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The 5′-untranslated region of the human muscle acylphosphatase mRNA has an inhibitory effect on protein expression

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Abstract The cDNA of the human muscle type acylphosphatase was isolated and characterized. The mRNA presents a very long 5′-untranslated region, covering the first half of the molecule: 175 bases of this part were cloned and prediction of the possible secondary structure showed that a very stable stem-loop structure could be formed in that region. Moreover, an additional AUG triplet was found upstream of the start codon of the protein, defining an open reading frame of 60 codons which overlapped that of acylphosphatase. The possible regulatory effect on translation of this part of the mRNA molecule was studied by means of transient transfection experiments: a 10-fold decrease in the expression of a reporter protein and a dramatic decrease in the corresponding mRNA was observed, due to the presence of the 5′-untranslated region of acylphosphatase mRNA. Mutagenesis of the upstream AUG triplet eliminated mRNA instability, leading to the hypothesis that the product of the upstream open reading frame could play a role in this mechanism.

Key words: Muscle acylphosphatase; 5′-Untranslated region

1. Introduction

Acylphosphatase (EC 3.6.1.7) is a small (11 kDa) cytosolic enzyme, widely distributed in animal tissues. Two structurally related isoenzymatic forms are known, called 'muscle type' (MT) and 'common type' (CT or 'erythrocytic') acylphosphatase respectively, showing about 60% identity in amino acid sequence. Both enzymes catalyze the in vitro hydrolysis of physiological acylphosphates such as 3-phosphoglyceroyl-phosphate, carbamoyl-phosphate and succinoyl-phosphate [1]. The structure of the MT isoform, determined with NMR techniques, is composed of two interleaved β-α-β packing units [2]. Acylphosphatase can hydrolyze the phosphoenzyme intermediate of different membrane pumps, particularly the Ca^{2+}-ATPase and Na^+/K^+-ATPase from red blood cell membrane, and the Ca^{2+}/Mg^{2+}-ATPase from sarcoplasmic reticulum of skeletal muscle [3,4]. Recently, we have demonstrated an unexpected in vitro activity of acylphosphatase: both isoforms are able to hydrolyze either DNA or RNA in acidic environment [5]. Little is known about the physiological function of acylphosphatase. An involvement of the enzyme in differentiation has been postulated; in fact, an increase of the MT isoform is associated with muscle differentiation [6]. Increases in both isoforms of acylphosphatase can also be observed in the K562 erythroid cell line after induction of differentiation [7]. The extent of this increase is very different between the two isoforms, depending on the differentiating agent used. This fact suggests that distinct regulations of the expression of the two proteins occur. Moreover, since the levels of the mRNAs of both isoforms always increase during differentiation, it is very likely that the regulation is at a post-transcriptional level. This fact also suggests that the two isoenzymes, despite the similar enzymatic activity, could play different roles in the cell. In another set of experiments it has been shown that treatment of K562 cells with T_3 hormone leads to an induction of the MT isoform alone. In this case transcriptional regulation of gene expression has been demonstrated, but a control at the translational level could not be excluded [8].

With a view to studying a possible regulation of both MT and CT isoforms of acylphosphatase at the translational level, isolation of specific cDNAs was performed. While the cDNA of the CT isoform has already been cloned [9], in this paper we describe the isolation of a still incomplete cDNA of the MT isoform and the peculiar structure of the 5′ untranslated region (5′-UTR) of acylphosphatase mRNA. Furthermore, results are presented indicating the involvement of the 5′-UTR region in the control of translation.

2. Materials and methods

Total RNA was purified from human erythroleukemia K562 cells using the method of Sacchi et al. [10]. Northern blot analysis was performed as already described [7,11]. The RACE technique was performed according to Frohman et al. [12]. Seven primers were synthesized for this purpose: oligo(dT) primer (5′-GACTCGAGTC-GACGAATTCT$_{(14)}$) containing also the restriction sites for *Xho*I, *Sal*I and *Eco*RI; primer 1 (5′-CTGTCACGGTGCCTTTGCTTG); primer 2 (5′-TCACCCAGCCAACCACTCCT); primer 3 (5′-ATG-TATACAGAAGATGAAGC); primer 4 (5′-GGCGGCGAGCCTC-TGTTGAG); primer 5 (5′-CCGGAGAGGGGCTCGGGG); primer 6 (5′-CTGCGGCGGCTGCGAGAGGA). The first strand of the cDNA was synthesized using 5 μg of total RNA, with *Tth* reverse transcriptase (Promega) and using primer 1 (or primer 4) by incubation at 70°C for 30 min. A stretch of dA was added to the 3′ end of the first strand cDNA by terminal deoxynucleotidyl transferase (Promega). The single strand cDNA was purified using Sephacryl-S300 column (Pharmacia) and ethanol precipitated. An aliquot of purified cDNA was used for PCR with primer 1 (or primer 4) and oligo(dT) primer, followed by a nested PCR with primer 2 (or primer 5) and oligo(dT) primer (95°C for 1 min, 55°C for 1 min, 72°C for 1 min, 30 cycles). The products were electrophoresed in 1.2% agarose gel, blotted onto Hybond N^+ nitrocellulose (Amersham), and hybridized with a ^{32}P-labelled specific probe (primer 3 or primer 6). PCR products were cloned using the TA Cloning System (InVitrogen).

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Abbreviations: MT, muscle type; CT, common type; RACE, rapid amplification of cDNA ends; 3′-UTR, 3′-untranslated region; 5′-UTR, 5′-untranslated region; ORF, open reading frame; uORF, upstream open reading frame; uAUG, upstream AUG

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Prediction of the mRNA secondary structure was obtained with the PC-GENE Program Package (IntelliGenetics). The 5′-UTR region of the MT acylphosphatase mRNA was cloned in the *Sal*I and *Bam*HI sites of the pEGFP-N1 eukaryotic vector (Clontech), upstream of the start codon for the EGFP protein: two oligonucleotides were synthesized (ApEr20, 5′-TTTTTGTCGACCCAGGCCCGCAG and ApEr21, 5′-TTTTTGGATCCGGCGGCGGCGAG), containing the restriction sites for *Sal*I and *Bam*HI respectively. Both primers were used in PCR experiments in which clone M1 and clone F5 were used as template DNA. PCR products were sequenced with the Sanger method. The unique restriction method for site directed mutagenesis, with minor modifications, was performed directly on the 5′-UTR-EGFP construct [13]. The AUG codon was replaced with the AUU codon using a synthetic mutation target oligonucleotide (5′-GCAAGCAGTCCCATTTGTCCCCTCCC) and the *Stu*I restriction site eliminating oligonucleotide (5′-GGCTTTTTTGGAGGGGTAGGCTTTTGC). The presence of the mutation was confirmed by nucleotide sequence analysis. Transient transfections were performed with the calcium-phosphate method [11]. Evaluation of fluorescent cells (2×10^5 cells washed twice in PBS) was performed in a Becton Dickinson FACScan according to the manufacturer's procedure [14]. Co-transfection experiments performed with a β-galactosidase expression vector together with the different constructs always gave an evaluation of transfection efficiency very similar to that obtained wuth the FACScan (number of events).

3. Results and discussion

3.1. Isolation of MT isoform cDNA

In a previous screening of a human heart cDNA library a partial acylphosphatase cDNA was isolated, containing most of the coding sequence starting from codon 20, and the complete 3′-UTR (Fig. 1A). Northern blot analysis was performed on total RNA from human K562 cells, using the cDNA as a probe: the experiment showed that the specific transcript is a single band of about 1.2 kbp in length (data not shown). Because the coding region should be 294 bases and the 3′-UTR was about 300 bases in length, it was possible to evaluate that the length of the 5′-UTR should cover about half of the entire mRNA sequence, with an approximate length of 600 bases. In order to isolate the complete human MT acylphosphatase cDNA, we used the RACE-PCR technique according to Frohman and al. [12]. In a first experiment two different oligonucleotides were used both complementary to the mRNA coding region and close to the 5′ end of the cDNA clone isolated from the human heart cDNA library (primer 1, complementary to the mRNA between codons 42 and 48, and primer 2 between codons 33 and 39). These oligonucleotides were used, as indicated in Section 2, in a retrotranscription reaction, using total RNA from K562 cells as template, and in the following set of nested PCR in combination with the oligo(dT) primer. Samples were subjected to Southern blot analysis using primer 3 (complementary to a region of mRNA immediately upstream of primer 2) as a probe. Hybridization revealed a specific product of about 300 bp in length. This fragment was cloned (clone F5) and sequenced. As expected the fragment contained the region encoding the first 40 amino acids plus 151 nucleotides of the 5′-UTR region of the mRNA. Since the deduced length of the 5′-UTR should be about 600 bases, a second round of RACE-PCR was performed with the same strategy as in the first experiment. For this purpose three other primers were synthesized on the basis of the sequence of clone F5 (primers 4, 5 and 6, which are complementary to the 5′-UTR region of clone F5). Cloning of the products of the second RACE-PCR experiments led to the isolation of clone M1, which was only 24 bases longer than clone F5. The use of several different conditions in order to optimize the RACE-PCR experiments did not allow the isolation of any other longer cDNA. The cDNA sequence is presented in Fig. 1A: two non-in-frame ATG start codons are present in positions 93 and 176 respectively. The downstream ORF encodes a polypeptide sequence identical to the known primary structure of the MT isoform [1]. The cDNA shows a putative polyadenylation site in position 759.

3.2. Structure of the 5′-UTR of the MT acylphosphatase mRNA

The analysis of the 5′-UTR sequence of MT acylphosphatase cDNA (175 bp in length) shows very interesting characteristics. The sequence has a high G+C content (73%) and the corresponding mRNA could form a peculiar structure in vivo. Computer modelling of the possible secondary structure was performed and the results are presented in Fig. 1B. The prediction shows the formation of a very stable ($\Delta G = -57.3$ kcal/mol) stem-loop structure. The presence of such a structure could explain the difficulty that we found in isolating the 5′ end of this cDNA: it is very likely that the stable secondary structure of the MT mRNA made it difficult for the reverse transcriptase to copy the 5′ end of this molecule. Moreover, the 5′-UTR presents an additional AUG triplet (uAUG), located 83 bases upstream and not in frame with the start codon of acylphosphatase. The uAUG codon defines a uORF of 60 amino acids in length (Fig. 1A) which overlaps the first 35 codons of the acylphosphatase ORF.

It has been demonstrated that the 5′-UTR region of a mRNA with such peculiar characteristics might play an important role in the regulation of gene expression by means of post-transcriptional or translational control. In fact, it has been shown that translational regulation is often exerted at the level of translation initiation, with the involvement of two elements within the 5′-UTR of a mRNA [15]: (i) stable secondary structure in the 5′-UTR and (ii) an AUG located upstream of the authentic start codon. It is known that stem-loop structures introduced between the cap and the AUG codon do not facilitate initiation. For example, it was demonstrated that the 5′-UTR of platelet derived growth factor, a region with a very stable secondary structure, acts as a translational inhibitor [16]. In the case of placenta growth factor mRNA, the 5′-UTR has a very high G+C content (73%) similar to that observed in the 5′-UTR of the acylphosphatase mRNA: in addition a short uORF is present having an inhibitory effect on translation [17]. Furthermore, the efficient translation of the mRNA encoding the 65 kDa regulatory subunit (PR65) of protein phosphatase 2A is prevented by an out-of-frame upstream AUG triplet and a stable stem-loop structure of the 5′-UTR [18].

In the light of these results, it appeared quite interesting to verify whether the peculiar sequences found in the 5′-UTR of MT acylphosphatase mRNA might play an important role in translational control.

3.3. Effects of the 5′-UTR on translation and mRNA stability

To directly demonstrate the possible involvement of the 5′-UTR of MT acylphosphatase mRNA in translation control, we fused this region of the cDNA to the coding region of the reporter protein EGFP (a mutated variant of the green fluorescent protein, GFP). EGFP emits bright green light when

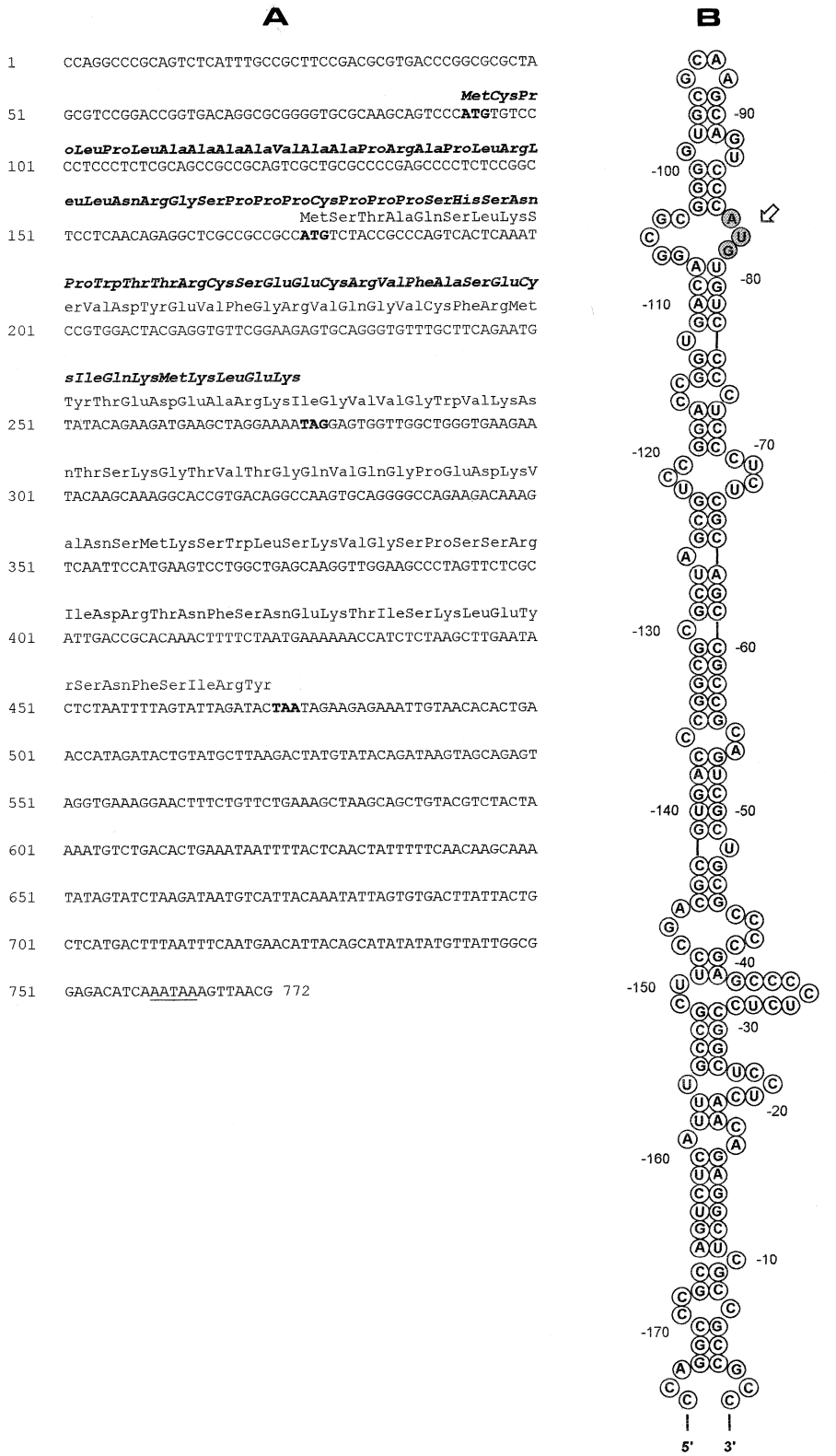

Fig. 1. A: Nucleotide sequence of muscle acylphosphatase cDNA and comparison with the CT isoform cDNA. ATG start codons and stop codons of MT isoform cDNA are in bold. Putative polyadenylation signals are underlined. Translations of ORFs are indicated (the upstream ORF is in bold). The sequence was submitted to the EMBL data bank, accession number X84195. B: Computer analysis of the secondary structure of the 5′-UTR of MT acylphosphatase. The upstream AUG triplet is indicated by the arrow. Position +1 corresponds to the A of the AUG acylphosphatase start codon (position 176 in the sequence in A).

exposed to UV or blue light, making it possible to measure the fluorescence by flow cytometry. In particular, the 5′-UTR region of the MT acylphosphatase mRNA was inserted into the pEGFP-N1 vector, upstream of the start codon of the

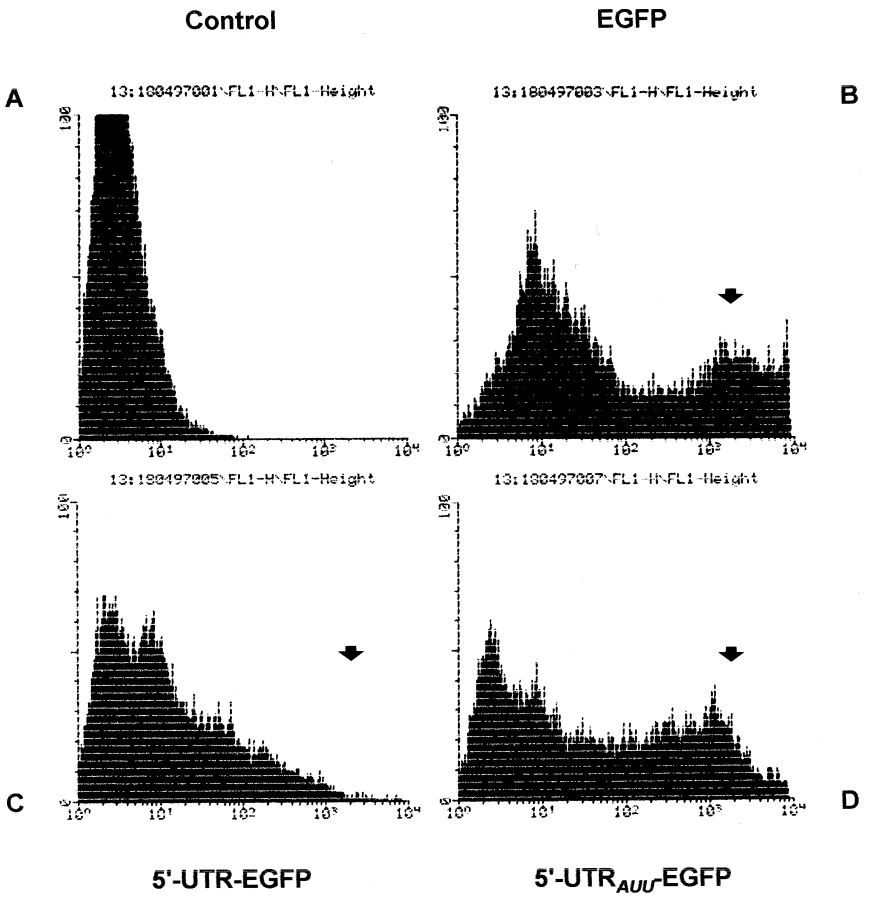

Fig. 2. FACScan analysis of EGFP expressing cells. Cytofluorimetric analysis of HeLa cells transfected with the different constructs. A: Negative control (no transfection). B: EGFP; as positive control. C: 5′-UTR-EGFP; containing the 5′-UTR of acylphosphatase. D: 5′-UTR$_{AUU}$-EGFP containing the AUG to AUU mutant. The X axis represents the fluorescence intensity while the Y axis represents the number of events evaluated with the FACScan. The arrows represent the position of maximum fluorescence intensity due to EGFP expression in the positive control. These data are representative of three independent experiments with overlapping results.

EGFP and under the control of the immediate-early promoter of CMV. The construct was created in such a way that the AUG start codon of EGFP is not in frame with the uAUG of the 5′-UTR region, as happens in the acylphosphatase mRNA. Furthermore, to investigate the importance of the uAUG, we created a mutant in which this codon was substituted with the AUU triplet. These constructs were used to transiently transfect HeLa cells. The results presented in Fig. 2 indicate that the 5′-UTR region of the MT acylphosphatase mRNA negatively influences translation of the EGFP protein. In fact, evaluation of the accumulation of the fluorescent protein in the cells, measured as fluorescence intensity 48 h after transfection, clearly shows an average 10-fold decrease (Fig. 2C) compared to a control experiment in which the pEGFP-N1 vector was used for transfection (Fig. 2B). On the other hand, the AUG to AUU mutant (Fig. 2D) shows a fluorescent intensity slightly lower than the positive control (Fig. 2B). The numbers of events evaluated with the FACScan were very similar in all the transfection experiments performed, confirming that transfection efficiency was quite reproducible in independent experiments and with different constructs (30–45%). The levels of the EGFP specific transcript in transfected cells were evaluated by Northern blotting. Total RNA was extracted from the same transiently transfected cells tested by flow cytometry and hybridized with the ^{32}P-labelled EGFP cDNA as a probe. The results (Fig. 3) show that the level of the specific mRNA dramatically decreases in cells transfected with the construct containing the 5′-UTR region of acylphosphatase, in comparison with the positive control

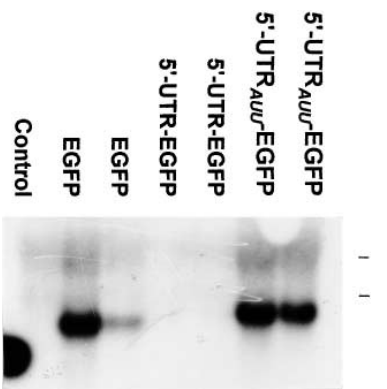

Fig. 3. Evaluation of the EGFP mRNA in HeLa cells transfected with different constructs. Northern blot analysis was performed with 10 μg of total RNA (1 μg in the third lane from the left) extracted from the same cell cultures tested by flow cytometry. Filters were hybridized with the ^{32}P-labelled cDNA of EGFP as a probe. Hybridization with an actin probe was performed for normalization (data not shown). 18S and 28S positions are indicated. These data are representative of three independent experiments with overlapping results.

cells, transfected with the pEGFP-N1 vector. On the other hand, when cells transfected with the construct containing the AUG to AUU mutant of the 5′-UTR of acylphosphatase were tested, the EGFP transcript level remained nearly unchanged with respect to the positive control.

These results indicate that the 5′-UTR region of MT acylphosphatase may exert a negative control of translation as a consequence of the presence of the uORF: we may hypothesize that the product of the uORF could be implicated in mechanisms leading to mRNA destabilization. This hypothesis could explain the fact that a single base mutation in the uAUG start codon is capable of restoring normal mRNA level and protein translation, and why the lack of translation of the uORF could lead to mRNA stabilization. In the light of these results it seems unlikely that the control of translation could be simply due to a phenomenon of competition between the uAUG and the acylphosphatase start codon. On the other hand, it is very unlikely that a single point mutation could dramatically change the 5′-UTR secondary structure.

Messenger RNA degradation is a process that plays an important role in the regulation of gene expression. Recent results indicate that an mRNA decay pathway regulates the abundance of both aberrant and wild-type transcripts. In particular, the stability of mRNAs that present uORFs might be regulated by this pathway. There is evidence that the introduction of an uORF into the 5′-UTR of an mRNA in yeast both inhibits translation and leads to accelerated degradation of the transcript. It is likely that the presence of a translatable uORF is the decisive factor that triggers events responsible for rapid decay of the mRNA [19].

In conclusion, it is clear that this portion of the 5′-UTR region of the MT acylphosphatase mRNA plays an important role in the regulation of the protein level through the mRNA instability. We may hypothesize that this region of the transcript influences mRNA stability with a consequent decrease in protein translation. It is intriguing that mRNA instability is not present when the uAUG codon is eliminated: this fact suggests that the product of the uORF could be involved in the control of mRNA stability.

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