Differential translational initiation of *lbp* mRNA is caused by a 5′ upstream open reading frame

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Abstract Expression of the luciferin-binding protein (LBP) from *Gonyaulax polyedra* is regulated by the circadian clock at the translational level. Here we report that in vitro translation of *lbp* mRNA results in the synthesis of two LBP variants of different sizes, which is shown to be due to translational initiation at different in-frame AUG codons on *lbp* mRNA. Differential initiation is caused by a small open reading frame (ORF, situated in the 5′ untranslated region of *lbp* mRNA), which gives rise to a leaky scanning mechanism. In *Gonyaulax*, only one of these variants, which is produced by initiation from the first AUG of the *lbp* ORF, exhibits a circadian rhythm and is far more abundant during night phase.

Key words: Circadian clock; Translational control; *Gonyaulax polyedra*

1. Introduction

Circadian rhythms occur widely from microorganisms to humans and regulate a variety of cellular processes such as conidiation in *Neurospora crassa*, eclosion in fruit flies and the human sleep–wake cycle [1–5]. They are defined as biological rhythms that persist under conditions of constant light and temperature with a period of about 24 h. *Gonyaulax polyedra* is a model unicellular organism for the study of circadian controlled cellular processes, such as photosynthesis, cell motility (aggregation), cell division and bioluminescence [6,7]. Bioluminescence has proved to be an especially favorable system for studying a circadian controlled process at the molecular level because the biochemical components of the reaction are known, well characterized and unambiguous.

The key components of the bioluminescence system in *Gonyaulax* are packaged in small (∼0.5 μm) organelles, called scintillons, which extend as cytoplasmic outpocketings into the vacuole [8]. These include the enzyme luciferase (LCF), its substrate luciferin and a luciferin-binding protein (LBP), which sequesters the substrate and thus prevents it from reacting with the enzyme [9,10]. The circadian expression of LBP is controlled at the translational level; the protein is synthesized at the beginning of the night and reaches its maximum cellular abundance in the middle of the night. However, the amount of its mRNA remains constant over the entire day-night cycle [11].

In general, initiation of translation is influenced by five aspects of mRNA structure: the (a) m7Gcap; (b) primary sequence or context surrounding the AUG codon; (c) location of the AUG codon, i.e. whether it is the first AUG in the message; (d) leader length; and (e) secondary structure both up- and downstream from the AUG codon [12]. In addition, the presence of upstream open reading frames (uORF) can effect translation of the downstream protein-coding sequences. uORFs are encountered in mRNAs encoding proteins involved in growth control of mammalian cells [12], and they occur at a strikingly high frequency in *Drosophila* mRNAs, where their significance has been little studied as yet.

Here we show that an uORF in the 5′ untranslated region of the *lbp* mRNA (5′ UTR) affects downstream initiation, resulting in the production of two major LBPs (LBP-A and -B). Interestingly, only one of the LBP forms (LBP-A) exhibits a dramatic circadian rhythm, being far more abundant (>10×) during the night phase.

2. Materials and methods

2.1. Preparation of plasmid constructs and in vitro mutagenesis

Cloning and sequencing of the *lbp* gene at both the genomic and cDNA levels has been published earlier [13]. It does not contain any introns. pMM10 was constructed by subcloning a *Aat*II–*Kpn*I fragment from LBP1.1 [11] into pDLA2 [13]. This fragment, which contains the entire *lbp* 3′ UTR and ≈40 bp from the poly(A) tail, replaced the *Aat*II–*Kpn*I fragment from pDLA2. pMM11 and pMM12 were made by in vitro mutagenesis of pMM10 applying the unique site elimination method of Deng and Nickoloff [14] along with the *Xmn*I primer for selection. The mutagenesis-primer was in one case a 39-mer, called *Pvu*II (5′-gtg agc ttc agc tga ttt gtc agg tac cca atc acc ttc-3′) resulting in pMM11 and in the other case a 37-mer, called *Hin*dIII (5′-gtg aat gcg gac aag caa gct tgc aac tcc aac acg c-3′) resulting in pMM12. The *Pvu*II primer introduces a *Pvu*II and *Kpn*I site into pMM10 at the third and fourth AUG of the *lbp* mRNA, respectively; it results in an amino acid change from Met into Leu in both cases. The *Hin*dIII primer introduces a *Hin*dIII site into pMM10 at the first AUG of the *lbp* mRNA. It eliminates the Met of the *lbp* uORF.

2.2. Preparation of RNA transcripts

The RNAs containing the entire *lbp* mRNA along with a part of its poly(A) tail were transcribed from the plasmids pMM10, pMM11 and pMM12, respectively. They were named M10, M11 and M12. The start site of the RNAs was determined by the T_7 promoter, and the ends were determined by digestion with *Sma*I, which cuts exactly after the poly(A). Capped transcripts were prepared by following the protocols of the supplier (mMessage mMachine Kit; Ambion).

2.3. In vitro translation

In vitro translation of the transcripts M10, M11 and M12 was

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carried out by using either a reticulocyte lysate or a wheat germ extract following the protocol of the suppliers (Ambion, BRL). The synthesized Met-^{35}S-labeled proteins were run on a 9% denaturing SDS-polyacrylamide gel [15], vacuum dried and autoradiographed. It should be noted that the Ambion and BRL reticulocyte lysates have been tested with appropriate Kozak constructs [[16], protocol of Ambion] and have been found to fulfill all the criteria to identify the appropriate AUG. In contrast, certain other commercial lysates do not discriminate efficiently between AUGs [16].

2.4. Isolation of the circadian LBP form (LBP-A)

LBP was isolated from *Gonyaulax* cells harvested in the middle of the night phase (LD 18) according to the method of Morse et al. [9] using fractionated ammonium sulfate precipitation and an anion exchange column. LBP-containing fractions from the anion exchange step were pooled together, ammonium precipitated and dialyzed against 10 mM Tris-HCl, 1 mM EDTA, 2 mM DTT (pH 8) on Millipore filters (VS; pore size 0.025 μm). The cysteines of the dialyzed protein were pyridylethylated, electrophoresed on 10% SDS-PAGE, electroblotted and subjected to laser scanning (see below).

2.5. Pyridylethylation of cysteines

LBP was reduced with 3% mercaptoethanol in 100 mM Tris-HCl (pH 8.5), 1 mM EDTA, and 6 M guanidine hydrochloride for 3 h at 50°C and then pyridylethylated with 3% of 4-vinyl pyridine (Sigma, Deisenhofen, Germany) in the same solution [17]. Afterwards proteins were desalted by reversed-phase HPLC.

2.6. Polyacrylamide electrophoresis and electroblotting

Samples were prepared and separated on SDS-containing polyacrylamide gels, essentially according to Laemmli [15]. The concentration of acrylamide was 10%, the acrylamide/*bis*-acrylamide ratio was 30:1. Acrylamide, *bis*-acrylamide and SDS were of electrophoresis grade (Bio-Rad, Munich). Immediately after electrophoresis separation the proteins were electroblotted onto a PVDF membrane (Immobilon PSQ, Millipore, Bredford, MA) as previously described [18]. Right after the electroblotting procedure the PVDF membrane was soaked in a saturated, aqueous succinic acid matrix solution for 15 min. The matrix-incubated membrane was allowed to dry afterwards [19].

2.7. Infra red-matrix assisted laser desorption/ionization mass spectroscopy (IR-MALDI-MS)

All mass spectra were obtained as previously described [19,20] using a home-built single-stage reflectron time-of-flight system. Ions were extracted with 12 kV acceleration potential under soft-extraction conditions (400 V/mm). The mass spectrometer was equipped with a post-acceleration detector using a 5–15 kV post-acceleration followed by a secondary electron multiplier (EMI 9643, Electron Tube Ltd.). An Er-YAG-laser (Spectrum GmbH, Berlin, Germany) at a wavelength of 2.94 μm and a pulse duration of 90 ns was employed in our investigations.

The detector signal was pre-amplified by a factor of 10 and digitized by a LeCroy 9400 (Chestnut Ridge, NY) at 10–80 ns time intervals depending on the mass range covered. The data were transferred to a PC which is used for mass spectra accumulation and further processing. Typically 10–20 single spectra were accumulated to increase the signal-to-noise ratio. Using Coomassie-stained lanes as a reference the non-stained matrix-incubated samples were cut with a razor blade and stuck onto the target by a double-sided adhesive, conductive tape. Spectra were calibrated with dot-blotted non-modified bovine serum albumin spotted onto the PVDF membrane.

3. Results and discussion

3.1. Translational initiation of lbp mRNA occurs from different in-frame AUGs

The first AUG of *lbp* mRNA ('1' in Fig. 1) is situated in the *lbp* 5′ UTR and is the start of a small uORF of only 87 nucleotides. Thus, the second AUG of the *lbp* mRNA ('2'), which codes for a protein with a molecular mass of 75 494 Da (the size of the major protein in extracts, which has been characterized in these studies as LBP-A), was considered to be the authentic start site of LBP [13]. It starts only 2 nt after the stop codon of the uORF, but contains both of the consensus nucleotides that ensure high fidelity of translational initiation. These consensus sequences are a purine (usually adenine) at position −3 (where the **A** of the **AUG** is considered +1) in most vertebrates (97% of 699 species) and plants (93% of 252 species), and a guanine residue at position +4 in 46% and 74% of vertebrates and plants, respectively [12]. The two other *Gonyaulax* mRNAs for which sequences are known [[21]; Li, L. and Hastings, J.W., unpublished] as well as one from *Cryptothecodinium cohnii* [22] are also in agreement. The third and fourth potential start sites on the *lbp* gene ('3' and '4'; 69 nt and 81 nt from '2', respectively), have neither of these consensus nucleotides, while the first start site ('1' in the 5′ UTR) has only one, and it is not the optimal one (Fig. 1).

In vitro translations of *lbp* mRNA were carried out in both reticulocyte lysate and wheat germ extract systems, using an in vitro transcribed capped RNA (M10-RNA) that contains the *lbp* 5′ UTR, the ORF, the 3′ UTR and about 40 nt of its poly(A) tail. Surprisingly, in vitro translations resulted in two major bands (Fig. 2), which migrate close together on a denaturing SDS-polyacrylamide gel, and are named LBP-A (∼75 kDa) and LBP-B (∼72 kDa).

It seemed possible that the third and/or fourth AUG of *lbp* mRNA might be used as translation start sites, since the predicted molecular weights of these proteins are 72 846 Da and 72 371 Da, respectively (Fig. 1). To check the validity of this hypothesis, we eliminated the third and fourth AUG of *lbp* mRNA by in vitro mutagenesis and performed in vitro translation with the altered *lbp* mRNA (M11-RNA) (Fig. 3A). We observed that the amount of LBP-B was greatly reduced (>90%). We conclude that LBP-B is produced primarily by initiation events at the third and/or fourth start site (AUG 3/4). The remaining LBP of this molecular weight (<10% of normal) might be due to a premature termination of translation of *lbp* mRNA or to proteolytic activity involving LBP-A.

3.2. uORF causes differential initiation on lbp mRNA

The existence of an ORF in the *lbp* 5′ UTR with its stop codon only two nucleotides before AUG 2 suggests a possible mechanism for bypassing AUG 2 and using AUG 3 and/or AUG 4 instead. In this mechanism, ribosomes that initiate at AUG 1 and terminate at the stop codon of this ORF would be unlikely to re-initiate at AUG 2 because of the short distance (i.e. intercistronic length) between the stop codon of the uORF and the start codon AUG 2 [23]. The ribosomes could thus resume scanning and some would initiate at AUG 3/4 despite the fact that neither of the two is in a good Kozak context.

To test this hypothesis, we eliminated AUG 1 by in vitro mutagenesis and translated the altered *lbp* mRNA (M12-RNA) in vitro. The amount of the smaller protein (LBP-B) was again reduced by >90% (Fig. 3B), suggesting that elimination of the uORF had indeed effectively eliminated initiation at both AUG 3 and AUG 4. In this case, all ribosomes initiate at AUG 2, which has a perfect Kozak context and is the first AUG on the altered M12-RNA.

3.3. The circadian regulated protein is initiated from AUG 2 of lbp mRNA

To determine whether the major circadian expressed LBP form in *Gonyaulax* is LBP-A or -B we isolated LBP from

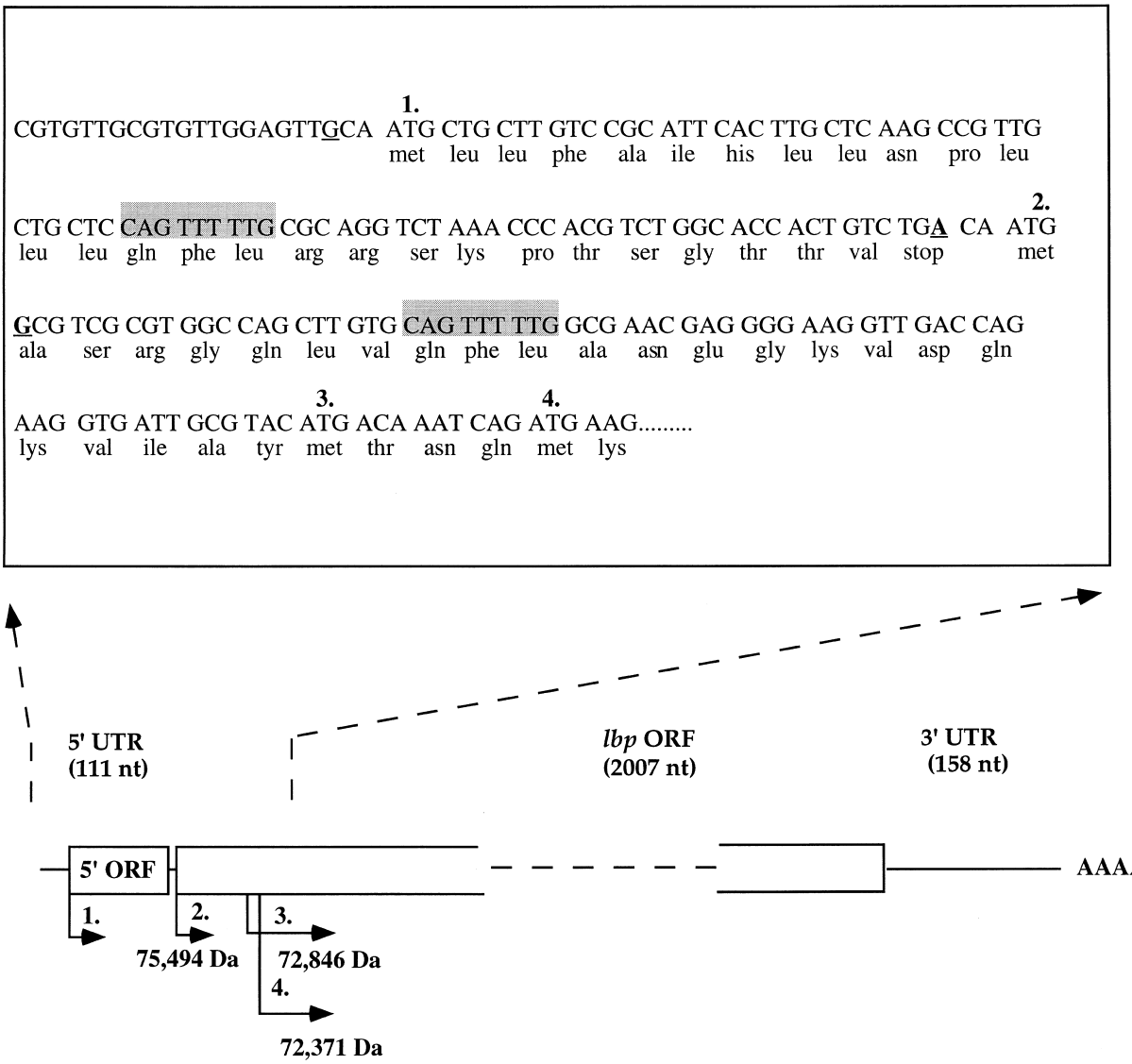

Fig. 1. Relevant features for translation initiation of *lbp* mRNA. The lower section shows a representation of the structure of *lbp* mRNA, whose sequence has been published earlier [13]. A small ORF of 87 nt is situated in the *lbp* 5′ UTR. Positions of the four AUGs (numbered 1–4) that may be relevant for the translational initiation of *lbp* mRNA are indicated, as well as the theoretical molecular weights of the resulting proteins. The upper section shows the nucleotide and amino acid sequences of the *lbp* 5′ end. Nucleotide positions (−3 and +4) around the AUGs that are relevant for translational initiation are underlined if they are favorable and in addition bold if they are highly favorable. The shadowed regions show repeat sequences that are situated at −39 and −40 in front of AUG 2 and AUG 3, respectively. It is unknown if these sequences are involved in the translational control of LBP.

Gonyaulax cells from the night phase (see Section 2), the time when the amount of circadian-expressed LBP is highest. A highly enriched LBP fraction was quantitatively alkylated by pyridylethylation of the 15 cysteines present in LBP, separated on a denaturing gel and transferred to a PVDF membrane. The mass of the alkylated LBP was obtained directly from the membrane by IR-MALDI-MS (see Section 2), and determined to be 77 100 ± 270 Da (Fig. 4). Quantitative alkylation of LBP was carried out since it is known that some of the cysteines present in a protein are modified non-quantitatively with β-mercaptoethanol [24] and that non-modified cysteines can interact with unreacted acrylamide in a gel forming cysteinyl-*S*-propionamide adducts [25]. In addition, it is known that the N-terminal of LBP is blocked, suggesting the presence of a formyl or acetyl group at the LBP N-terminus. Subtraction of the molecular mass of the alkyl groups from the 15 cysteines (15×10^5 Da) as well as of a potential formyl group (28 Da) or acyl group (42 Da) results in a molecular mass of 75 497 ± 270 Da or 75 483 ± 270 Da, which is in agreement with the calculated value of LBP-A of 75 494 Da. It can therefore be concluded that the circadian expressed LBP represents LBP-A and derives from translational initiation on AUG 2 of *lbp* mRNA.

3.4. *Differential expression of LBP-A and -B during the circadian cycle*

The results described above allow us to interpret more completely the anti-LBP Western blots of *Gonyaulax* proteins previously reported. These Western blots (Fig. 1 in Morse et al. [11]) show two bands migrating close together, the more

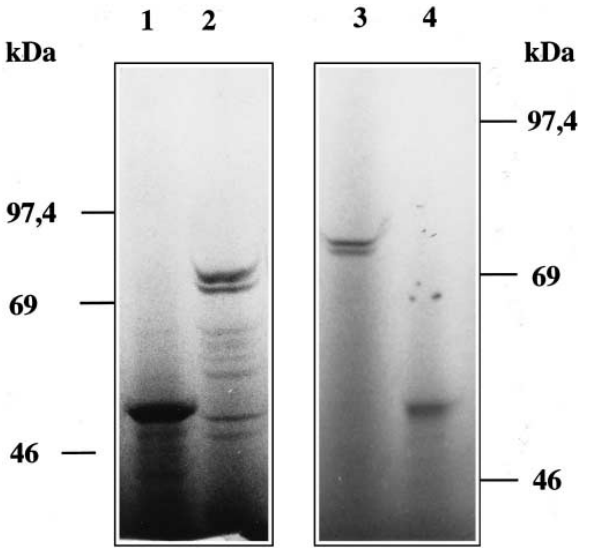

Fig. 2. In vitro translation of in vitro transcribed *lbp* mRNA (wild type) and an in vitro transcribed mRNA of the *Xenopus* elongation factor 1-alpha (XeF-1; positive control). In vitro translation was carried out using a reticulocyte lysate (lanes 1, 2) and a wheat germ extract (lanes 3, 4), respectively. The protein products were electrophoresed on NaDodSO4 polyacrylamide gel and autoradiographed. Lanes 1 and 4 show the control protein (XeF-1), which has a molecular weight of 50426 Da, and the others show the in vitro translated LBP (lanes 2, 3).

abundant corresponding to a molecular mass of ~75 kDa (erroneously estimated as 72 kDa in that paper). The lower and less intense band of a molecular weight of ~72 kDa was not discussed by Morse et al. [11], who considered that the ~75 kDa band represents the circadian controlled LBP because its amount increased dramatically at the beginning of the night phase, reached a maximum in the middle of the night and decreased again at the end of the night. On the basis of the data presented here, we now confirm the identity of the 75 kDa band as LBP-A and propose that the ~72 kDa band is LBP-B (derived by translation starting at AUG 3 and/or AUG 4). The protein constituting this band reacts in the Western blots with anti-LBP antibodies, and its molecular mass agrees with the value determined by sequence analysis for LBP-B. The presence of two LBP variants in vivo in *Gonyaulax* thus corresponds to the situation in vitro with both the reticulocyte lysate and the wheat germ system.

During the day phase the amounts of LBP-A and LBP-B present in cell extracts are equal (Fig. 1 in Morse et al., [11]). While LBP-A dramatically increases at the beginning of the night, LBP-B is either constant in its amount or may be less abundant during the night. This is difficult to ascertain precisely because of the large amount of the ~75 kDa component, which spills over into the 72 kDa region.

3.5. Translational regulation of lbp mRNA

Translational regulation occurs in many systems and can be mediated by a variety of mechanisms [26]. Here, we have shown that a uORF situated in the *lbp* 5′ UTR causes differential initiation by utilizing a downstream AUG. Quite recently it has been reported that mRNAs of the *Drosophila* clock gene *timeless* (*tim*) and the *Neurospora* clock gene *fre-*

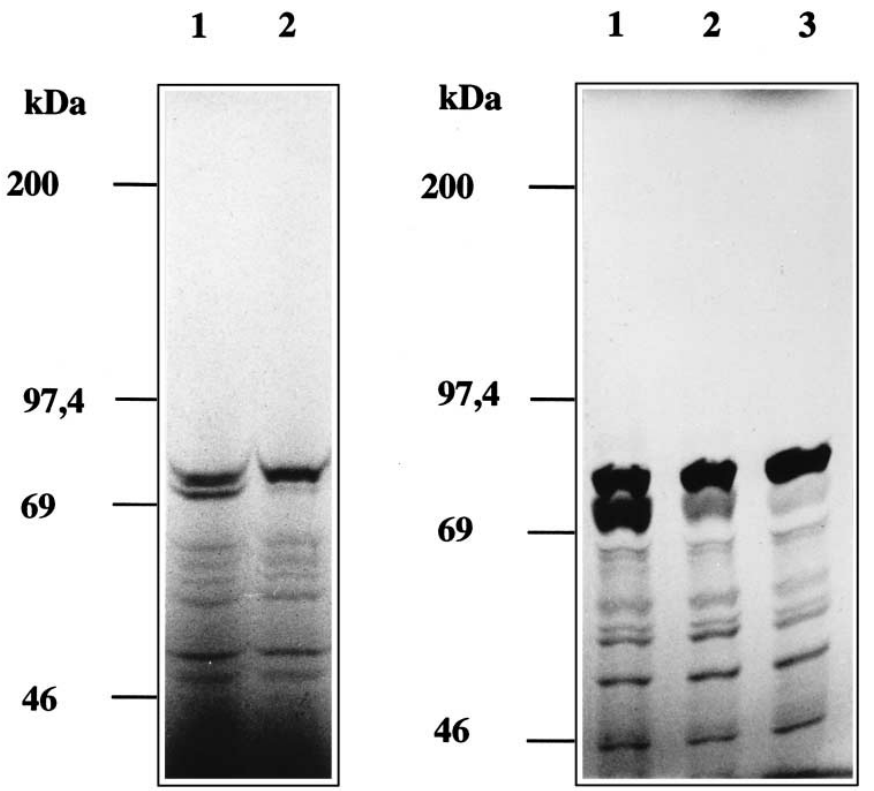

Fig. 3. In vitro translation using a reticulocyte lysate of in vitro transcribed *lbp* mRNA (wild type) and of altered *lbp* mRNAs with AUG 3/4 and AUG 1 eliminated, respectively. In vitro translation of wild-type *lbp* RNA serves as control and is always shown in the first lane. Lanes 2 show in vitro translations with altered *lbp* mRNA with AUG 3 and AUG 4 exchanged into CUGs (leu). B: Lane 3 depicts an in vitro translation with altered *lbp* mRNA with the first AUG being changed to AGC. This change eliminates the presence of the *lbp* uORF.

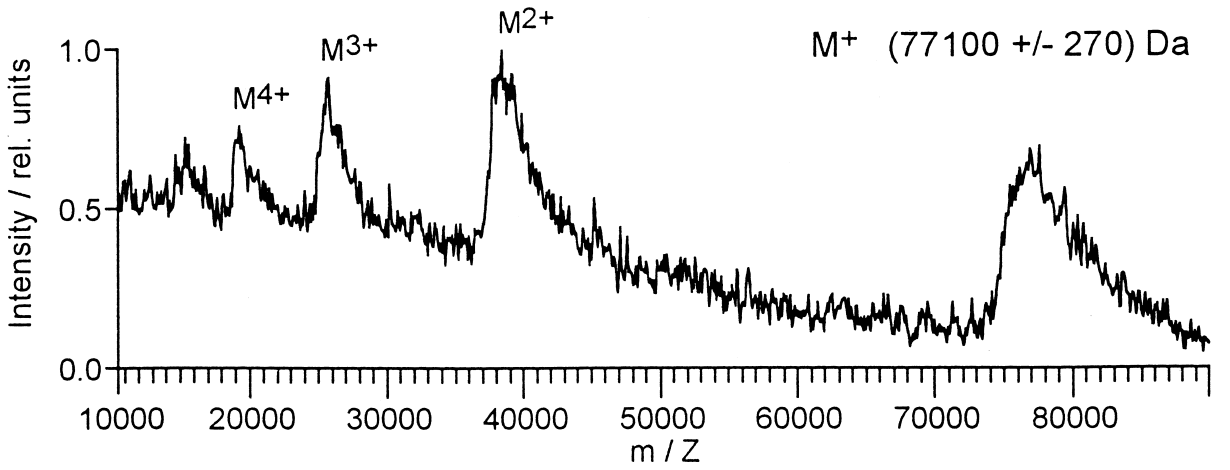

Fig. 4. IR-MALDI mass spectrum (sum of 15 single spectra; IR-laser wavelength: 2.94 μm, succinic acid matrix) from the circadian LBP-variant (LBP-A), which peaks during the night. Spectrum from LBP-A with quantitative alkylation (pyridylethylation of cysteines) prior to SDS-PAGE separation. Molecular mass determined is 77 100 ± 270 Da. Subtraction of the molecular mass of the alkyl groups from the 15 cysteines in LBP (15 × 105 Da) as well as of an potential formyl group (28 Da) or acyl (42 Da) at the LBP N-terminus (LBP is N-terminal blocked) results in a molecular mass of 75 497 ± 270 Da or 75 483 ± 270 Da, which fits nicely with the calculated molecular mass of LBP-A of 75 494 Da.

quency (*frq*) can also be initiated at a downstream AUG [27–29]. In *D. simulans*, *D. yakuba* and certain strains of *D. melanogaster* the first potential start site of the *tim* ORF seems not to be utilized. Instead a second start site, which is situated 23 codons downstream of *tim* ORF is used [27]. With *frq* mRNA in *N. crassa*, the first and third (100 codons downstream) AUGs of the *frq* ORF are utilized as translational start sites [28]. In addition, uORFs have been found in the 5′ UTRs of the mRNAs of both *tim* and *frq*. The *tim* 5′ UTR contains two uORFs and the *frq* 5′ UTR six uORFs, but in both cases it has not yet been determined if the uORFs are involved in the differential initiation process, as shown with *lbp* mRNA. However, with *frq* mRNA it has been shown that the use of the initiation sites differs depending on temperature [29]. At a lower temperature AUG 3 of the *frq* ORF is favored while at a higher temperature more FRQ is produced and AUG 1 is favored. It is not known whether temperature changes can also affect the use of initiation sites with *lbp* mRNA.

Recently, we have reported that translational control of LBP synthesis involves the 3′ untranslated region (3′ UTR) of *lbp* mRNA, where a short, 'UG'-containing region was shown to serve as a *cis*-element to which a *trans*-acting factor binds in a circadian manner [30,31]. The binding activity of this protein factor (named CCTR: circadian controlled translational regulator) is high during the day phase and decreases just at the beginning of the night, when the onset of LBP-A synthesis occurs, indicating that CCTR acts as a translational repressor. Repression of translation by a 3′ UTR-binding protein is known in several cases [32–34].

The finding that 3′ UTRs can control the decapping rate of mRNAs, in combination with their ability to influence the initiation of translation, suggests that 3′ UTRs act through a direct or indirect interaction with the 5′ ends of mRNAs [34]. Quite recently it has been suggested that mRNAs could be circularized in vivo [35]. These findings are of special interest in the case of *lbp* mRNA, in which both ends of the mRNA seem to be involved in its translational control. During the night phase large amounts of LBP-A are formed. To be compatible with our results, the CCTR would have to block access to AUG 1 during the night phase, and only then, so that AUG 2 with its favorable Kozak context is encountered first. While there is no precedent for such a mechanism, and no easy way to envision it on the basis of what we now know, no other cases of such complex circadian regulation of translation have been previously investigated.

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