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Effect of Transcription Terminators on Expression of Human Lipocortin-1 in Recombinant *Saccharomyces cerevisiae*

KIM, BYUNG MOON, SOO WAN NAM, BONG HYUN CHUNG*, YOUNG HOON PARK, AND SANG-KI RHEE

Genetic Engineering Research Institute, KIST, P.O. Box 115, Yusong, Taejon 305-600, Korea

The vector systems for the expression and secretion of human lipocortin-1 (LC1) from *Saccharomyces cerevisiae* were constructed with *GAL10* promoter and the prepro leader sequence of mating factor-α1. They were further constructed to contain three different transcription terminators; *GAL7* terminator, LC1 terminator and a fused form of these two terminators. The expression and secretion levels of LC1 were compared to investigate the effect of transcription terminators on the LC1 gene expression. For the expression cassettes employing the *GAL7* terminator or the terminator of fused form, the expression levels of LC1 were measured by scanning the immunoreactive LC1 protein bands, and were found to be 0.27 g/l and 0.32 g/l, respectively. The highest expression level of 0.54 g/l was obtained with the expression vector containing the LC1 transcription terminator. In all expression cassettes, the majority of LC1 proteins expressed were retained intracellularly, indicating a low secretion efficiency of about 5%. The high expression level of LC1 was explained by the great content and stability of LC1 mRNA transcribed from the LC1 terminator-employing vector. The results of this study demonstrate that the LC1 transcription terminator functions for the expression of LC1 in *S. cerevisiae* better than the *GAL7* terminator.

The yeast *Saccharomyces cerevisiae* has been widely used as a host for the expression of heterologous proteins because of the easy handling, availability of strong controllable promoters and lack of pathogenicity. Among the controllable promoters used in yeast, the galactose-inducible promoters such as *GAL1*, *GAL7* and *GAL10* have been more frequently used to construct a variety of expression vector systems for the production of heterologous proteins. In general, a cassette for the expression and secretion of protein in yeast consists of "promoter - signal sequence - heterologous gene - terminator". On constructing the expression vector, one should consider a number of factors such as plasmid stability, plasmid copy number, accuracy of transcription and translation, codon usage, mRNA stability, and translational efficiency. The terminator is known to play an important role to enhance the expression level. Deletion of 3′ termination sequence from the *CYC1* gene resulted in a longer mRNA and a dramatic decrease in mRNA level (41). Therefore, a suitable terminator sequence should be chosen and inserted in the downstream of heterologous DNA sequence to enhance the expression level by the efficient formation of mRNA 3′ end.

A number of expression cassettes have been constructed to produce both homologous and heterologous proteins in *S. cerevisiae* with a variety of yeast gene terminators such as *PGK* (4), *TRP1* (4), *TRP5* (26), *URA3* (28), *ADH1* (34), *ADH2* (8), *SUC2* (13), *GAL7* (1), *GAL10* (18), *GAP* (31), *ARG3* (7), *CYC1* (43), *TPI* (37), 2μm *FLP* (19,22) and mating factor-α1 (4, 5, 27, 36). In some cases, the expression level of heterologous gene was increased by using certain terminators (1, 18, 41). Transcriptional terminators of yeast mRNAs are poorly understood compared to those of bacteria and higher eukaryotes. Bacterial transcription terminates at the 3′ end of mature mRNA. In higher eukaryotes, the mRNA 3′ end formation involves the cleavage and polyadenylation of precursor mRNAs that extend several hundreds of nucleotides beyond the coding region. Contrary to earlier ideas, it appears that the yeast mRNAs follow the same pattern of termination, processing and polyadenylation of pre-mRNA as those in higher eukaryotes. However, in yeast these processes are closely coupled and occur within a short distance near the 3′ end of the gene (6).

Lipocortin-1 (LC1) has been implicated as a glucocorticoid-induced, Ca^{++}-dependent and membrane-bi-

*Corresponding author
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nding protein which mediates the anti-inflammatory function through phospholipase A_2 (PLA_2) inhibition (40).

A cDNA coding for human LC1 has been cloned and expressed in *Escherichia coli* (20,39) and *S. cerevisiae* (29).

In this work, we constructed three different LC1 expression vectors with *GAL7* terminator, LC1 terminator and a hybrid form of these two terminators, respectively. The effect of transcription terminators on the expression of LC1 was investigated by cultivating each yeast transformants and by comparing the expression levels of LC1. Furthermore, the content of LC1 mRNA was measured to elucidate the detailed effect of terminator on the transcription level.

MATERIALS AND METHODS

Yeast Strain and Culture Conditions

Saccharomyces cerevisiae SEY2102 (*MATα ura3-52 leu2-3,-112 his4-519 suc2-△9*) was used as the host cell for the gene expression and secretion of LC1 in this study (12). A YPD medium (1% yeast extract, 2% Bacto-peptone and 2% glucose) was used for the cultivation of host and yeast transformants. A YNBDLH medium (0.67% yeast nitrogen base without amino acids, 2% glucose, 0.003% leucine and 0.002% histidine) was used for the selection of yeast transformants and for the seed culture. For the induction of LC1 gene, the yeast transformants were grown on a YPDG (1% yeast extract, 2% Bacto-peptone, 0.4% glucose and 2% galactose) medium at 30°C.

Recombinant DNA Techniques

Restriction endonucleases, T4 DNA ligase, Taq DNA polymerase, dNTP's, and a DIG DNA Labeling and Detection Kit were obtained from Boehringer Mannheim. Nitrocellulose membrane, nylon membrane, and a prestained protein marker were from BIO-RAD. *Escherichia coli* JM109 was used for the construction and amplification of the plasmids. General DNA manipulation was performed as described by Sambrook *et al.* (33). DNA fragments required for the plasmid construction were purified from an agarose gel using GENECLEAN and MERMAID kit (Bio101).

Construction of LC1 Expression Vectors

Plasmids pLCI-S and YEGα-HIR525 have been previously described (20, 9). Plasmid YEp352 (17) was used for the backbone of the general yeast expression and secretion vectors. For the secretion of LC1 protein into the culture medium, the prepro leader sequence (ppL; 85 amino acids) of mating factor-α1 (MFα1) was connected between the *GAL10* promoter and the LC1 cDNA (9). The ATG start codon of LC1 was connected next to ^{84}Lys-^{85}Arg codon (AAA AGG) of ppL, which is the

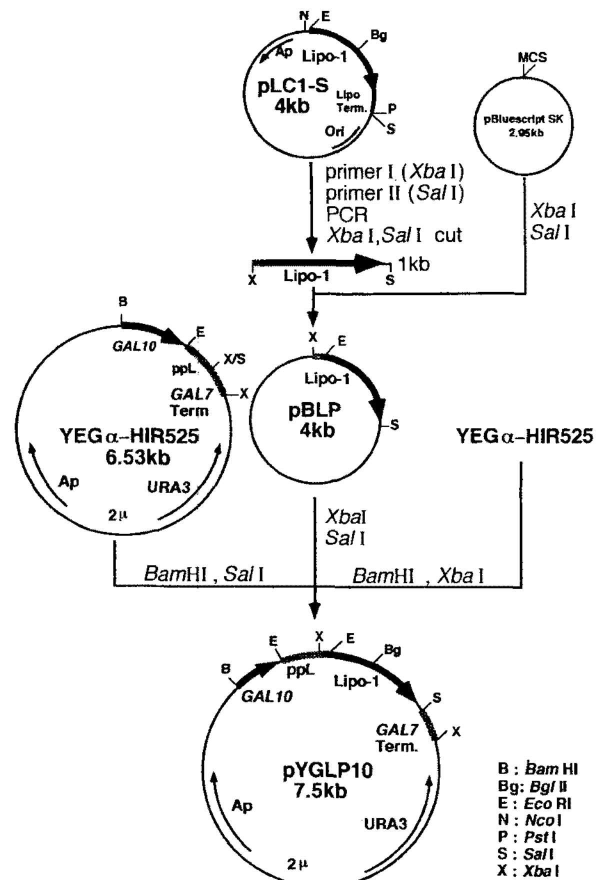


Fig. 1. Construction scheme for the human lipocortin-1 expression vector pYGLP10.
GAL10, *GAL10* promoter; ppL, prepro leader sequence of mating factor-α; Lipo-1, lipocortin-1; Lipo Term., lipocortin-1 transcription terminator; GAL7 Term., *GAL7* transcription terminator.

cleavage site of the yeast *KEX2* gene product (23).

Fig.1 and 2 show the construction schemes of expression vectors used in this work. The full length of LC1 gene was amplified by PCR, using primer I (5'-CGC CGT CTA GAT AAA AGG ATG GCA ATG GTA TCA G-3'), primer II (5'-TGT CGA CCA TCA AGG GAA TGT TTA GTT TCC TCC ACA-3'), and pLC1-S as the template. The resulting 1050 bp fragment containing LC1 cDNA has unique *Xba*I and *Sal*I sites at 5' and 3' ends, respectively, and it subcloned into pBluescript SK vector (Stratagene Co.). The resulting plasmid, pBLP, was digested with *Xba*I/*Sal*I, and then ligated with *Bam*HI/*Sal*I and *Bam*HI/*Xba*I fragments of pYEGα-HIR525 plasmid. The plasmid obtained, pYGLP10, was further digested with *Bam*HI/*Sal*I, and then ligated with *Bam*HI/*Sal*I fragment of YEp352. The pYGLPT plasmid was constructed so as not to contain any terminator sequences. The pYGLP10 plasmid was further digested with *Bgl*II/*Sal*I, and ligated with *Bgl*II/*Sal*I fragment of pLC1-S. As a consequence, both the LC1 and *GAL7* terminators were co-

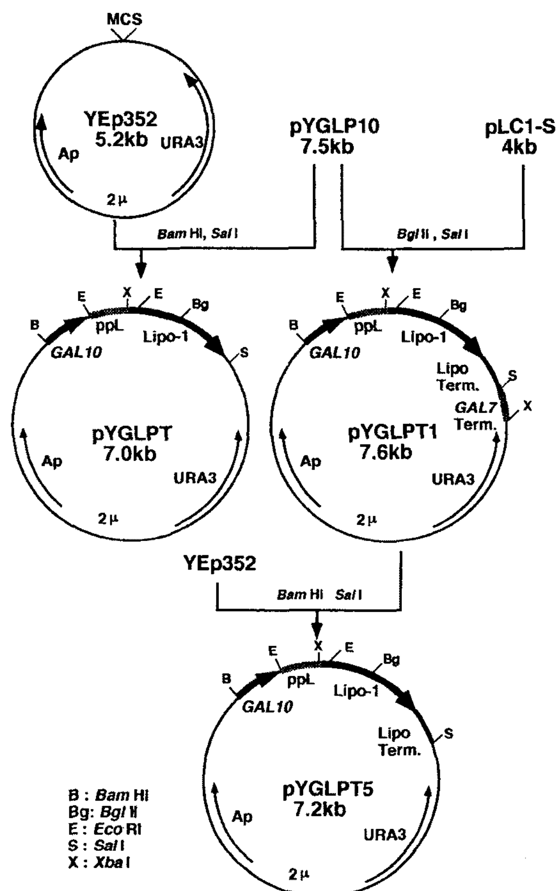


Fig. 2. Construction scheme for the human lipocortin-1 expression vectors pYGLPT, pYGLPT1, and pYGLPT5.

ntained in the plasmid pYGLPT1. The pYGLPT1 plasmid was treated with *Bam*HI/*Sal*I, and ligated with YEp352 which had been previously digested with *Bam*HI/*Sal*I. The resulting pYGLPT5 plasmid contained the LC1 terminator. *S. cerevisiae* SEY2102 was transformed with the above LC1 expression plasmids by the lithium-acetate method (21).

SDS-PAGE and Western Blotting

After centrifugation of 1 m*l* culture broth, the culture supernatant was precipitated by the addition of 100 μl of 100% trichloroacetic acid and 100 μl of 5% deoxycholate, and the precipitates (ppts) were collected after centrifugation at 15,000 rpm for 5min. After washing the ppts with cold acetone, ppts were suspended in 50 μl of lysis buffer (25), and were boiled for 5 min. An amount equivalent to 100 μl culture medium was subjected to 10% SDS-PAGE.

For the analysis of the cellular proteins, yeast cell pellets from 1 m*l* culture broth were suspended in 1 m*l* of cell breakage buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl of pH 8.0 and 1 mM EDTA) and broken by vortexing with glass beads (0.4~0.5 mm) for 5 min. After the addition of 330 μl 4X lysis buffer, 5 μl was loaded on a 10% SDS-PAGE. Extracellular and cellular proteins, separated by SDS-PAGE, were transferred to a nitrocellulose membrane by electroblotting, and immunoblotted with a rabbit anti-LC1-polyclonal antibody (20) and with the Promega ProtoBlot System (goat anti-rabbit IgG (H+L or Fc)-alkaline phosphatase conjugated [GAR-AP]). The amount of LC1 was quantified by measuring the staining intensity of recombinant LC1 on a nitrocellulose filter with a densitometer (Biomed Instrument SCR 2D/1D, USA), using the purified LC1 as a standard.

To investigate the localization of LC1 protein within the yeast cell, the yeast cells were converted to spheroplasts by treating them with 1 μg/μl Zymolyase 100T (Seikagaku Kogyo, Japan) for 30 min at 30°C. After centrifugation at 3,000 rpm for 5 min, the supernatant containing the cell wall fraction, periplasmic fraction, was separately collected. The residual cell pellets, spheroplasts, were lysed in a 50 mM Tris-HCl buffer solution (pH 7.5) containing 10 mM EDTA for 15 min on ice. The soluble fraction of cytoplasm was obtained in the supernatant after centrifugation at 13,000 rpm at 4°C for 10 min. The periplasmic and cytoplasmic fractions were analyzed by SDS-PAGE and Western blotting.

Northern Blotting Analysis

Yeast transformants were grown on a 200 m*l* YPDG medium and harvested when the cell density (OD_{600}) reached 10. About 6 mg of total RNA was isolated from 2 g of wet cells by the hot phenol method (24). 100μg of total RNA was subjected to 1% agarose gel or 1% formaldehyde agarose gel (3.5% formaldehyde, 20 mM MOPS pH 7.0, 5 mM sodium acetate and 1 mM EDTA) electrophoresis and transferred to nylon membrane. The membrane was illuminated under UV for 15 seconds and hybridized with an LC1 DNA probe. The LC1 DNA probe (1,050 bp) was obtained by *Eco*RI/*Sal*I digestion of pBLP plasmid. Hybridization, DIG-labeling and detection were nonradioactively performed by the *In situ* Hybridization System (Boehringer Mannheim).

RESULTS AND DISCUSSION

LC1 Expression Vectors with Various Terminators

Fig. 3 shows the schematic of LC1 expression vectors constructed in this study. The cleavage site of *KEX2* protease (23), Lys-Arg, was artificially created in the junction between ppL sequence and LC1 cDNA, directing the full length LC1 to be expressed. The terminator sequences of *GAL7* and LC1 are shown in Fig. 4A and Fig. 4B, respectively, in which the poly A signal (AATAAA) is underlined. *S. cerevisiae* SEY2102 was transformed with four different expression plasmids, and the resulting

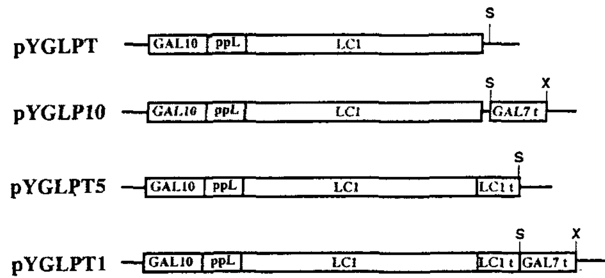


Fig. 3. Schematic diagram of the human lipocortin-1 expression vectors pYGLPT, pYGLP10, pYGLPT1, and pYGLPT5.
GAL10, *GAL10* promoter; ppL, prepro leader sequence of mating factor-α; LC1, lipocortin-1cDNA; GAL7 t, *GAL7* transcription terminator; LC1 t, lipocortin-1 transcription terminator; S, *Sal*I; X, *Xba*I.

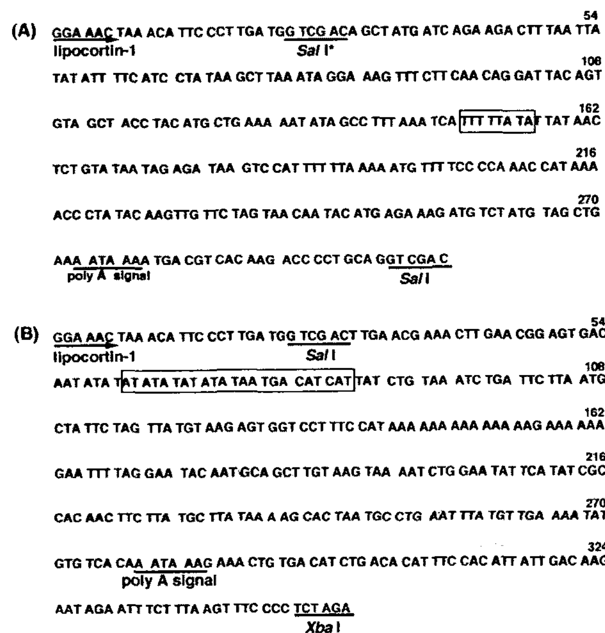
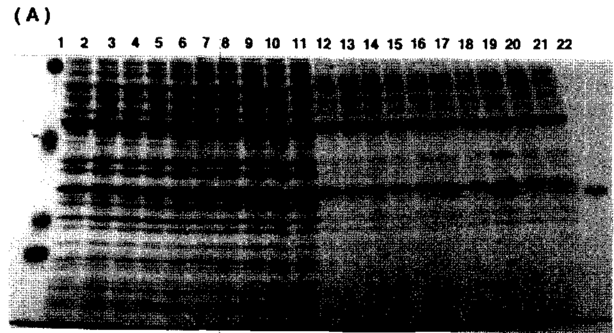


Fig. 4. Nucleotide sequences for the 3′-region of lipocortin-1 gene (A) and *GAL7* gene (B).
The boxed region in (A) is an element of transcription terminator (TTTTTATA) for lipocortin-1. The boxed region in (B) is an element of transcription terminator (AT-rich region) for *GAL7*. The original sequence GTCTCA in the 3′-noncoding region of lipocortin-1 was changed to GTCGAC (*Sal*I*) sequence by the site-directed mutagenesis.

transformed cells were designated as YGLP10, YGLPT, YGLPT1 and YGLPT5, respectively.

Expression and Secretion of LC1

The host and transformant cells (YGLPT, YGLP10, YGLPT1 and YGLPT5) were grown in YPD and YPDG media at 30°C for 72 hr. Recombinant LC1 protein secreted into the culture medium was analyzed by SDS-PAGE (Fig. 5A). In both media, no LC1 protein bands were detected in the culture medium of host and YGLPT cells (lanes 12, 13, 14 and 15 in Fig. 5A). In the repressed medium (YPD), the LC1 protein was not observed in the extracellular culture medium of YGLP10, YGLPT1 and



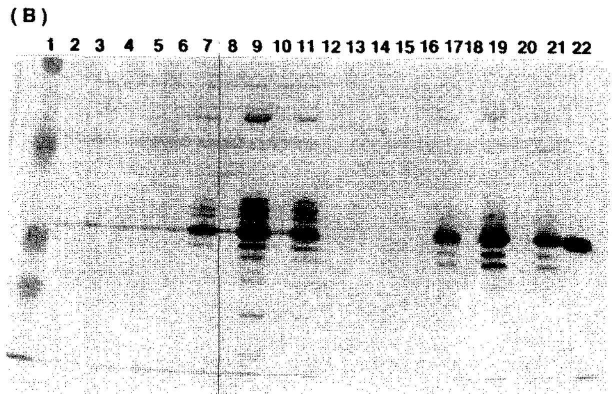


Fig. 5. SDS-PAGE (A) and Western blotting (B) analysis for cellular (lane 2-11) and extracellular (lane 12-21) proteins of yeast transformants grown on YPD (lanes with even number) and YPDG (lanes with odd number) media.
lane 1; protein molecular weight marker (80, 50, 33, 27 kDa), lane 2, 3, 12, 13; host cell, lane 4, 5, 14, 15; pYGLPT-containing cell, lane 6, 7, 16, 17; pYGLP10-containing cell, lane 8, 9, 18, 19; pYGLPT5-containing cell, lane 10, 11, 20, 21; pYGLPT1-containing cell, lane 22; purified LC1 (5 μg).

YGLPT5 cells (lanes 16, 18 and 20 in Fig. 5A). However, the extracellular LC1 of 37 kDa was clearly observed in the culture medium of YGLP10, YGLPT1 and YGLPT5 cells grown on a YPDG medium (lanes 17, 19 and 21 in Fig. 5A). The recombinant LC1 in the cellular fraction was not clearly detected by the Coomassie blue staining of SDS-PAGE (lanes 2-11 in Fig. 5A). Therefore, the extracellular and cellular proteins were further analyzed by Western blotting with 10% SDS-PAGE (Fig. 5B). As shown in Fig. 5B, the immunoreactive bands with the anti-LC1 polyclonal antibody were clearly detected in both the extracellular (lanes 17, 19 and 21) and cellular (lanes 7, 9 and 11) fractions of YGLP10, YGLPT5, and YGLPT1 cells grown on the YPDG medium.

The lower molecular weight (MW) bands of 30~35 kDa appear to be the partial degradation products of LC1 by yeast proteases during the secretion. The higher MW bands of 40~50 kDa might be glycosylated and/or unprocessed LC1. Since the LC1 protein treated with Endo H gave mobility identical to that of the untreated

Table 1. Expression and secretion levels of lipocortin-1 in recombinant yeast strains. The recombinant *S. cerevisiae* SEY2102 cells containing the plasmid were cultivated on YPDG media for 72 hr at 30°C.

Plasmids	Lipocortin-1 (mg/L)		Secretion
	Medium	Whole Cell	Efficiency (%)
pYGLPT	-	-	-
pYGLP10	10	260	3.7
pYGLPT1	15	300	4.8
pYGLPT5	40	500	7.4

LC1 in Western blotting, it appeared that N-linked glycosylation did not occur in the LC1 protein (data not shown). The MW of α-factor leader sequence (ppL) predicted from the nucleotide sequence is about 10,000 Da. Therefore, the larger LC1 shown in Western blotting may be the precursor of LC1, which is the fused form of α-factor ppL and LC1. An immunoreactive protein of 85 kDa was observed in the cellular fractions of YGLP10, YGLPT5, and YGLPT1 cells (Fig. 5B). The protein is not yet identified but it is assumed to be an LC1 dimer.

The amounts of recombinant LC1 secreted into the culture media were about 10 mg/*l*, 15 mg/*l* and 40 mg/*l* for YGLP10, YGLPT1 and YGLPT5 cells, respectively (Table 1). The total amounts of cellular LC1 expressed were found to be about 0.26 g/*l* (YGLP10), 0.3 g/*l* (YGLPT1) and 0.5 g/*l* (YGLPT5) when the peak intensities of immunoreactive LC1 bands were scanned with a densitometer. Therefore, the secretion efficiency of LC1 was estimated to be about 4~7%. After many experiments with various hosts and culture media, the highest expression and secretion level of LC1 was obtained with the expression vector pYGLPT5 employing the LC1 terminator (detailed data not shown).

Localization of Recombinant LC1

To further investigate the localization of unprocessed LC1, the cellular fraction of YGLP10 was divided into periplasmic and cytoplasmic fractions, and analyzed by immunoblotting. As shown in Fig. 6, most LC1s of above 40 kDa were localized in the cellular fraction (spheroplast), while LC1 of 37 kDa was found in the periplasmic fraction (zymolyase-released supernatant). From the scanning of immunoreactive LC1 bands, about 70% of LC1 in the cellular fraction was retained in the spheroplast. The recombinant LC1 protein in the spheroplast, periplasmic space and culture medium accounted for 67%, 29% and 4% of the total LC1 expressed, respectively.

According to Zsebo et al. (44), small proteins such as β-endorphin (31 amino acids) and calcitonin (32 amino acids) were completely secreted into the culture medium with the ppL of MFα1, while 95% of the consensus interferon (166 amino acids) was retained in the cell

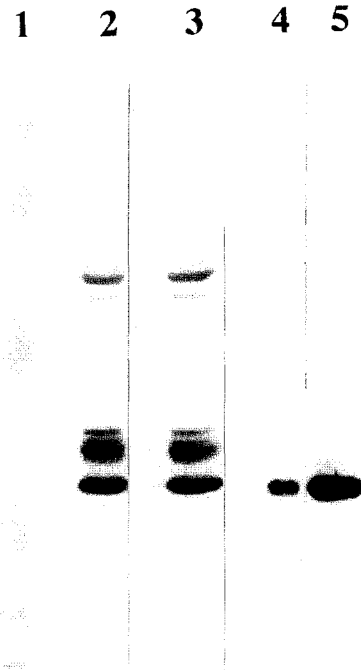


Fig. 6. Localization of lipocortin-1 expressed in pYGLP10-containing cell.
lane 1; protein molecular weight marker (106, 80, 50, 33, 27 kDa), lane 2; whole cell fraction, lane 3; cytoplasmic fraction, lane 4; periplasmic fraction, lane 5; purified LC1.

during the exponential phase as well as the stationary phase. In the expression of α-1-antitrypsin using the ppL of MFα1, the secretion efficiency was about 20% (27). In general, it has been reported that it is difficult to secrete the heterologous proteins of above 20 kDa into the yeast culture medium, and thus they mainly remained in the periplasmic space and/or yeast secretory pathway such as ER or Golgi apparatus (10, 35, 36). Our observation is consistent with the previous results, indicating that MFα1 leader sequence (ppL) is not so efficient for the secretion of LC1 (37 kDa).

Content and Stability of LC1 mRNA

It was of very interest that three different plasmids resulted in the marked differences in the expression and secretion levels of LC1, even though the plasmids had the same plasmid backbone except for the transcription terminator. To investigate whether these differences resulted from the transcription terminators or not, the transcription levels of LC1 gene from all expression vectors were analyzed. Total RNA was isolated from the transformed cells at the exponential growth phase in YPDG medium, and the mRNA levels of LC1 were analyzed. Total RNA of high purity showed two distinct bands (28S and 18S ribosomal RNA) co-migrating with DNA fragments of 1.0 and 0.7 kb, respectively. In addition, a smear of low molecular weight RNA co-migrating

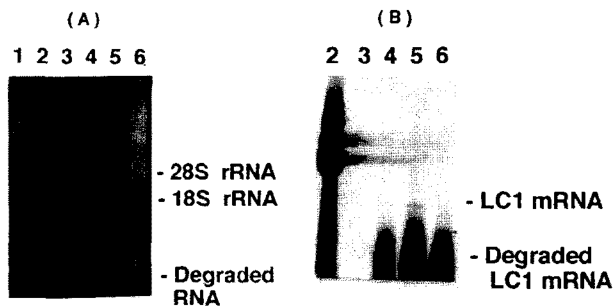


Fig. 7. Agarose gel electrophoresis (A) and northern blotting (B) analysis for LC1 mRNA.
lane 1; *Hind*III DNA marker, lane 2; pLC1-S plasmid DNA (0.1 μg), lane 3; mRNA from pYGLPT-containing cell, lane 4; mRNA from pYGLP1 0-containing cell, lane 5; mRNA from pYGLPT5-containing cell, lane 6; mRNA from pYGLPT1-containing cell.

with 50-200 bp DNA fragments was also observed (Fig. 7A). The amount of low MW RNA was much more than that of high MW RNA. The LC1 mRNA and its degraded mRNA were detected (Fig. 7B). YGLP10, YGLPT5 and YGLPT1 cells showed clear LC1 mRNA bands (lanes 4, 5 and 6 in Fig. 7B), while YGLPT cell did not display any detectable LC1 mRNA band (lane 3 in Fig. 7B). The patterns shown in LC1 mRNA levels were identical to those in LC1 expression level, i.e., increased transcription level resulted in an increase in LC1 expression.

A variety of sequences have been proposed to direct a 3'-end formation in yeast cells. One of these proposed terminator elements, TAG...(T-rich)...TA(T)GT...(AT-rich)... TTT (3, 42), was not found in the downstream sequence of LC1 cDNA. In cases of the *CYC1* and *GAL7* genes, the high AT-content (80%) is also known to be an element for the 3'-end formation (2, 30). This AT-richness was observed in the *GAL7* terminator region (Fig. 4B, open box), but not in the 3'-noncoding sequence of LC1 DNA. Another proposed terminator element is 5'-TTTTTATA (14, 15). This element was found in the 150 bp downstream of C-terminus of LC1 DNA (Fig. 4A, open box). The remainder of LC1 DNA contained no other sequences which were considered to play a role in the 3'-end formation. The highest content of LC1 transcript was observed for the pYGLPT5 vector employing the LC1 terminator (Fig. 7B, lane5), indicating that 5'-TTTTTATA element is the most effective for the specifying 3'-end formation of LC1 mRNA. However, further experiments should be carried out to elucidate in detail the significance of TTTTTATA sequence in the termination of transcription and polyadenylation of LC1 mRNA in yeast.

An adequate transcription termination is also an important factor in mRNA stability (41, 42). The potential importance of mRNA stability in a mechanism for regulating the gene expression has been recognized (32). However, the structure and mechanism involved in the determination of individual mRNA decay rates have not been elucidated so far. The parameters such as poly(A) length, mRNA size and codon bias can affect the mRNA stability, translational efficiency and post-translational processing (16, 32). Therefore, our results cannot rule out the possibility of rapid mRNA decay rate for the LC1 transcripts derived from pYGLP10 and pYGLPT1 plasmids.

Although it has been originally suggested that the presence of a yeast 3' termination sequence in the immediate vicinity of foreign gene is a prerequisite for the efficient expression of a gene (19), it is now clear that the heterologous 3' noncoding sequences often contain the effective transcriptional termination signals as in the cases of human interferon-α (38), human interferon-γ (11) and *Drodophila ADE8* genes (15). As in the above genes, the 3'-noncoding region of LC1 DNA functions well and is very efficient for the transcription termination (3'-end formation), resulting in the high expression level of LC1 in yeast *S. cerevisiae*.

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