IS THERE A LINKER HISTONE IN THE YEAST *KLUYVEROMYCES LACTIS*?

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ABSTRACT

In all eukaryotic cells nuclear DNA is organized in a highly-ordered nucleoprotein complex called chromatin. Along with DNA, essential structural and functional components of chromatin are histone proteins: core histones and linker histones. The latter are involved in both the maintenance of higher chromatin structures and together with core histones in regulation of gene expression. It has to be mentioned, however, that both functions of linker histones are more presumed than proved and therefore are subject of disputes.

The aim of the current research is to go in more details of the functions of linker histones. We have explored the yeast Kluyveromyces lactis as a model organism. In silico analysis revealed a single open reading frame (ORF) in K. lactis genome, with homology (around 48%) to linker histone genes of different organisms. The predicted amino acid sequence of the putative K. lactis H1 protein (KlH1p) showed 40% identity. Interestingly, an expression of mRNA from the gene was not detected. Knockout of KlH1 gene has not shown great impact on the cellular viability. In order to answer whether KlH1 is a true gene coding for a linker histone or it is a pseudogene several phenotypic features of KlH1 knockout cells were examined. Based on the obtained results we determined the significance of KlH1 for K. lactis.

Key words: *chromatin, linker histone, gene expression, K. lactis*

INTRODUCTION

Nuclear DNA of eukaryotic cells is organized in a highly-ordered nucleoprotein complex called chromatin. The basic units of chromatin are nucleosomes composed of 146 bp of DNA wound around a histone octamer consisting of two molecules of each of the core histones H2A, H2B, H3 and H4. DNA between two nucleosomes, termed linker DNA, could vary in length from 10 to 70 bp. Linker histones (H1 histones) bind the linker DNA and the nucleosome and thus contribute to the formation and maintenance of higher-order chromatin structures which is prerequisite for proper gene expression (Happel & Doenecke, 2009). It has been reported that linker histones are involved in several cellular processes such as regulation of gene expression, differentiation, DNA repair and recombination, apoptosis, and senescence. However, what is the particular role of linker histones and how precisely they modulate chromatin structure and function yet remains badly understood.

Yeasts are single-celled eukaryotes that have been widely used by researchers to study a number of molecular processes conserved in all eukaryotic cells. *Kluyveromyces lactis* is the best-established non-*Saccharomyces* yeast valuable for both fundamental research and biotechnology applications and has been approved as GRAS (*g*enerally *r*egarded *a*s *s*afe) organism (summarized in Breunig&Sreensma, 2003). *K. lactis* genome is completely sequenced and well developed tools for genetic analysis and manipulation in this organism are available. However, with the exception of few reports concerning the structure of some particular chromatin regions, e.g. centromeres (Heus *et al.,* 1993), the cryptic α-locus (Astrom *et al*., 2000) and the *K. lactis* nucleosome repeat length (Heus *et al*., 1993), the global organization of chromatin in *K. lactis* remains largely unknown.

Exploiting the yeast *K. lactis* as a model system we aim to shed more light on the role of linker histones as epigenetic factors, their contribution to the organization and maintenance of the higher-order chromatin structure and gene expression.

Materials and Methods

Strains, media, culture conditions and growth tests

K. lactis strains CBS2359, *MATa metA1-1*; PM6-7A, *MATa ade-T600 uraA1-1*; JA6, *MATα ade1-600 adeT-600 trp1-11 ura3-12*, and 2-19B, *MATα argA lysA uraA1*; *Saccharomyces cerevisiae* strain ABR, *MATa/α ade2 arg(RV) leu2-3,112 trp1-289 ura2-52*. Yeast cell cultures were grown in standard rich medium YPD (1% Yeast extract, 2% Peptone, 2% dextrose) at 30°C. Yeast cell cultures density (OD_{600}) was determined spectrophotometrically at 600 nm.

Molecular Biology techniques

Agarose gel electrophoresis of nucleic acids was performed in general as described in Sambrook *et al*. (1989). Analysis of total RNA was performed on 1% agarose gels. Depending on the size of DNA fragments we used gels with 0.8% -1.3% agarose in 0.5 x TBE.

PCR and Reverse transcription (RT) PCR. Total RNA was extracted from yeast cells using hot acidic phenol and then was treated with RNase-free DNase I to remove trace amounts of genomic DNA. For RT-PCR, RevertAid™ H Minus kit (Fermentas) was used according to manufacture's recommendations. DNaseI, Taq DNA polymerase and DNA ladders (Fermentas) were used according to the provided instructions. Oligonucleotides used for PCR analyses of *KlH1* gene and reverse transcription were **F1**, 5'-ATGGCTGCCAAAGTTGTTAAGAC-3'; **R1**, 5'-TTATTTCTTGGACTTCTTAACGGC-3'; **F2**, 5'-ACTTCGACACCCAATTCAACCT-3'; **R2**, 5'-CACCAACTGGGTATTTTTCCTTG-3'. Primers used for PCR analysis of *KlACT1* were **F**, 5'-ACGTTGTTCCAATCTACGCC-3' and **R**, 5'-CGGACGATTTCTCTTTCAGC-3'.

Yeast Comet Assay (YCA) was carried out in general as described previously (Peycheva *et al.*, 2009). Chromatin Yeast Comet Assay (ChYCA) was performed according to Georgieva *et al*. (2008) with 5 units of DNaseI for chromatin digestion. After staining with SYBR Green I (Molecular Probes) yeast comets were observed under epi-fluorescent microscope (Leitz). Parameters for comets scoring were: the percentage of comets, the length of comets' tails and percentage of DNA in the tail.

***In silico* analyses.** To search for histone H1 homologue amongst sequenced genomes we used Homologene, a resource in NCBI and WU-BLAST2 application in SGD (*Saccharomyces* genome database).

Results and Discussion

Comparison of chromatin compaction in K. lactis and S. cerevisiae - sensitivity to MNase and DNaseI

Micrococcal nuclease (MNase) and deoxyribonuclease I (DNaseI) are endonucleases routinely applied for chromatin studies. Difference in nucleosome ladders of *K. lactis* and *S. cerevisiae* after digestion of chromatin with MNase was reported by Heus *et al*. (1993). According to these authors the nucleosome repeat length of *K. lactis* was estimated to be 16 bp longer than that of *S. cerevisiae* – 176 and 160 bp, respectively. Interestingly, the nucleosome repeat length of another yeast species *S. pombe* is only 156 bp. Obviously, the NRL of *K. lactis* is of comparable length to those of most of the vertebrate cells for which the average NRL values fall between 175 bp and 190 bp (Woodcock *et al*., 2006) and is not shorter as in other yeast species. Moreover, a robust linear relationship between H1 stoichiometry (H1 molecules per nucleosome) and NRL has been demonstrated, with values ranging from 0.14 H1/nucleosome, NRL 160 bp (in *S. cerevisiae* cells), through 0.83 H1/nucleosome, NRL 196 bp (wild-type mouse thymus) to 1.04 H1/nucleosome, NRL 201 bp (in rat glia) and 1.3 H1/nucleosome, NRL 212 bp (in chicken erythrocytes). If this relationship is true then the calculated 176 bp NRL for *K. lactis* suggests the possibility that *K. lactis* cells have a linker histone homologue.

In order to compare chromatin organization in *K. lactis* and *S. cerevisiae* we studied the general accessibility of chromatin to DNaseI performing Chromatin Yeast Comet Assay (ChYCA). ChYCA revealed differences in the comets produced by *K. lactis* and *S. cerevisiae* after DNaseI treatment (Fig. 1). Chromatin loops released by DNaseI in *K. lactis* cells had an average length of

188 kb while those of *S. cerevisiae* were 270 kb in average. These observations suggest distinctive types of organization of chromatin loops in the two yeast species. As mentioned above linker histones promote higher-order chromatin compaction and the length of the nucleosomal repeat correlates with the amount of H1 molecules per nucleosome. Therefore, observed differences in the nucleosome repeat length and the chromatin compaction in *K. lactis* and *S. cerevisiae* raise an interesting issue about the search for a linker histone homolog in *K. lactis*.

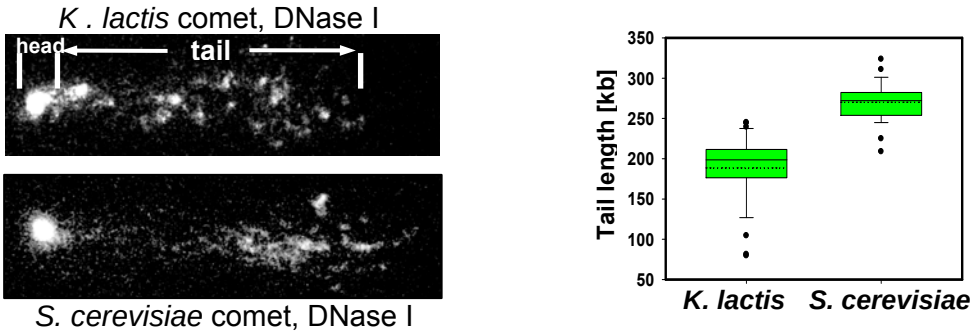

Figure 1. Sensitivity of *K. lactis* and *S. cerevisiae* chromatin to DNaseI assessed by Chromatin Yeast Comet Assay (ChYCA). Comparison uncovered differences in comet tail length and respectively in chromatin loops of *K. lactis* and *S. cerevisiae* cells.

In silico identification and analysis of histone H1 encoding gene in K. lactis genome

The nucleotide sequence of the *S. cerevisiae HHO1* (*YPL127C*) gene coding for linker histone was aligned against *K. lactis* genome. By the algorithms of WU-BLAST2 one sequence producing significant identity was identified in the *K. lactis* genome. This sequence - *KLLA0D06743g* we designated as *KlH1*. No other sequence showing homology to *ScHHO1* was detected in *K. lactis* genome. Thus, we concluded that there is a single ORF in *K. lactis* genome that could encode a histone H1 homologue.

The identified *KLLA0D06743g* open reading frame (ORF) consists of 873 bp and maps to *K. lactis* chromosome D from 581750 to 582586 bp. The predicted *KlH1* gene has an exon-intron structure unusual for H1 histone genes (Fig. 3). The mature transcript, mRNA, resulting from the splicing of the two exons should be 517 bp long and encodes a putative protein product of 171 amino acid residues.

KLLA0D06743g displayed significant homology to linker histone encoding genes of different eukaryotic organisms (Table 1).

Table 1. Identity of KlH1 with H1 histones.

Organism	gene	mRNA	protein
S. kluyveri	49.0	61.3	56.2
S. cerevisiae	37.2	50.4	41.9
K. thermotolerans	48.9	51.0	41.5
G. galus	42.0	49.2	43.0
H. sapiens	41.4	47.3	39.9

The putative KlH1p has a tri-domain structure typical for mammalian H1 histones with well defined N-terminal, globular and C-terminal domains (Fig. 2). Searching in conservative domains database (CDD, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) we identified a region of *Kl*H1p that showed 50% identity to the conservative domain H1/5 (cd00073) characteristic of the linker histone family. The identified region comprises amino acid residues from 27 to 98 of KlH1 polypeptide (Fig . 2).

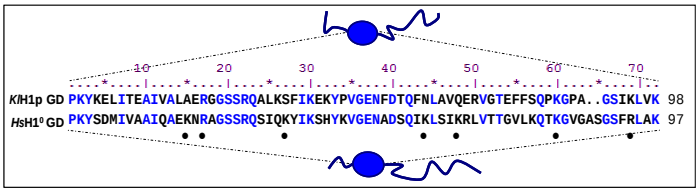

Figure 2. Conservatism of globular domains of hH1° and the predicted KlH1p. Identical amino acids in globular domains (GD) are in grey. Dots mark amino acid residues of S1 and S2 binding sites in H1° GD.

Reverse transcription PCR analysis of KlH1 gene expression

Interestingly, the *KlH1* sequence annotated in databases possesses a single nucleotide insertion at position 244 that could lead to a frameshift. This finding opens the questions whether the predicted *KlH1* is expressed, is it a true gene or a pseudogene?

To examine the transcription of *KlH1* gene we carried out two steps RT-PCR of total RNA isolated from *K. lactis* cells. Oligonucleotide R1 or R2 (see Materials and methods) were used in the first strand cDNA synthesis. For the subsequent PCR amplification primer pairs F1/R1, F1/R2 and F2/R2 were employed (Fig.3).

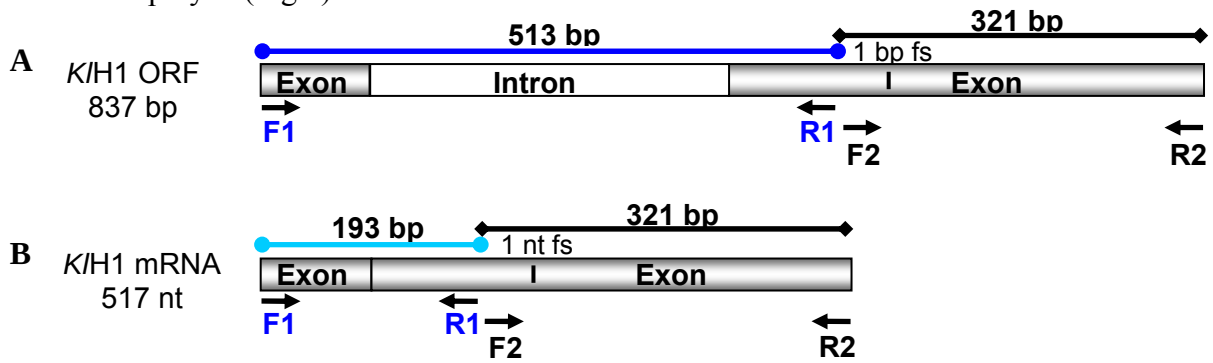

Figure 3. Scheme of *KlH1* ORF (**A**) and mRNA (**B**). The predicted *KlH1* gene has an exon-intron structure. Position of the primers used for RT-PCR analyses and the length of the expected PCR products (in base pairs, bp) is depicted. "1 bp fs" stands for a single nucleotide insertion that could lead to a frameshift.

In the presence of a spliced *KlH1* mRNA the expected amplified product using the primer pair F1/R1 should be 197 bp in length (Fig.3). As shown on Fig. 4, A, a 200 bp amplicon was revealed in three of *K. lactis* strains examined.

If a full KlH1 mRNA is present in the cell the product amplified with the primer pair F1/R2 would be 517 bp long (Fig.3). No band of the expected size (approximately 500 bp) was observed when total RNA from *K. lactis* was analyzed by RT-PCR (Fig 4, B)**.**

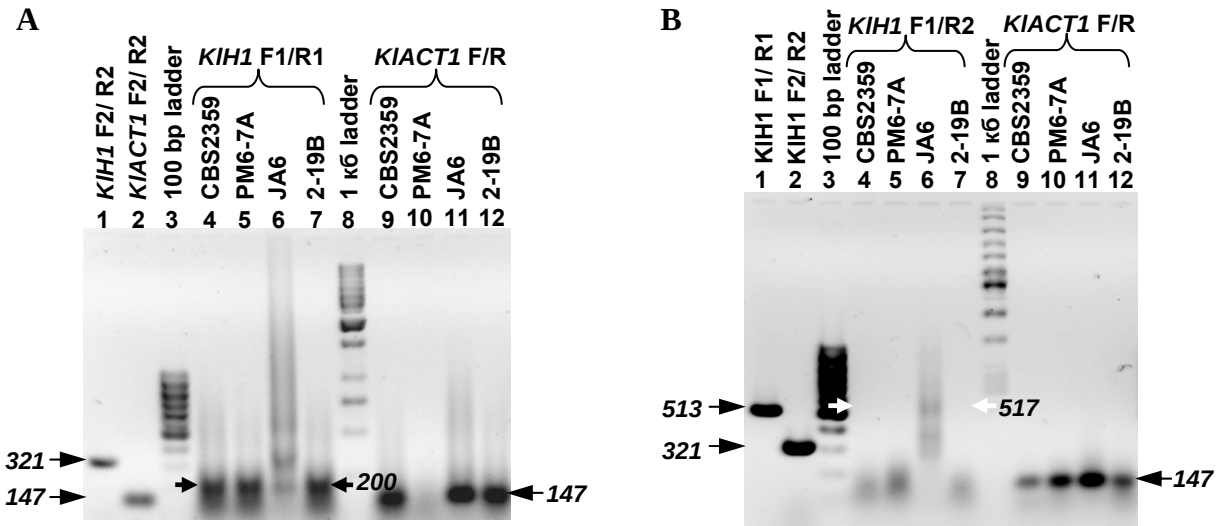

Figure 4. Analyses of *KlH1* gene expression. Total RNA isolated from *K. lactis* strains CBS2359, PM6-7A, JA6 and 2-19B was analyzed by two-steps RT-PCR using *KlH1*F1/R1 (**A, lanes 4-7**) or *KlH1*F1/R2 (**B, lanes 4-7**) primer pairs. The 147 bp of the *KlACT1* transcript amplified with oligonucleotides *KlACT1*F/R (**A**, lanes 9-12 and **B**, lanes 9-12) was used as a control. Arrows point the position and length of the expected and obtained PCR products (in base pairs). 100 bp (**A** and **B**, lane 3) and 1 kb (**A** and **B**, lane 8) DNA ladders were run for bands size determination.

According to the results of RT-PCR analyses it could be assumed that *K. lactis* cells do not contain an entire KlH1 mRNA transcript but only a shortened one. This suggests that the predicted *KlH1* is not fully transcribed. The absence of functional *KlH1* in the wild type strain needs additional experimental evidence. If being proved, *K. lactis* will be the first evidence for an eukaryotic organism in which the histone H1 encoding gene had been pseudogenized.

Conclusions

The *K. lactis* genome contains only one ORF showing significant homology to the linker histone encoding gene, *HHO1,* of *S. cerevisiae.* The predicted KlH1 showed considerable similarity at nucleotide (in average 48%) and amino acid level (40%) to linker histones from different eukaryotes.

Based on the results from RT-PCR analyses we concluded that the predicted histone H1 encoding gene is not transcribed in full length in *K. lactis* cells.

K. lactis is a promising organism to be developed as a model system for studying linker histones. Wild type as well as the constructed *KlH1* knock-out strain could be exploited as a "clean room" for investigating the individual role of mammalian linker histone subtypes in chromatin organization and functions.

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