Introns Regulate the Rate of Unstable mRNA Decay*

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The expression of neutrophil-specific chemokines is known to be regulated via adenine-uridine-rich sequence elements in the 3′-untranslated regions of their mRNAs that confer a high degree of mRNA instability. Although the presence of intron sequences in eukaryotic genes is known to enhance expression, the effect of intron content on the rate of mature, translatable mRNA degradation has not been demonstrated. In this study, we have determined the effects of intron content on the rate of decay of the chemokine CXCL1 (KC) mRNA. The half-life of KC mRNA was markedly prolonged when the primary transcript was obtained from a genomic clone containing three introns as compared with the half-life observed with sequence-identical KC mRNA derived from an intron-free cDNA construct. The effect of intron content was achieved with a single intron, and neither the intron sequences nor the intron positions were critical determinants of the outcome. The intron content produced the same effect when expressed in multiple cell types and when the sequences were stably integrated into the genome. The differential decay rates were not a consequence of differential nuclear to cytoplasmic transport. The intron content of the primary transcript did not influence the rate of KC mRNA translation and did not modulate the ability of interleukin-1 stimulation to stabilize the otherwise unstable mRNA. The intron effect on mRNA decay was seen with mRNAs containing two distinct instability determinants. These findings document that intron content marks the mRNA sequence leading to enhanced stability that is particularly evident in short lived ARE-containing mRNAs.

Inflammation, a process essential in protection against the consequences of injury and infection, has the potential to produce unnecessary tissue damage and hence must be stringently controlled (1). This regulation occurs in part through the modulation of gene expression and is achieved mechanistically at multiple levels that include transcription, mRNA stability, and mRNA translation (2–5). The induction of inflammatory gene expression frequently necessitates a rapid burst of transcription, and the features controlling this process have been well studied. It is becoming increasingly recognized, however, that post-transcriptional regulation at the level of mRNA stability is also a vitally important mechanism to control gene expression during inflammation (6–10).

Adenylate-uridylate-rich elements (AREs), located in the 3′-untranslated region (3′UTR) of many short lived mRNAs are the most widely recognized cis-acting elements involved in regulating mRNA decay (11, 12). These sequences have been associated not only with mRNA instability but also with stimulus-induced stabilization of mRNAs and control of translational efficiency (12–17). The pathophysiologic impact of this kind of regulation is perhaps best illustrated by the systemic inflammatory disease seen in mice bearing mutations affecting the half-life of tumor necrosis factor-α mRNA (18, 19).

Genes in most higher eukaryotes contain introns that must be accurately removed from the primary transcripts to create translatable mRNAs. Introns not only allow multiple proteins to be produced from a single gene through alternative splicing but have also been shown to make important contributions to the regulation of gene expression (20–22). It is generally recognized that the presence of an intron in a primary transcript will promote more abundant gene expression, and this may act at many steps in the process, including transcription (22, 23), processing of primary transcripts (24, 25), transport of mRNAs from nucleus to cytoplasm (26, 27), translational efficiency (22, 28, 29), as well as detection and elimination of mRNAs with nonsense-coding errors through the process of nonsense-mediated mRNA decay (NMD) (27, 30–32). A recent report suggests that there is correlation between intron content and mRNA stability (33), but to date there has been no experimental documentation of an effect on the process of mRNA decay, particularly involving highly unstable ARE-containing sequences.

Using the mouse chemokine gene CXCL1 (also known as KC) as a model, we demonstrate that mRNA derived from a primary transcript containing introns is significantly more stable than that derived from an intron-free primary transcript. Only a single intron is required to produce this effect, and the intron position and sequence do not appear to be important. Although the presence of at least one intron modulates the rate of mRNA decay, it does not modulate the nuclear/cytoplasmic distribution, the rate of translation, or the ability of extracellular stimuli to stabilize the mRNA.

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2The abbreviations used are: ARE, adenine-uridine-rich element; KC (CXCL1), mouse CXC family chemokine ligand 1; 3′UTR, 3′ untranslated region; Dox, doxycycline; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NMD, nonsense-mediated mRNA decay; IL, interleukin; gKC, genomic KC; MOPS, 4-morpholinepropanesulfonic acid.
**EXPERIMENTAL PROCEDURES**

**Reagents**—Dulbecco’s modified Eagle’s medium, Dulbecco’s phosphate-buffered saline, and antibiotics were obtained from Central Cell Services of the Lerner Research Institute (Cleveland, OH). Fetal bovine serum was purchased from BioWhittaker (Walkersville, MA). G418, puromycin, formamide, MOPS, salmon sperm DNA, diethyl pyrocarbonate, and Nuclei EZ Prep kit (NUC-101) were purchased from Sigma. Hygromycin was obtained from Invitrogen. Doxycycline (Dox) and the vector pTRE2 were obtained from Clontech. SuperFect transfection reagent was obtained from Qiagen (Valencia, CA), TRI Reagent was purchased from Molecular Research Center (Cincinnati, OH), and nylon transfer membrane was purchased from Micron Separation (Westboro, MA). Recombinant human IL-1β was obtained from R&D Systems (Minneapolis, MN). PerkinElmer Life Sciences was the source of [α-32P]dCTP. Protogel and related buffers were obtained from National Diagnostics Inc. (Atlanta, GA). Protein assay reagents were purchased from Bio-Rad. A rabbit polyclonal antibody to histone H3 was obtained from Cell Signaling Technology (Danvers, MA), and a mouse monoclonal antibody specific for GAPDH was purchased from Chemicon International (Temecula, CA).

**Cell Culture and Transient or Stable Transfection**—HEK293 C6 cells stably expressing the tetR-VP-16 fusion protein (293tet-off) were prepared as described previously (34) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, G418, and puromycin. 293tet-off cells stably expressing KC cDNA and gKC (described below) were first selected for hygromycin resistance and subsequently for stable expression of Dox-sensitive KC. 293tet-off cells were transfected using Superf ect transfection reagent according to the manufacturer’s protocol. RAW264.7 Cells were prepared as described previously (35). Transient transfections of RAW264.7 Cells were done using the Amaxa Nucleofector Kit V (AMAXA Inc., Gaithersburg, MD) according to the manufacturer’s protocol.

**Plasmids**—Radiolabeled cDNA probes for use in Northern hybridization analysis were prepared from plasmids containing fragments of GAPDH and KC in the Bluescript vector as described previously (36). Plasmids used to drive expression of Dox-sensitive KC. 293tet-off cells were transiently transduced as described above. Twenty four hours after transfection, the supernatants were removed and replaced by fresh medium. Following a 3-h incubation, the supernatants were harvested and saved for later determination of KC protein secretion by ELISA prior to the termination of transcription. The plates were washed, and fresh medium containing Dox was added for 3 h to allow mRNA decay to occur in the absence of Dox initiation. The supernatants were discarded, and cultures were washed extensively prior to addition of fresh medium containing Dox with or without stimuli for a final 3 h, and supernatants were harvested again for determination of KC protein. The ratio of protein secretion before and after the addition of Dox provides a quantitative estimate of residual RNA.

**Preparation and Fractionation of Cell Extracts**—Pools of 293tet-off cells were transiently transfected as indicated. Twenty four hours after transfection, cells were trypsinized and harvested by centrifugation. Cell pellets were washed twice with ice-cold PBS and resuspended in 6 volumes of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and allowed to sit on ice for 15 min. The cell suspension was transferred to the Dounce homogenizer, and the cells were disrupted with 26 strokes. Cytoplasmic extracts were obtained by centrifugation at 500 × g for 5 min at 4°C. After removing a portion for subsequent Western analysis to assess the purity of the subcellular fractionation, the remaining was used to isolate RNA using TRI Reagent. The nuclear pellet was washed with 1 ml of Nuclei EZ Lysis buffer (Nuclei EZ Prep kit, Sigma) followed by washing with 1 ml of buffer A. Nuclei were recovered by centrifugation.
at 500 g for 5 min at 4 °C and resuspended in 6 volumes (according to the original cell pellet) of buffer A. A portion of the nuclear fraction was saved for Western analysis, and the remainder was used to isolate RNA. RNA from both fractions was resuspended in an equal volume of diethyl pyrocarbonate-treated water, and equal portions were used to determine KC and GAPDH mRNA levels by Northern blot hybridization. For determination of the fraction purity, comparable amounts of protein from each fraction were subjected to Western blot analysis as described previously using 15% gels (37). Blots developed using antibodies specific for histone H3 and GAPDH were used to quantify the purity of subcellular fractions.

RESULTS

Intron-containing and Intron-free Transcripts Produce mRNAs with Different Half-lives—Both genomic (gKC) and cDNA (KC cDNA) versions of the KC (CXCL1) gene were placed under control of a tetracycline-regulated promoter (see Fig. 1 for details of plasmid constructs). Twenty four hours after transient transfection of these constructs in 293tet-off cells, Dox was added to terminate transcription, and total RNA was prepared and used to determine the remaining levels of KC mRNA after the indicated time periods by Northern hybridization. For determination of the fraction purity, comparable amounts of protein from each fraction were subjected to Western blot analysis as described previously using 15% gels (37). Blots developed using antibodies specific for histone H3 and GAPDH were used to quantify the purity of subcellular fractions.

FIGURE 1. Schematic representation of reporter constructs. All the constructs contain the full 5′ UTR and coding region of KC mRNA linked with the 3′ UTRs from KC or CCR2 mRNAs as indicated. Transcription is driven by the tet-responsive element in pTRE2. RBG represents the intron from the rabbit β-globin gene. The details for preparation of each construct are provided under “Experimental Procedures.”

One Intron Is Sufficient to Stabilize mRNA, Regardless of Sequence and Position—Based on the comparison of gKC and KC cDNA-derived KC mRNAs, it is apparent that intron sequence within the primary transcript can influence the half-life of the derivative KC mRNA. Because the KC gene contains three introns, we first asked whether the number of intron sequences was a critical determinant. To accomplish this, three additional plasmid constructs were prepared in which individual intron sequences were deleted. These include gKC(−11) containing two introns (introns 2 and 3) and gKC(−11,2) or gKC(−11,3) each containing only 1 intron (introns 3 or 2, respectively). When these constructs were transfected in 293tet-off cells and their half-lives determined following the addition of Dox, no difference was observed in the decay rates for mRNAs derived from constructs containing 3, 2, or 1 intron.

plasmids and a tet-regulated luciferase expression plasmid to allow normalization for transfection efficiency. 24 hours after transfection, the levels of KC mRNA derived from each construct were determined by Northern hybridization, quantified by analysis of the autoradiographs, and normalized to the levels of both luciferase activity and GAPDH mRNA in each sample (Fig. 2C). The genomic, intron-containing construct clearly results in a significantly higher level of KC mRNA as compared with the construct containing the intron-free cDNA.

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FIGURE 2. Introns influence KC mRNA decay. A, 293tet-off cells were transfected with the plasmid constructs KC cDNA and gKC (each at 2 μg/dish), and each transfection pool was separated into five individual Petri dishes and cultured overnight. Dox (1 μg/ml) was added, and total RNA was prepared after incubation for the indicated times prior to determine the levels of KC and GAPDH mRNA by Northern hybridization. B, 293tet-off cells were transfected with the indicated amounts of plasmid DNA for constructs KC cDNA and gKC. After overnight culture, Dox was added, and total RNA was prepared using the times indicated in A and used to determine the levels of KC and GAPDH mRNA by Northern hybridization and autoradiography. The autoradiographs were quantified using the NIH Image software, and the ratios of KC to GAPDH were determined for each time point. Half-lives for each construct in each experiment were determined as described under “Experimental Procedures.” C, 293tet-off cells were co-transfected with either KC cDNA or gKC plasmids (2 μg per dish) and a plasmid encoding luciferase under control of a tet-responsive element promoter. After overnight culture, total RNA was prepared and used to determine levels of KC and GAPDH mRNA as above. An identical culture for each plasmid was used to prepare a cell extract for determination of luciferase activity. The levels of each mRNA were quantified as above, and the ratio of KC to GAPDH in each sample was normalized for the content of luciferase in each sample.

Introns and mRNA Half-life Is Also Seen in RAW264.7 Cells—KC is a mouse gene that is normally expressed in multiple cell types (39). To determine whether the intron content of the KC gene could modulate the half-life of the derived mRNA in cells other than HEK293 and particularly in those of mouse origin, we examined the decay of the full set of constructs described above in the mouse macrophage-like RAW264.7 tet-off cell line. This cell line does not express the endogenous KC mRNA and thus allows assessment of transfected KC mRNA in the absence of endogenous gene background (35). RAW264.7 tet-off cells were transfected and divided into separate culture dishes for different treatments. Twenty four hours later, the cells were treated with Dox for the indicated times, and residual KC and GAPDH mRNA levels decay. It remains possible that all three introns contained some common sequence determinant that might contribute to this behavior. To test this final possibility, we created a construct in which all three KC intronic sequences were deleted, and intron 1 was replaced by a sequence derived from intron 2 of the rabbit β-globin gene (38) [gKC(I1-RBG)]. Not surprisingly, mRNAs derived from this construct showed similar behavior with the full-length wild type gKC construct (Fig. 2, A and B). These results eliminate the idea that introns stabilize mRNA via a unique intron sequence or a specific intron position.

Intron-dependent Control of mRNA Half-life

FIGURE 3. Intron position and sequence do not influence mRNA decay. A, 293tet-off cells were transfected in separate pools with plasmid constructs KC cDNA, gKC, gKC(-I1), gKC(-I1,2), gKC(-I1,3) and gKC(I1-RBG) (see Fig. 1) each at 2 μg/dish, and after 3 h each transfection pool was separated into five individual Petri dishes and cultured overnight. Dox (1 μg/ml) was added, and total RNA was prepared following further incubation for the indicated times. KC and GAPDH mRNA levels were determined in each sample by Northern hybridization as described under “Experimental Procedures.” B, autoradiographs shown in A as well as two additional sets from separate experiments were quantified using the NIH Image software, and the levels of KC mRNA were normalized to those of GAPDH mRNA in each sample. Half-lives were calculated as described under “Experimental Procedures.” Values represent the mean of three experiments ± 1 S.D.
were determined by Northern hybridization. Similar to our findings in 293tet-off cells, KC mRNA derived from all intron-containing constructs decayed more slowly than that obtained from the no-intron cDNA construct (Fig. 4, A and B). Interestingly, in these cells, the half-lives for all constructs were considerably shorter than seen in 293tet-off cells.

The Intron-dependent Difference in mRNA Decay Does Not Reflect Altered Nuclear/Cytoplasmic Distribution—Following completion of splicing and polyadenylation, the mature mRNA is exported to the cytoplasm. If mature mRNA were to be retained within the nuclear compartment, exposure to the degradation machinery within the cytoplasm would be limited and would appear to prolong the half-life. Nuclear-cytoplasmic mRNA transport has been reported to occur more efficiently with mRNAs derived from intron-containing transcripts (26), and therefore, such messages should not exhibit nuclear retention relative to mRNAs from intron-free transcripts. Nevertheless, we wished to determine the extent to which the observed differences in decay might reflect differences in subcellular location. Hence we performed biochemical fractionation of nuclear and cytoplasmic compartments to determine whether there were differences in the nuclear-cytoplasmic distribution for KC mRNA derived from either gKC or KC cDNA constructs. The great majority of KC mRNA was localized in the cytoplasmic fraction, and we did not observe significant differences in the nuclear-cytoplasmic distribution of RNAs derived from either intron-free or intron-containing transcripts (Fig. 5A). Because the cultures are assumed to be in steady state, the relative abundance of RNA in each fraction should be a measure of the relative time spent in each subcellular location. The nuclear-cytoplasmic distribution of GAPDH mRNA was comparable with that of KC mRNA (Fig. 5A). The purity of subcellular extracts was demonstrated by immunoblot analysis that showed that the nuclear marker histone H3 and the cytoplasmic marker GAPDH were appropriately partitioned into nuclear and cytoplasmic fractions, respectively (Fig. 5B). Thus, the differential decay does not appear to result from unequal distribution between these two compartments.

Translational Efficiency Is Not Altered by Intron Content—It has been reported that translational efficiency may be enhanced in mRNAs from intron-containing transcripts (22, 29). To determine whether translational control for KC mRNA is impacted by the intron content of the originating DNA, 293tet-off cultures transfected with KC cDNA or gKC constructs were washed and subsequently incubated with fresh medium for 3 h. KC protein secretion into the culture medium during the 3-h period was determined by ELISA. Total cellular RNA was then isolated and used to assess KC mRNA levels by Northern blot and quantified using the NIH Image software package. The
translational efficiency, presented as the ratio of protein/RNA (arbitrary value) was not different for mRNA from intron-containing (ratio of 3.1) and intron-free (ratio of 3.2) transcripts, indicating that translational control is not influenced by this parameter.

Stabilization of KC mRNA by IL-1α Is Not Altered by Intron Content—Although constitutively unstable, KC mRNA can be markedly stabilized in cells treated with various pro-inflammatory stimuli, including IL-1α (14, 35, 40). To determine whether stimulus sensitivity would be altered in mRNAs derived from intron-free or intron-containing transcripts, we compared the sensitivity for IL-1α-mediated KC mRNA stabilization in 293tet-off cells stably transfected with either cDNA or a wild type genomic construct. Although the difference in half-life between the two constructs was maintained in the stable transfectants, both showed a significant stabilization following stimulation with IL-1α (Fig. 6, A and B).

Intron Content Influences Half-life for Other Instability Determinants—Finally, we wished to determine whether the effects of intron content of a primary transcript on mRNA stability was specific for the instability determinants present in KC mRNA or could also modulate the rapid decay dependent on 3′UTR sequences from other unstable mRNAs. To test this, we chose to examine the effects of intron content on the half-life of a chimeric mRNA containing an instability determinant of the C-C motif chemokine receptor 2 (CCR2). CCR2, the receptor for monocyte chemoattractant protein-1, is a seven-transmembrane-spanning G protein–coupled receptor that has been shown to exhibit a short half-life (41, 42). We replaced the 3′UTR of KC with a 474-bp fragment from the 3′UTR of the CCR2 mRNA both in the intron-free cDNA or the construct containing rabbit β-globin intron 2 in position 1 (KC-CCR2 and gKC(I1-RBG)-CCR2; see Fig. 1). We have recently shown that this segment of the CCR2 3′UTR, which contains no ARE motifs, is responsible for the instability of CCR2 mRNA. When these constructs were transiently transfected in 293tet-off cells, the CCR2 3′UTR sequence conferred enhanced mRNA decay (half-life = 100 min) (Fig. 7, A and B). The presence of the β-globin intron, however, produced a 2-fold reduction in the decay rate. These results were also confirmed by comparing the amount of reporter KC protein secreted from transfected cells before and after the addition of Dox (Fig. 7C).

DISCUSSION

Introns are present in most protein-coding genes in higher eukaryotes, and their presence is known to influence the metabolism of the primary transcript resulting in significantly higher levels of expression (20–22). Enhanced expression from intron-containing genes has been shown to result from effects at multiple levels, including primary transcript processing, nucleocytoplasmic transport, sensitivity to nonsense-mediated mRNA decay, and efficiency of mRNA translation (21, 22). Mature cytoplasmic mRNAs exhibit a broad range of half-life,
and it is now well accepted that regulation of their degradation is an important mechanism for the selective control of gene expression (6–10). Indeed, mRNAs encoding cytokines associated with the inflammatory response are well known to exhibit constitutive instability and stimulus-sensitive stabilization that depends upon cis-acting ARE sequence motifs located within their 3’UTRs (6–8). Although it has been reported that the presence of introns did not influence the rate of RNA decay (22), a recent report comparing mRNA half-lives and intron content from several published data bases suggests that intron content correlates with greater mRNA stability (33). To date, however, the impact of intron content on the half-life of short lived mRNAs has not been experimentally evaluated. Using the mouse KC (CXCL1) gene as a model, the present results clearly show that mRNAs containing identical sequence are markedly more stable if they are derived from primary transcripts containing intron sequence that required splicing as part of the maturation process. This effect is independent of individual intron sequences or position and requires only a single intron. Although the mRNA half-lives are significantly prolonged, neither the translational yield nor sensitivity to stabilization by IL-1 is altered by intron presence.

Although introns have been documented to have a large positive effect on gene expression, there are varied reports regarding the importance of specific intron sequence and position. For example, different intron sequences positioned identically elicited entirely opposite effects on protein expression at the translational level (28), whereas in another report, the intron-mediated enhancement of mRNA accumulation was independent of intron sequence (43). In some cases, the same intron sequence placed into different positions could also have dramatically different consequences on gene expression (20, 22, 28). In the present study, the modulation of mRNA stability was found to be independent of intron sequence and position, and a single intron was sufficient to produce the effect.

There is an extensive literature describing the contribution of intron position toward mRNA degradation involving the NMD. A termination codon is recognized as premature if located upstream of a splicing-generated exon-exon junction, within a distance of at least 50–55 nucleotides, leading to rapid degradation (30–32). NMD is recognized as an important mechanism not only because it functions as a quality control to eliminate abnormal transcripts, but also because it may modulate the levels of naturally occurring transcripts (30). According to the results of data base and microarray analyses, an estimated one-third of naturally occurring, alternatively spliced mRNAs should be targeted for NMD, which has led to the proposal that NMD is widely used by mammalian cells to achieve proper levels of normal gene expression (30, 44, 45).

The degradation of mature cytoplasmic mRNAs is also a well documented means for regulation of gene expression generally associated with mRNAs that contain ARE motifs within their 3’UTRs. Although as many as 4000 ARE-containing mRNAs have been identified, representing as much as 5–8% of the human genome (46), whether these all exhibit significant instability has not been determined. Although intron position is clearly the most important determinant for NMD, there has been no experimental documentation to date of an effect of intron content on ARE-mediated mRNA decay. This study provides a direct demonstration that intron content markedly lengthens the half-life of a model ARE-containing mRNA. Moreover, the intron-dependent modulation of mRNA half-life was also observed using a chimeric mRNA containing an instability determinant derived from the human CCR2 gene 3’UTR, a sequence also known to exhibit instability (41, 42). Although KC mRNA is well documented to contain multiple ARE motifs (14, 36), the CCR2 mRNA instability determinant contains no ARE motifs. This suggests that the intron effect is not selectively linked with a particular subset of ARE instability determinants.

Because the mRNAs derived from intron-containing and intron-free transcripts contain identical sequence, their differential decay rates suggest that the excision of an intron may leave a mark in spliced mRNA that will influence its subsequent cytoplasmic metabolism. During splicing, an exon junction complex is deposited on the RNA 20–24 nucleotides upstream of splice junctions (47). This complex, which influences RNA splicing, export, cytoplasmic localization, and NMD (32, 47, 48), is bound to the mRNA until being displaced by the ribosome during the pioneer round of translation (49). Several proteins exhibiting binding specificity for ARE sequence motifs have been shown to promote rapid decay of such mRNAs by recruitment of mRNA decay machinery via protein-protein interactions (50, 51). Therefore, it is possible that some residual component of the exon junction complex can influence the function of such decay-promoting ARE-binding proteins resulting in reduced rates of degradation as seen here. Interestingly, whereas intron-containing sequences have been shown previously to exhibit more efficient nuclear cytoplasmic transport and translational efficiency, these properties did not appear to be altered in the present study (21, 22). The rate-limiting step in most mRNA decay appears to be deadenylation or removal of the poly(A) tail (52), and hence the rate of deadenylation is likely to be reduced for mRNAs derived from intron-containing transcripts as compared with those from intron-free transcripts.

The evidence presented here demonstrates yet another mechanism by which intron content may impact on the efficiency of gene expression. Previous reports examining RNA decay have not identified changes in mature mRNA decay. This might indicate that only a subset of short lived mRNAs is sensitive to the prolongation of half-life or that the effect of introns, although operative on long lived mRNAs, has a relatively more modest impact than seen with short lived mRNAs where stabilization will be readily detected and significantly affect the magnitude of protein production as well. Given the growing recognition of the importance of mRNA half-life in regulating transient biologic responses like inflammation, the recognition that intron content and the processing of primary transcripts can appreciably alter the post-transcriptional function should be considered in future study of the mechanisms regulating these processes.

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