from the *KlADH4* gene of *K. lactis*. This promoter, also present in multicopy vectors, allows regulated or constitutive expression of heterologous genes depending on the host strain used. In nonfermenting strains (Rag$^-$), which are unable to produce intracellular ethanol from fermentable carbon sources, the transcriptional activation of the *KlADH4* promoter is dependent on the addition of ethanol to the culture medium. Nonfermenting *K. lactis* mutants can be easily generated from fermenting strains with particular industrially relevant properties (i.e., biomass yield and secretion efficiency) by disrupting genes which affect ethanol production.

Another interesting aspect of the regulation of this promoter is its insensitivity to glucose repression. This property, combined with the specificity of ethanol as the activator, allows the expression of cloned genes after the addition of the inducer, independently of the carbon source present in the medium.

We identified an ethanol-responsive element (UAS$_E$) which is located within a 200-bp region spanning from -953 to -741 of the promoter. Deletions of UAS$_E$ render the *KlADH4* promoter no longer inducible in the presence of ethanol. On the

other hand, UAS$_E$ constitutes an interesting "transportable" *cis*-acting element for the construction of a regulated chimeric promoter. In fact, UAS$_E$ can confer ethanol-dependent promoter activation, such as of the *S. cerevisiae PGK* and *K. lactis LAC4* promoters, which are usually not induced by this substrate.

Finally, we studied the production of rHSA under the control of the *KlADH4* promoter in *K. lactis* cells grown in complex and defined media under different cultivation conditions. Considering the results obtained in terms of production per liter of culture, high biomass production (70 to 80 g [dry-cell weight]/liter) was obtained either in both complex and defined media, and a significant amount of rHSA production was observed in fed-batch cultures (1 g/liter) as compared to batch cultures (0.2 to 0.3 g/liter). These values are similar to those obtained under the same conditions with the HSA gene under the control of the *PGK* and *LAC4* promoters (18).

On the basis of other studies, it should certainly be possible to increase the level of production of rHSA to at least 2 g/liter by working on the fermentation parameters. As an example, under the culture conditions used, the oxygen uptake rate never exceeded 2 mM/min.

In conclusion, we believe that the yields of rHSA obtained can be improved and that the process can be scaled up.

ACKNOWLEDGMENTS

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