ARTICULOS ORIGINALES CORTOS

EXPRESSION OF HEPATITIS B SURFACE ANTIGEN IN *Kluyveromyces lactis* USING A REPLICATIVE VECTOR

Eduardo Martínez, Jorge Aguiar, Juan Morales, Viviana Falcón, Mario Callejo, Denia Gutiérrez, Julio Sánchez, Gerardo Ferbeyre

Center for Genetic Engineering and Biotechnology, P.O.Box 6162, Ciudad Habana, Cuba.

Recibido en febrero de 1992. Aprobado en agosto de 1992.

Key words: *K. lactis,* hepatitis vaccine, recombinant.

SUMMARY

LAC 4 gene sequences that regulates the expression of ß-galactosidase and the pKD1 plasmid from *Kluyveromyces drosophilarum* were used to construct a replicative expression vector for the regulated expression of hepatitis B surface antigen (HBsAg) in *Kluyveromyces lactis.* Yeasts transformed with this vector express the HBsAg up to 0.5% of total cell protein. The expression was dependent on the addition of the inducer (lactose). Evidences for the entry of the HBsAg in the secretory pathway are discussed.

RESUMEN

Se uso la secuencia del gen LAC 4 que regula la expresion de la B-galactocidasa y el plasmidio pKD1 de *K. drosophilarum* para construir un vector replicativo para la expresión regulada del antígeno de superficie de la hepatitis B (HBsAg) en *K. lactis.* Las levaduras transformadas con este vector expresan el HBsAg a un 0.5% del total de proteína celular. La expresión fue dependiente de la adición del inductor (lactosa). Se discuten algunas evidencias de la entrada del HBsAg en la via secretora.

INTRODUCTION

K. lactis is a very promising yeast for the expression of heterologous genes due to its well known secretory properties and its ability to reach high cell densities on fermentors. These characteristics were demonstrated for the case of calf prochymosin (Van den Berg et al., 1990). The system reported for expression in *K. lactis* is based on the regulatory regions of the ß-Galactosidase gene, (LAC 4), with different signal sequences. More recently, high level expression of fungal rennin has been reported using basically the same strategy (Ferbeyre et al., 1991).

The expression systems described above depend on the integration of the expression cartridge into the yeast chromosomes and higher levels of expression have been associated with multiple integration events (Van den Berg *et al.*, 1990 and Ferbeyre *et al.*, 1991). The copy number of the expression cartridge can be increased using replicative vectors instead of integrative vectors. 2 μm derivatives have been used succesfully in *Saccharomyces cerevisiae* (Hollemberg, 1982). But unfortunately, these plasmids are very unstable in *K. lactis.* However Chen *et al.*, (1986) isolated pKD1, a 2μ m analogue, from K. drosophilarum that replicates with high stability in K. lactis. Using this plasmid, Fleer *et al.*, (1991a and b) have constructed expression vectors for secreting high levels of human serum albumin and human interleukin-1ß respectively.

In this work, we have used pKD1 plasmid and the regulatory sequences of the *K. lactis* LAC 4 gene to construct a vector able to replicate in this yeast and express the HBsAg.

MATERIALS AND METHODS

Strains, media, growth conditions and transformation were done as refered by Ferbeyre *et al.*, (1992).

Plasmids and constructions

General cloning procedures and bacterial transformation were as described, (Maniatis *et al.*, 1982). Plasmid pCXJ is a pKD1 derivative (Bianchi *et al.*, 1987) used as starting point to construct our vector. This plasmid was digested with Cla I, filled in with Klenow, dephosphorylated with calf intestine phosphatase (CIP) and ligated to the band obtained after digestion of pDLH2(constructed in our lab) with EcoR I and Bgl II and blunted with Klenow. This band corresponds to the expression cartridge and possesses the S gene flanked by the LAC 4 promoter and terminator. The resulting plasmid was named pGMH1 (figure 1).

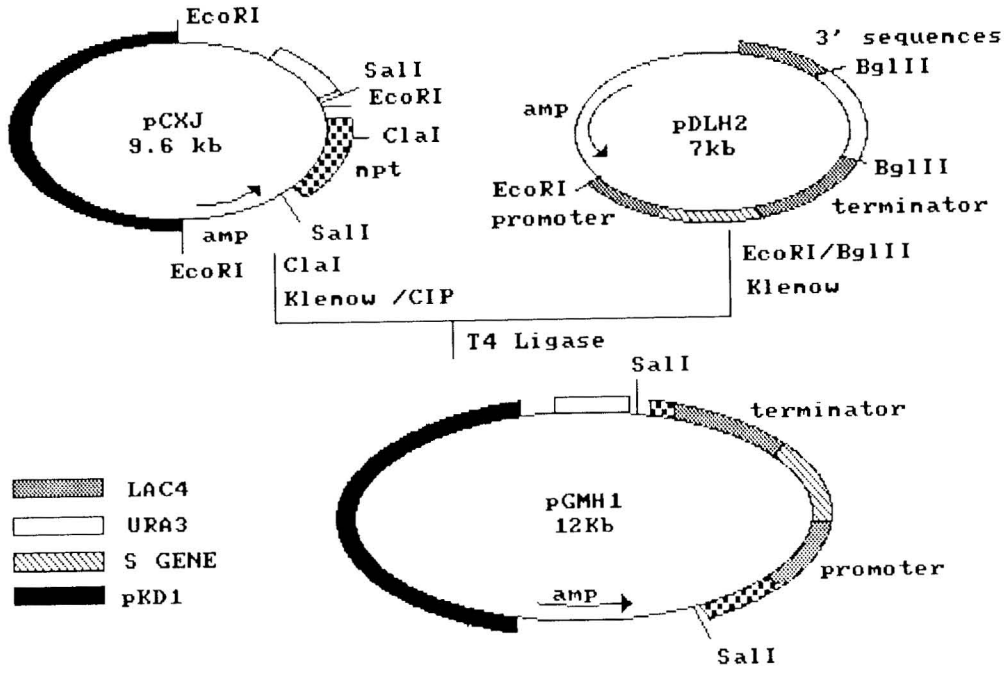

Fig. 1 Construction of the replicative plasmid pGMH1.

Protein Analysis and preparation of cell extracts

Protein concentration was determined by the method described by Bradford, (1976). HBsAg was measured by ELISA (Gonzalez *et al.*, 1989). Cell extracts were prepared as described previously Ferbeyre *et al.*, 1991).

Electron Microscopy

Cells were proccesed as described by Spurr (1969), then they were exposed to a gold-labeled mAb specific for S-HBsAg and observed in Transmission Electronic Microscopie JEOL-JEM-2000 EX.

Enzymes and Chemicals

Restriction enzymes, T4 ligase and DNA polymerase (Klenow fragment) were from ENZIBIOT, (CIGB-Havana, Cuba). Calf instestine phosphatase (CIP) was from Bochringer Mannheim, (Germany); zymolase 100 T was from Seikagaku Co. Ltd, (Japan). lactose was from BDH, Ltd, U.K.

RESULTS AND DISCUSSION

pGMH1: A replicative vector for the expression of HBsAg in *K. lactis*

Plasmid pGMH1 constructed as described in Materials and Methods, consists of pKD1 sequences that confer replication ability in K. lactis, pUC19 sequences that confer replication ability and ampicillin resistance in *E. coli,* and the S gene from the HBsAg under the control of LAC 4 regulatory sequences, (see figure 1).

This plasmid transformed *K. lactis* with a frequency of 10^3 ura+ transformants/μg of DNA, similar to that obtained with pCXJ. The transformants were 100% stable after being cultured for 50 generations in non selective medium using glucose as carbon source. However, they were unstable when lactose was used as inducer (only 13% of the cells contained the plasmid after 18 generations). This could be due to toxicity of HBsAg for the cells. Fleer *et al.*, (1991) reported plasmid unstability when expressed the Met-interleukin-1ß. They also suggested that intracellular accumulation of interleukin-1ß has a toxic effect on *K. lactis.*

Expression of HBsAg in *K. lactis*

The expression of HBsAg was assessed in flask cultures using YEP medium. Lactose was required at concentrations of at least 0.5% in order to obtain good expression levels, 0.5% (4ug/ml) of the total cell protein. The S-HBsAg synthesized has an apparent molecular mass of 23 kDa and it was able of forming particles similar to those found in asymptomatic carriers (data not shown).

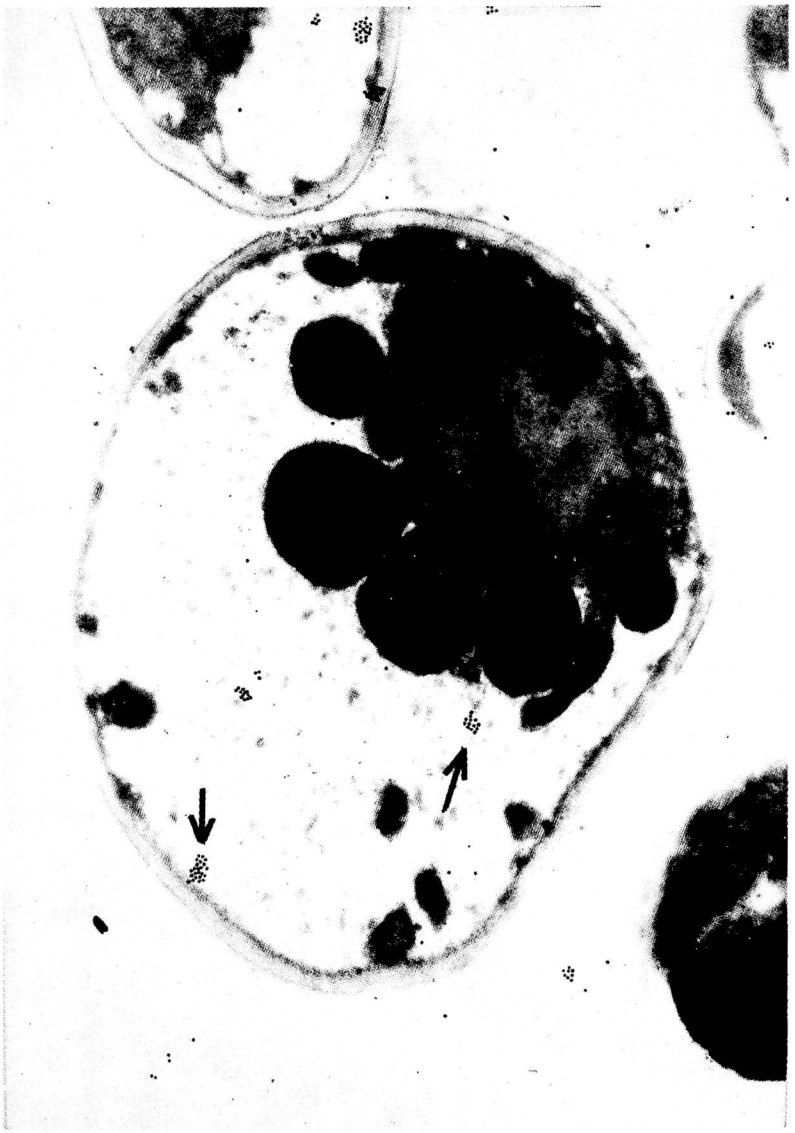

Fig.2 Immunogold labeling of a thin section of yeast cells expressing HBsAg with mAb anti-S-HBsAg. (20 000 X). Nucleo (N), Vacuole (V). Arrows indicate S-HBSAg protein.

The presence of the HBsAg into yeast cell was confirmed by immunocytochemistry (figure 2).

HBsAg particles enter the secretory pathway

We have evidences for the entry of the S-protein into the secretory pathway. First, we found around 50 ng/ml of S-HBsAg antigen in the culture medium; then, we measured the levels of S-HBsAg in the periplasmic space (supernatant obtained following treatment of the cells with zymolase) and we found 130 ng/ml of antigen. In addition using electron microscopy we have noticed morphological changes in the endoplasmic reticulum of the cells that produce S- HBsAg (not shown).

This phenomenon was not observed in the control strain VD1. Biemans *et al.*, (1991), found similar results for the large surface protein in *S. cerevisiae* and Shen *et al.*, (1989) reported the secretion of the M-protein in *Hansenula polymorpha*, but up to now, the entry of the S-protein in the secretory pathway of yeasts has not been reported. We think that the secretion of the S-antigen in K. lactis is related with particular secretory properties of this yeast.

REFERENCES

Bianchi, M.M.; Falcone, C.; Jie, C.X.; Wesslowsk-Louvel, M.; Frontali, L. and Fukuhara, H. (1987). Transformation of the Yeast Kluyveromyces lactis by new vectors from the 1,6m circular plasmid pKD1. *Current Genet* **12**:185-192.

Biemans, R.; Thines, D.; Rutgers, T.; Wilde, M. and Cabezon, T. (1991). The large surface protein of Hepatitis B Virus is retained in the yeast Endoplasmic Reticulum and provokes its unique enlargement. *DNA and cell biology* **10**:191-200.

Bradford, M.M. (1976). A Rapid and Sensitive Method for the quantitation of micrograms quantities of protein utilizing the principle of dye binding. *Anal. Biochem.* **72**:248-254

Chen, X.J.; Saliola, M.; Falcone, C.; Bianchi, M.M. and Fukuhara, H. (1986). Sequence organization of the circular plasmid pKD1 from the yeast Kluyveromyces drosophilarum. *Nucleic Acids Res.* **14**:4471-4481.

Ferbeyre, G.; Aguiar, J.; Villareal, A.; Torrens, I.; Santizo, C. and Morales, J. (1991). La inducción de alcohol oxidasa en levaduras metilotroficas. Biotecnologia Aplicada 8:242-247.

Ferbeyre, G.; Martinez, E.; Aguiar, J.; Torrens, I.C.; Gonzalez, T.; Silva, A. and Morales, J. (1992). Secretion of recombinant fungal rennin in Kluyveromyces lactis. *Biotecnología Aplicada* **8**:335-344.

Fleer, R.; Yeh, P.; Amellal, N.; Maury, I.; Fournier, A.; Baccetta, F.; Baduel, P.; Jung, G.; L'Hote, H.; Becquart,J.; Fukuhara, H. and Mayaux, J-F. (1991,a). Stable multicopy vectors for high-level secretion of recombinant human serun albumin in Kluyveromyces yeast. *Biot/Technology* **9**:968-975.

Fleer, R.; Chen, X.J.; Amellal, N.; Yeh, P.; Fournier, A.; Guinet, F.; Gault, N.; Faucher, D.; Folliard, F.; Fukuhara, H. and Mayaux, J-F. (1991,b). High-level secretion of correctly processed recombinant human interleukin-1ß in *Kluyveromyces lactis. Gene* **107**:285-295.

Gonzalez, M.; Ramirez, G.A.; Vega, G.I.; Salgado, B.A.; Diaz, R. and Izquierdo, M. (1989). Proocedings of the international simposium on Viral Hepatitis. Madrid, p-22.

Hollemberg, C.P. (1982). Cloning with 2m DNA Vectors and the expression of foreign genes in Saccharomyces cerevisiae. Current Tops. *Microbiol. Immunol* **96**:119-144.

Maniatis, T.; Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning. A laboratory manual. Cold Spring Harbor, New York, USA.

Shen, S-H.; Bastien, L.; Nguyen, T.; Fung, M. Slilaty, S. (1989). Synthesis and secretion of hepatitis B middle surface antigen by the methylothophic yeast Hansenula polymorpha. *Gene* **84**:303-309.

Spurr, A.R.J. (1969). Ultrastruct, Res. 26:31.

Van den Berg, J.; van der Laken, K.J.; van Ooyen, A.J.J.; Renniers, T.C.H.M.; Rietveld, K.; Scharp, A.; Brake, A.J.; Bishop, R.J.; Schultz, K.; Moyer, D.; Richman, M. and Shuster, J.R. (1990). Kluyveromyces as a host for heterologous gene expression: Expression and secretion of prochymosin. *Bio/technology* **8**:135- 139.