Analysis of the rabbit cardiac/slow twitch muscle sarcoplasmic reticulum calcium ATPase (SERCA2) gene promoter

Drew A. Sukovich, Junaid Shabbeer¹ and Muthu Periasamy^{1,*}

Department of Physiology and Biophysics, University of Vermont, College of Medicine, Burlington, VT 05405 and ¹Molecular Cardiology Laboratory, Division of Cardiology, University of Cincinnati College of Medicine, ML 542, 231 Bethesda Avenue, Cincinnati, OH 45267, USA

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ABSTRACT

The rabbit cardiac/slow twitch muscle sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA2) gene encodes a Ca^{2+} transport pump whose expression is regulated during skeletal/cardiac muscle development and by different pathophysiological states of the heart. This study was designed to delineate *cis*-acting regulatory elements involved in SERCA2 gene expression. A series of unidirectionally deleted fragments of the upstream 1,460 bp SERCA2 promoter were linked to the chloramphenicol acetyltransferase (CAT) reporter gene. Transient DNA transfection experiments performed with these constructs in C2C12 muscle cells and NIH3T3 fibroblasts revealed a 17 bp upstream promoter element (UPE) important for transcription of the SERCA2 gene in skeletal muscle cells. These studies have also identified a strong (muscle specific) negative regulatory region located upstream of nucleotide –658. Gel mobility shift and southwestern analyses using the 17 bp UPE have revealed a specific DNA binding complex referred to as Ca^{2+} ATPase promoter factor –1 (CaPF1). The binding factor has an approximate M_r of 43 kDa. Comparison of CaPF1 with known transcription factors suggests that the CaPF1 complex may be a novel DNA-binding transcription factor which plays a role in SERCA2 gene regulation *in vivo*.

INTRODUCTION

The rabbit cardiac/slow twitch muscle sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2) gene encodes a mammalian Ca^{2+} transport protein (1), the function of which is to transport calcium from the cytoplasm into the sarcoplasmic reticulum (SR) lumen. The calcium ATPase protein plays a critical role in the regulation of the contraction/relaxation cycle of cardiac, skeletal and smooth muscles (2). The SERCA2 gene belongs to a family of three recently identified SR Ca^{2+} ATPase genes (1,3,4) which are

regulated in a developmental and tissue specific manner. The SERCA2 gene is expressed at high levels in embryonic and fetal fast skeletal muscle. However, in adult fast twitch skeletal muscle tissue, SERCA2 mRNA expression is down regulated to a very low level (5,6). Recent work from our lab has shown that the SERCA2 gene is not expressed in proliferating C2C12 myoblasts, but is induced during their differentiation into myotubes (1,6). Other work has shown that primary fibroblasts derived from cardiac tissue do not express any detectable levels of the SERCA2 mRNA transcript.

In experimental animal models, we and others have previously shown that the relative levels of SERCA2 mRNA level are down regulated in pressure overload induced hypertrophy, but up regulated in T3 induced cardiac hypertrophy (8, 19). A decrease in levels of SERCA2 mRNA and protein were also observed in failing human hearts (10). These findings suggest that transcriptional control of SERCA2 gene expression plays an important role in modulating SR function, although other mechanisms might be equally important. Thus, the SERCA2 gene is an excellent model system to study the complexity of transcription control, since its expression is regulated developmentally, in a tissue specific manner and responds to specific pathophysiological conditions of heart muscle.

Transcription is a primary control point in the regulation of gene expression. The cellular transcriptional machinery integrates regulatory information through the binding of *trans*-acting factors to *cis*-acting DNA elements. These DNA elements are found both proximal and distal to the transcription initiation site, and are involved in the activation or repression of transcription. The *cis*-acting regulatory elements required for expression of muscle specific genes during myogenesis have been studied for many muscle specific genes, such as skeletal and cardiac α -actin (11,12), myosin light chain (13,14), and muscle creatine kinase (MCK) (15,16). These and other studies have identified several *cis*-acting elements important for regulated muscle-specific gene expression. This includes the M-CAT sequence in cardiac troponin T (17,18), the CarG box element (11), a related A/T

* To whom correspondence should be addressed

rich element which binds MEF-2/RSRF/TARP (19–21), and the E-box which binds a family of myogenic determination factors and basic helix-loop-helix (BHLH) proteins (22–24).

Towards understanding the transcriptional regulation of the SERCA2 gene, this study was designed to identify *cis* and *trans*-acting elements critical for its expression in skeletal muscle cells. A 1.1 kb fragment of 5'-flanking DNA of the rabbit SERCA2 gene has been shown to confer transcription upon the chloramphenicol acetyl transferase (CAT) reporter gene *in vitro* (1). We have cloned a series of deletion fragments of the 5' region, upstream of CAT, and used these constructs in transient transfection assays in both muscle and non-muscle cell lines. We report the identification and characterization of a 17 bp upstream promoter element (UPE) located between nucleotides –284 and –267, important for high level transcription of the SERCA2 promoter in muscle cells *in vitro*. Using the gel mobility shift assay, we have identified a DNA–protein complex referred to as CaPF1 (Calcium ATPase Promoter Factor 1), which specifically binds the 17 bp UPE. The approximate molecular weight of this factor was determined by southwestern analysis. Comparison of CaPF1 to known vertebrate transcription factors suggests that the CaPF1 factor may be a novel transcription factor which plays a role in the regulation of the SERCA2 gene in muscle cells.

MATERIAL AND METHODS

Construction of the SERCA2/CAT gene chimeric constructs

A 1,460 bp DNA fragment containing the 5'-flanking region of the SERCA2 gene was excised from λ RbCA3-3 (1) using EcoRI and SmaI endonucleases. The 1,460 bp fragment was blunt ended with Klenow enzyme, and ligated with BamHI linkers using standard methods (25). The resulting fragment was ligated in a 5' to 3' orientation into the unique BamHI site of the chloramphenicol acetyltransferase (CAT) expression vector pBLCAT3 (26). The resulting vector, pD1110, was used as a template for 5' to 3' exonuclease III digestion (Erase-a-base kit, Promega). This produced a series of promoter constructs (figure 1), which were used in transfection studies. The 5' ends of the deleted constructs were confirmed by dideoxynucleotide sequencing (27).

Cell culture and DNA transfections

C2C12 mouse myoblasts (28) were maintained in growth medium (DMEM supplemented with 20% fetal bovine serum and 60 mg/l of kanamycin sulfate) at subconfluent densities. Differentiation was induced by switching cells to fusion medium (DMEM supplemented with 5% horse serum and 60 mg/l of kanamycin sulfate). Myotube formation was visible within 36 hrs; cells were maximally fused after 48–60 hours. NIH 3T3 fibroblasts were propagated in DMEM supplemented with 10% fetal bovine serum and 60 mg/l of kanamycin sulfate. Transient transfections with the SERCA2 constructs were performed using the calcium phosphate co-precipitation method (29). Briefly, duplicate dishes of cells (5×10^5 cells/dish) were transfected with 15 μ g of the SERCA2 construct, plus 5 μ g of MSV β GAL. DNA used for transfections was purified by two successive CsCl₂ density gradient centrifugations. After 10–14 hrs, cells were washed with PBS buffer (137 mM NaCl, 27 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄) and glycerol shocked for 1–2 minutes. Cells were then washed twice with PBS, and fresh

growth medium was added. For C2C12 myoblasts and 3T3 fibroblasts, cells were harvested 48 hr post transfection. For C2C12 myotubes, cells were allowed to propagate in growth medium for 8–10 hours, then growth medium was removed and replaced with differentiation medium. C2C12 myotubes were harvested 36–48 hr post fusion medium change. For CAT assays, cells were harvested in PBS buffer, centrifuged at 3000 rpm, and the cell pellet resuspended in 100 μ l of 0.25 M Tris–HCl pH 7.8. Cells were lysed by three cycles of freeze thawing, and the resulting supernatants assayed for β -galactosidase and CAT activity.

CAT assays

β -galactosidase assays (30) were performed on 30% of the cell extract to provide values of relative transfection efficiency. The resulting values were used to normalize the amount of extract added to the subsequent CAT assay reactions. For each experiment, duplicate determinations of CAT activities of each construct was performed by the method of Nordeen (31). All constructs were tested in at least three separate transfection experiments with at least two different plasmid preparations.

Protein extraction and gel mobility shift assays

Nuclear extracts from C2C12 and 3T3 fibroblast cells were prepared essentially as described by Dignam et al (32). The protein concentration was determined by the Bradford assay (33). The DS1/2 probe which consisted of the 17 bp UPE (nucleotides –284 to –267) flanked by BamHI recognition sites was produced by annealing two complementary oligonucleotides with the sequences: 5'-gatcGAGCGCTCGCCCTCCGC-3' and 5'-gatcGCGGAGGGCGAGCGCTC-3'. The resulting double stranded fragment was purified from a 12% acrylamide gel and end labeled with (α -³²P) dGTP using DNA polymerase (Klenow fragment) (30). Approximately 15,000 cpm (.1 ng) of probe was incubated with 3–5 μ g of nuclear extract in a 20–25 μ l reaction mixture containing 40mM KCl, 15mM Hepes Ph 7.9, 1mM EDTA, 0.5mM dithiothreitol, 0.5mM PMSF, 20% glycerol, and 0.1–0.2 μ g/ μ l poly (dA-dT) for 30 minutes at room temperature (29). Competition experiments were performed with 100–1000 fold molar excess of specific or non-specific unlabeled DNA fragments. The reaction mixtures were loaded directly onto a 6% polyacrylamide gel (30:1 polyacrylamide-bisacrylamide) and electrophoresed in 50mM Tris-base, 50mM, boric acid, 1mM EDTA. Gels were run at 225 V for 3–4 hours at 4°C, fixed in 30% methanol/10% acetic acid for 30 minutes, then dried on 3 M Whatman paper. Gels were exposed overnight at 70°C with an intensifying screen.

SDS–PAGE and South/Western blot analysis

Nuclear extracts and standards were electrophoresed on a 10% polyacrylamide gel with 3% stack according to the method of Laemmli (34). Proteins were then electroblotted onto a nitrocellulose filter in transfer buffer containing 25 Mm Tris and 192 Mm glycine. The filter was washed briefly in transfer buffer, and then incubated in 5% non-fat dry milk powder, 50mM Tris–HCl Ph 7.5, 50mM NaCl, 0.1mM EDTA, and 1mM DDT with gentle swirling for 60 minutes at room temperature. The filter was next washed twice for 5 min. at 4°C with binding buffer (10mM Tris–HCl Ph 7.5, 50 mM NaCl, 1mM EDTA and 1mM DDT). The membrane was then incubated for 60 minutes in binding buffer containing 5×10^7 cpm of ³²P labeled DNA

recognition site probe (DS1/2). The filter was briefly washed three times with binding buffer, air dried and then autoradiographed.

RESULTS

Sequences within 284 nucleotides of the SERCA2 transcription initiation site confer transcriptional activation in muscle cells

To begin to identify promoter sequences that regulate SERCA2 transcription, we used deletion fragments of the 5' promoter (extending upstream to 1.1 kb from nucleotide +358 of the 5' untranslated region) linked to the CAT reporter gene (Fig. 1). These unidirectionally deleted promoter CAT constructs were introduced by calcium phosphate mediated transfection into C2C12 muscle cells and NIH 3T3 fibroblast cells. Following transfection, C2C12 myoblasts were allowed to remain in serum rich medium (10% fetal calf serum) or switched to differentiation medium. The CAT activity data from these transfection analyses is presented in Fig. 2 as relative CAT activities (SERCA2-CAT construct activity/pBLCAT3 CAT activity).

We repeatedly observed that the promoter construct pD284 gave the maximal levels of CAT activity, in both C2C12 myoblasts and myotubes, 25-fold and 77-fold respectively over the promoterless pBLCAT3 vector. As expected, the C2C12 myotubes gave the highest CAT expression, suggesting that muscle differentiation upregulates this gene. Interestingly, in both C2C12 myoblasts and myotubes, there was a large decrease in CAT activity when a 17 bp region located between -284 and -267 was deleted (compare construct pD284 to pD267). In myoblasts, a 50% reduction in CAT activity is observed when this region is deleted, while in the myotubes there is a 75% reduction in CAT activity. In contrast, there is little difference in the level of CAT activity generated by constructs pD284 and

pD267 when transfected into 3T3 fibroblast cells. The CAT activities generated by each of the SERCA2-CAT constructs in fibroblast cells is approximately 5-fold over the background activity of the promoterless pBLCAT3, except for construct pD72 which is not expressed at any appreciable levels in either C2C12 cells or fibroblasts. This result is in contrast to that observed for the SV40 promoter/enhancer containing plasmid pSV2CAT, which is expressed at similar levels in all three cell types. From these results, we may conclude that sequences within 284 nucleotides are sufficient for conferring transcriptional activation in skeletal muscle cells. These experiments also suggest that the 17 bp element located between -284 and -267 is an important upstream promoter element (UPE) for SERCA2 expression in C2C12 cells *in vitro*.

The larger constructs pD1110 and pD811 produced low levels of CAT activity compared to pD284 in muscle cells. This loss of activity (greater than 90% in myotubes and 80% in myoblasts) is not observed in 3T3 fibroblast cells. This result suggests that the SERCA2 gene promoter may contain one or more negative cis-regulatory elements located between nucleotides -1110 and -811. Promoter construct pD658 produced much higher CAT activity in both C2C12 myoblasts and myotubes (12-fold and 25-fold, respectively). This increase in CAT activity is not observed in 3T3 fibroblasts, suggesting that this region may also contain additional DNA elements important for the SERCA2 gene expression in muscle. The results of these transfection experiments demonstrate that the SERCA2 gene contains multiple positive and negative cis-acting elements that function in a muscle specific environment, but not in the fibroblast cell environment.

Transcriptional activation of the SERCA2 gene promoter in muscle cells is myogenin independent

The up-regulation of the SERCA2 gene during *in vitro* C2C12 muscle cell differentiation (1,6) suggests that this gene may be regulated by the HLH family of myogenic regulators, specifically MyoD or myogenin. This hypothesis is supported by the observation that there is an E-box consensus site present at

Figure 1. SERCA2-CAT constructs used in transient transfection studies a) Schematic representation of the 5'-flanking region of the SERCA2 gene containing the transcription start site (+1) and the translation initiation codon ATG (+528). b) The SERCA2-CAT constructs, named according to the number of nucleotides each contains upstream of the transcriptional initiation site. Constructs were generated by exonuclease III and S1 digestion (see material and methods) and fused to CAT expression vector pBLCAT3.

Figure 2. Transcriptional activity of the SERCA2-CAT constructs in muscle and non-muscle cells. Data represent the CAT activities of the SERCA2-CAT constructs divided by the activity of the promoterless CAT expression vector, pBLCAT3. pSV2CAT expression data is included as a positive control. Each error bar represents the standard error of the mean.

Figure 3. Gel mobility shift analysis of the 17 bp UPE. The DNA probe is a 25 bp fragment (DS1/2, see material and methods) containing the 17 bp UPE. Gel shift assays with the DS1/2 probe and 5 μ g of C2C12 myotube nuclear extract (lanes 2–7 and 14,15) and 3T3 fibroblast extract (lanes 8–13). Competitions with unlabeled DS1/2, Sp1 and non-specific (NS) oligonucleotides at the molar concentrations are shown. The positions of the retarded CaPF1 complex and the unbound probe are shown. B) Distribution of the CaPF1 complex in C2C12 and 3T3 fibroblasts, Gel shifts were prepared with the DS1/2 probe and 5 μ g of nuclear extracts from C2C12 myoblasts, C2C12 myotubes and 3T3 fibroblasts. Competition with the unlabeled DS1/2 oligonucleotide at 25, 50 and 100 molar excess is shown.

nucleotide –54 in the SERCA2 promoter and in promoter constructs pD284 and pD267 which demonstrate up-regulation of CAT protein expression during *in vitro* C2C12 cell differentiation. To test this experimentally, each of the Ca²⁺ ATPase promoter constructs were cotransfected into C3H 10T1/2 cells with the MyoD or myogenin expression vectors following the method described in Chakraborty et al (35) in Dr Olsons laboratory. All of the SERCA2 promoter constructs

Figure 4. Southwestern analysis with the DS1/2 probe. Nuclear extracts were from 3T3 fibroblasts (lane 2), C2C12 myoblasts (lane 3) and C2C12 myotubes (lane 4). Molecular weight standard (Lane 1).

produced low levels of CAT activity equivalent to that seen in 3T3 fibroblasts (Data not shown). These results are in contrast to the MCK-CAT constructs which were upregulated in C3H 10T1/2 when either MyoD or myogenin was cotransfected (data not shown). These results suggest that SERCA2 promoter is regulated by an E-box independent mechanism during *in vitro* C2C12 cell myogenesis. Furthermore, the fact that both constructs pD284 and pD267 contain a consensus E-box at nucleotide –54 and are not activated in response to the overexpression of either MyoD or myogenin suggests that this site is not a functional E-box binding site.

Gel-mobility shift analysis of the 17 bp UPE

Gel mobility shift analysis was used to determine if *trans*-acting factors recognize and bind to the 17 bp UPE, (determined to be important for transcriptional regulation of SERCA2-CAT constructs in C2C12 cells *in vitro*). Using a 25 bp probe (DS1/2) containing the 17 bp UPE, a shifted complex was observed in assays using C2C12 and 3T3 nuclear extracts (Fig. 3A). The shifted complex was competed by the addition of specific, unlabeled oligo, but not by the addition of equivalent amounts of the Sp1 consensus binding site oligo, or a non-specific unlabeled oligo. This shifted complex observed in muscle cells has been named Ca²⁺ ATPase promoter binding factor-1 (CaPF1). Nuclear extracts from NIH-3T3 cells gave a similar gel shift complex, but produced additional higher mobility complexes (Fig. 3B). Further, these complexes were not readily competed out by the unlabelled specific oligo, as seen in muscle cells. This data suggests that multiple factor(s) are binding to this sequence in 3T3 cells. It is unclear from this analysis whether the CaPF1 factor is present in both muscle and non-muscle cells.

Interestingly, addition of 1000-fold molar excess of the Sp1 consensus binding site oligo can compete out protein binding to the 17 bp UPE. This may be due to the similar GC content in both the 17 bp UPE and Sp1 consensus binding site, although other well characterized DNA-binding elements, such as Oct1 or Ap2, do not compete out binding of CaPF1 at similar molar concentration (data not shown). The gel mobility shift experiments

Figure 5. A) Comparison of the 17 bp UPE in the rabbit and rat SERCA2 genes. The upper line is the sequence of the 17 bp UPE located between nucleotides -284 and -267 in the rabbit SERCA2 gene. The lower line contains 17 bp region of the rat SERCA2 (36) gene located between nucleotides -293 and -276. The contiguous 13 bp 100% homologous region is indicated. B) Nucleotide sequence of the 5' flanking region of the rabbit SERCA2 gene. The 17 bp UPE located between nucleotides -284 and -267 is in bold face. Three consensus Sp1 sites located downstream of the 17 bp UPE are capitalized. The consensus CAAT and TATA box located at nucleotides -78 and -24, respectively are capitalized.

establish that the binding of this complex which we refer to as CaPF1, is specific for the 17 bp UPE. *The CaPF1 factor is a 43 kDa protein.*

To determine the approximate molecular weight of CaPF1, southwestern analysis was performed. Nuclear extracts from 3T3 cells, and C2C12 myoblasts and myotubes were separated by SDS-PAGE, and blotted onto nitrocellulose, then probed with the DS1/2 oligo. We observed a single band which corresponds to a protein of approximate molecular weight 43 kDa, and which appears to be present in all three nuclear extracts (figure 4), although 3T3 fibroblast nuclear extracts produce a fainter signal. This may be a reflection of the differences observed in the gel shift experiments utilizing fibroblasts extracts. These results also suggest that a single protein is sufficient to provide for CaPF1 DNA-binding, since proteins are denatured by SDS-PAGE.

DISCUSSION

The SERCA2 gene serves as a target for diverse regulatory mechanisms in skeletal and cardiac muscle. It has been shown that SERCA2 gene expression is regulated in a developmental, and tissue specific manner in skeletal and cardiac muscle (5,6) and is also altered by pathophysiological conditions of the heart (7-10). It is unknown whether these different regulatory influences are directed at a common control sequence, or are associated with multiple regulatory regions associated with the SERCA2 gene. Our results from this study indicate that SERCA2 gene expression may be governed by multiple (both positive and negative) regulatory elements.

In the present study, we demonstrate that sequences located within the first 284 nucleotides of the SERCA2 promoter are sufficient for high level CAT expression in muscle cells. Further, we show that a 17 bp element located between positions -284 and -267 is a positive regulatory element, whose deletion results in loss of CAT activity in muscle cells, but not in 3T3 fibroblast

cells. Comparison of the 5' flanking regions of the rabbit and rat (36) SERCA2 genes shows that the rat contains a similar 17 bp DNA sequence between positions -293 and -276, and has 88% sequence homology within the 17 bp UPE. In addition, the rabbit SERCA2 proximal promoter region contains three consensus SP1 sites (fig. 5b) downstream of the 17 bp UPE, and two of these sites are also present in the rat SERCA2 promoter (36). The function of these sites remains to be tested.

Transcriptional activity of the SERCA2 promoter decreased when upstream DNA sequences were included in the CAT construct. DNA sequences upstream of position -658 have a large negative effect upon the activity of the SERCA2 promoter in C2C12 muscle cells, but not in fibroblasts. In contrast, in a parallel study in which, the promoter constructs bearing -658 and -1110 bp of upstream sequences were introduced into fetal cardiocytes, high level expression of the reporter construct was produced (Fisher et al. unpublished). This result clearly indicates that the sequences upstream of -658 serve as negative regulatory elements in skeletal muscle. During in vivo development of skeletal muscle, SERCA2 gene expression is selectively down regulated in fast twitch skeletal muscle, but up regulated in slow twitch muscle and cardiac muscle (6). In addition to positive elements, many genes have been shown to contain negative regulatory elements that are thought to repress gene expression by binding negative regulatory transcription factors (37-39). A perfect example of this type is the Vimentin gene, the expression of which is down regulated during muscle development by a negative regulatory mechanism (39).

An unanticipated outcome of this study was the finding that the SERCA2 promoter is not transactivated by either MyoD or myogenin transcription factors, despite the fact that the SERCA2 gene is transcriptionally activated during C2C12 in vitro myogenesis. Regulation of the SERCA2 promoter by other regulatory factors activated by myogenin/MyoD is not ruled out by this study. It has been shown that several other genes, including β MHC,(40) Cardiac Troponin-T (18) and cardiac MLC-2 (41) do not require myogenin for their expression, and it is probable that the SERCA2 gene belongs to this group.

A factor present in nuclear extracts from C2C12 myoblasts, myotubes and NIH 3T3 fibroblasts has been shown to interact with the 17 bp UPE, a positive regulatory element of the SERCA2 gene. A similar size protein complex was detectable in cardiomyocyte nuclear extracts (Fisher et al. unpublished). It is unclear whether this factor is muscle specific, but it is expressed at higher levels in C2C12 myotubes than myoblasts and fibroblasts. Our southwestern blot analyses indicates that this protein complex can bind DNA in a monomeric form, since a single protein of approximately 43 kDa can bind to this 17 bp UPE.

It is also interesting to note that binding of the 43 kDa protein was completely inhibited by 100 fold molar excess of the nonradioactive probe in C2C12 myoblasts or myotubes but there appeared to be still some binding present in the 3T3 nuclear extracts tested with equivalent competitor. It is quite possible that this variation in competition reflects differences in transcriptional activity of these constructs in muscle cells versus non-muscle cells. Possible hypotheses are that the 43 kDa protein is a member of a family of transcription factors which exhibit tissue specificity and have varying affinities for their cognate binding sequence.(5) An alternative possibility is that post-translational modification or the presence of specific accessory factors which do not bind to DNA themselves, but promote the affinity of the 43 kDa

protein to its target sequence and are themselves tissue specific. Recently, Grueneberg et al (42) described a homeodomain protein that enhances the binding activity of the serum response factor to its cognate binding site but exhibits no autonomous DNA binding function. Therefore, any of these possibilities may be responsible for differences in the binding affinity of the 43 kDa protein complex to the 17 bp UPE in muscle versus fibroblast nuclear extracts.

Our analyses indicate that this protein may represent a novel transcription factor yet to be characterized. Presently, studies are underway to further determine the biochemical identity of the 32 kDa protein, which may help understand the role this protein plays in the transcriptional regulation of the SERCA2 gene.

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