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Synthesis, secretion and processing of α-factor-interferon fusion proteins in yeast

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ABSTRACT

A gene fusion consisting of 960 base pairs of 5'-flanking region of the yeast MFα1 gene, 257 base pairs coding for α-factor prepro sequence, and a modified human IFN-α1 gene was constructed. MATα cells containing the chimeric gene synthesized and secreted active IFN-α1 into the growth medium. The secreted interferon molecules contained the last 4 amino acids of α-factor prepro sequence and the amino acids encoded by the DNA modifications introduced at the beginning of IFN-α1 gene. DNA sequences coding for these amino acids were removed by oligonucleotide-directed in vitro mutagenesis. Yeast cells transformed with expression plasmids containing the altered junction synthesized and secreted human IFN-α1 with the natural NH_2-terminus.

INTRODUCTION

Many proteins destined for secretion are synthesized in precursor forms containing NH_2-terminal extension called the signal sequence. The signal sequence is removed by proteolytic cleavage during or after the export to yield the mature protein. Experiments with hybrid fusion proteins have provided evidence for some common features of protein export in both the eucaryotes and the procaryotes. Talmadge and coworkers (1) found that either the bacterial penicillinase signal or the eucaryotic preproinsulin signal is sufficient to transport rat proinsulin polypeptide across the procaryotic cytoplasmic membrane (2). Studies with heterologous expression systems containing cDNAs for human pre-interferons have shown that the eucaryotic microorganism Saccharomyces cerevisiae can recognize human secretion signals (3). Although processing was not always correct and only about 30 percent of the interferon activity was secreted, a significant proportion of the interferon molecules isolated from the growth media had the same amino termini as the natural mature interferons (3).

The yeast Saccharomyces cerevisiae secretes only a limited number of proteins into the culture medium. One of the proteins that is found in the

medium is α-pheromone or α-factor (4). Duntze and coworkers (5,6) and subsequently other groups (7,8,9) determined that α-factor is a family of four oligopeptides of 12-13 amino acid residues having the basic sequence: H_2N-(Trp)-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met(or MetSO)-Tyr-COOH. Yeast contains two genes coding for the α mating pheromone. One of the two genes, MFα1 (10,11) codes for a precursor protein of 165 amino acids containing 4 copies of the α-factor, whereas the other gene, MFα2 (11), codes for a precursor of 120 amino acids containing 2 copies of the pheromone. In this report we describe experiments which show that the MFα1 promoter and the α-factor secretion signals can direct the synthesis and secretion of the human IFN-α1 (leukocyte interferon D). We have determined the processing of the natural α-factor prepro-IFN-α1 fusion protein. In addition, we have deleted sequences at the junction of the chimeric gene and shown that altered junctions may be used to synthesize and secrete proteins with desired NH_2-terminus.

MATERIALS AND METHODS

Yeast and Bacterial Strains.

Yeast strain 20B-12 (α trp1 pep4) (12) was obtained from the Yeast Genetics Stock Center. Yeast strain A88 (a trp1) is from our culture collection. E. coli K-12 strain 294 (endA thi$^-$ hsr$^-$ hsm$^+$) (13) was used for bacterial plasmid transformation. E. coli strain JM101 (14) was used for experiments involving bacteriophage M13.

Growth Media.

The routine yeast growth medium contained 1 percent Bacto-yeast extract, 2 percent Bacto-peptone and 2 percent dextrose. Yeast minimal medium (YNB) contained 0.67 percent Bacto-yeast nitrogen base without amino acids, 2 percent dextrose. The minimal medium supplemented with 1 M sorbitol and 3 percent agar was used for yeast transformations. Yeast transformants were grown on YNB containing 0.5 percent casamino acids. Bacterial growth medium for strain 294 was LB (15) which was supplemented with 20 μg/ml ampicillin when used for transformation. E. coli JM101 was grown on 2YT (15).

Transformations.

E. coli 294 was transformed as described before (16). E. coli JM101 cells were transformed as described by Messing (14). Yeast were transformed essentially as described (17,18).

DNA Preparations.

Synthetic oligodeoxynucleotides were prepared by the phosphotriester

method (19). The 24-mer oligonucleotide 5'-AGGGAGATCACATCTTTTATCCAA-3' was used for site-directed mutagenesis and for the screening of the mutant recombinant phages. The 15-mer oligonucleotide 5'-TGCCAGGAGCATCAA-3' was used as a sequencing primer.

Plasmid DNAs were prepared by the cleared lysate method (20) and were purified by Bio-Rad Agarose A-50 column chromatography. A quick-screening procedure (21) was used to obtain small amounts of plasmid DNAs from individual E. coli transformants. DNA restriction fragments were isolated by electroelution from a 1 percent agarose gel followed by phenol/chloroform extraction and ethanol precipitation.

In Vitro Mutagenesis.

A variation of previously described protocols (22) was used for oligonucleotide-directed deletion mutagenesis. Single-stranded DNA template was prepared from the recombinant M13mp8 phage containing the appropriate insert. This template was annealed with the phosphorylated synthetic oligonucleotide of 24 bases in length. This primer-template intermediate was subjected to extension and ligation reaction at 23°C for 2 hours in the presence of 500 mM dATP, 100 mM dTTP, 100 mM dGTP, 100 mM dCTP, 20 mM dATP, 3 units DNA polymerase (Klenow), and 400 units T4 DNA ligase in 10 mM Tris pH7.4, 50 mM NaCl and 10 mM MgSO4. Then additional 3 units of DNA polymerase (Klenow) and 400 units of T4 DNA ligase was added and mixture incubated for 2 hours at 23°C followed by incubation at 14°C for 15 hours. Aliquots of this mixture were used to transform E. coli JM101.

Screening of Phage Plaques.

^{32}P-labeled (23) 24-mer oligonucleotide described above was used to screen recombinant M13 phages by in situ plaque hybridization (24). Filters were hybridized overnight at 42°C in 10 mM Tris (pH 7.5), 6 mM EDTA, 0.8 M NaCl, 1X Denhardt's solution, 0.5 percent NP-40, and 0.1 mg/ml E. coli tRNA. Filters were washed 3 times for 20 min in 6X SSC, 0.1 percent SDS at 30°C. Dried filters were exposed to Kodak XR-2 X-ray film with DuPont Lightning-Plus intensifying screen at -80°C.

DNA Sequence Determination.

DNA sequence analysis was carried out by the chain termination method (25) using recombinant phage M13mp8 as a source of single stranded template DNA prepared as described (14). A synthetic pentadecanucleotide complementary to IFN-α1 coding strand near the region of interest was used for priming E. coli DNA polymerase I (large fragment) in the presence of

dideoxynucleotide triphosphates using α-^{32}P dCTP for labeling synthesized chains.

Interferon Assay and Purification.

Individual colonies of the transformants were grown at 30°C in 20 ml YNB+CAA to an A_{660} of approximately 10. For assay, a 10 ml aliquot was centrifuged at 7K rpm for 10 minutes in a Sorval SM24 rotor. Various dilutions of supernate (media) and cell extracts prepared by vortexing with glass beads as described before (3) were assayed for interferon activity by comparison with interferon standards using a vesicular stomatitis virus challenge of MDBK (bovine kidney) tissue culture cells (26). The interferon activity is expressed as units relative to the NIH leukocyte interferon standard G-203-901-527.

Interferon was purified from culture medium from which cells had been removed by centrifugation. Frozen media were concentrated and dialyzed against 25mM Tris, 10mM EDTA, pH 8.0 in a 2.5 liter Amicon stirred cell (Amicon 2000) using a YM-5 ultrafiltration membrane. One ml of the concentrated medium was precipitated with 4 ml acetone, spun in a microfuge and washed with acetone. The pellet was resuspended in 0.1 percent TFA and further purified by HPLC on a Synchropak RP-P column. The column was eluted with a linear gradient of 0 to 100 percent acetonitrile in 0.1 percent TFA in 60 minutes. A 12 µg sample of purified IFN-α1 was chromatographed as a standard. The peaks of absorbance at 280 nm were collected and sequenced.

Determination of NH2-terminal Amino Acid Sequences.

Sequence analysis was based on Edman degradation (27). Liquid samples were introduced into the cup of a modified Beckman 890B spinning cup sequencer. Polybrene™ was used as a carrier in the cup. Reagents used were Beckman's sequence grade 0.1 molar Quadrol buffer, phenyl-isothiocynate, and heptafluorabutyric acid. Norleucine was added during each cycle with the Quadrol buffer to serve as an internal standard. The presence of PTH-norleucine in each chromatogram aided in the identification of PTH amino acids by retention time.

RESULTS

Construction of MFα1-IFN-α1 Fusion.

A 1230 bp DNA fragment that contains 960 bp of MFα1 5'-flanking sequence and the sequence coding for the prepro portion of the α-factor was fused with a modified human IFN-α1 gene, as outlined in Fig. 1. The DNA sequences coding for the α-factor peptides were removed from the MFα1 clone, p53

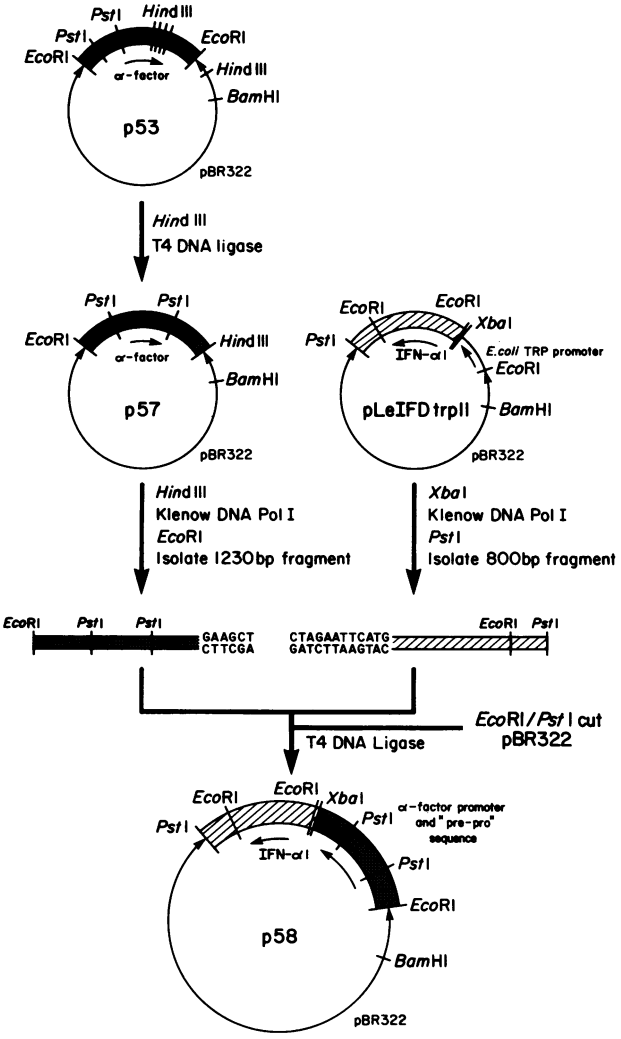

Figure 1. Construction of MFα1-IFN-α1 chimeric gene. The plasmid p53 has been described before (11). Plasmid pLeIFDtrp11 is identical to plasmid pLeIFD3 (28).

(11). The resulting plasmid, p57, contained the promoter sequences and the sequence corresponding to 89 amino acids of the α-factor prepro protein. This sequence was joined with human IFN-α1 gene to form the plasmid p58. The human IFN-α1 gene (28) was modified such that DNA sequences corresponding to Leu-Glu-Phe had been added before the initiating methionine codon. These

modifications resulted in the following sequence at the junction:

α-factor prepro sequence	Modified IFN-α1 gene
...Lys Arg Glu Ala Glu Ala	Leu Glu Phe Met Cys Asp ...
...AAA AGA GAG GCT GAA GCT	CTA GAA TTC ATG TGT GAT ...
...TTT TCT CTC CGA CTT CGA	GAT CTT AAG TAC ACA CTA ...

Expression of IFN-α1.

An expression plasmid, p60, shown in Fig. 2, was constructed using the gene fusion described above. This plasmid contains the pBR322 DNA needed for its selection and replication in E. coli (29). In addition, it contains the yeast TRP1 gene on an EcoRI to PstI fragment from chromosome IV and a yeast origin of replication on a PstI to EcoRI fragment from the endogenous 2μ plasmid DNA. These two DNA fragments from yeast allow for its selection in yeast and for its autonomous replication and maintenance as a plasmid. The 2μ DNA also provides a transcription termination/polyadenylation signal (30). The p60 plasmid was introduced into the yeast strain 20B-12 (α pep4-3 trp1) (12) and growth medium and cell extracts of the trp^+ transformants were assayed for interferon activity. We found $100x10^6$ units of interferon activity per liter of growth medium. The cell extracts also yielded interferon at the rate of $100x10^6$ units per liter of culture. As expected, the yeast strain A88 transformants containing p60 produced no detectable interferon activity, indicating that normal mating type regulation is maintained.

Processing of Secreted Interferon.

The interferon activity was purified from the growth medium and

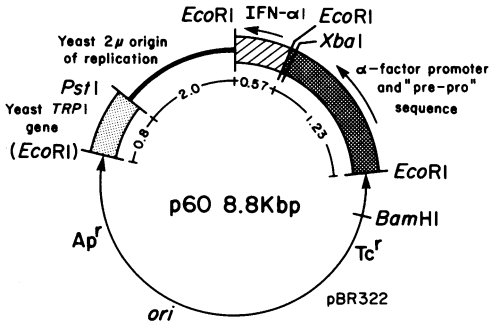

Figure 2. Plasmid for expression of IFN-α1. The EcoRI fragment containing MFα1-IFN-α1 fusion was isolated after partial EcoRI digestion of p58 (Fig. 1) and inserted into the EcoRI site of YEp9T. The YEp9T had been previously made by replacing the EcoRI-SalI fragment in plasmid YEp1PT (3) with the EcoRI-SalI fragment from pBR322.

Table 1
NH_2-Terminal Sequence Analysis of IFN-α1 from the Growth Medium of Yeast Strain 20B-12 Containing Plasmid p60.

Cycle	Residue	Yield (nanomoles)
1	Glutamic acid	.44
2	Alanine	.38
3	Glutamic acid	.24
4	Alanine	.26
5	Leucine	.20
6	Glutamic acid	.18
7	Phenylalanine	.15
8	Methionine	.08
9	Cysteine* (no modification)	--
10	Aspartic acid	.06
11	Leucine	.11
12	Proline	.02
13	Glutamic acid	.05

*Unmodified cysteine yields no detectable PTH-amino acid derivation from the spinning cup sequencer.

NH_2-terminal amino acid sequence was determined by the method of Edman degradation (27). The amino acid sequence analysis (Table 1) showed only one species of interferon molecule with the NH_2-terminal sequence: NH_2-Glu-Ala-Glu-Ala-Leu-Glu-Phe-Met-Cys-Asp... The sequence was determined by analyzing the pattern of increase, over background amino acids, of specific amino acids in each cycle of Edman degradation. Other amino acids, which were detected in various amounts in different cycles, could not be arranged to form any peptide that could be derived from the sequence (deduced from the DNA sequence of the gene fusion) of the hybrid protein. Thus the protein produced contains 8 extra amino acids, 4 (Leu-Glu-Phe-Met) encoded by the DNA sequence added to the interferon gene, and 4 (Glu-Ala-Glu-Ala) from the prepro sequence of α-factor. It should be noted that the NH_2-terminal amino acid of natural interferon is cysteine; the initiator methionine codon (ATG) had been added to IFN-α1 gene in pLeIFDtrp11 plasmid (Fig. 1) in order to produce this protein in *E. coli* (28). As indicated above, the polypeptide containing this 8 amino acid NH_2-terminal extension retains interferon activity.

Oligonucleotide-Directed Deletion Mutagenesis of MFα1-IFN-α1 Junction Sequences.

To test whether enzymes involved in the processing of α-factor precursor would function if the junction between the α-factor prepro and IFN-α1 gene were changed, we created an *in vitro* deletion such that the removal of the

modified prepro sequence would result in the release of a mature interferon molecule containing the natural N-terminus. We therefore decided to delete DNA sequences coding for the 8 amino acids mentioned above. Such a deletion creates the following sequence at the junction:

From α-factor leader	Mature IFN-α1
...Ser Leu Asp Lys Arg	Cys Asp Leu Pro Glu ...
...TCT TTG GAT AAA AGA	TGT GAT CTC CCT GAG ...
...AGA AAC CTA TTT TCT	ACA CTA GAG GGA CTC ...

An outline of the procedure to obtain appropriate junction between α-factor prepro and IFN-α1 sequences is shown in Fig. 3. A DNA fragment

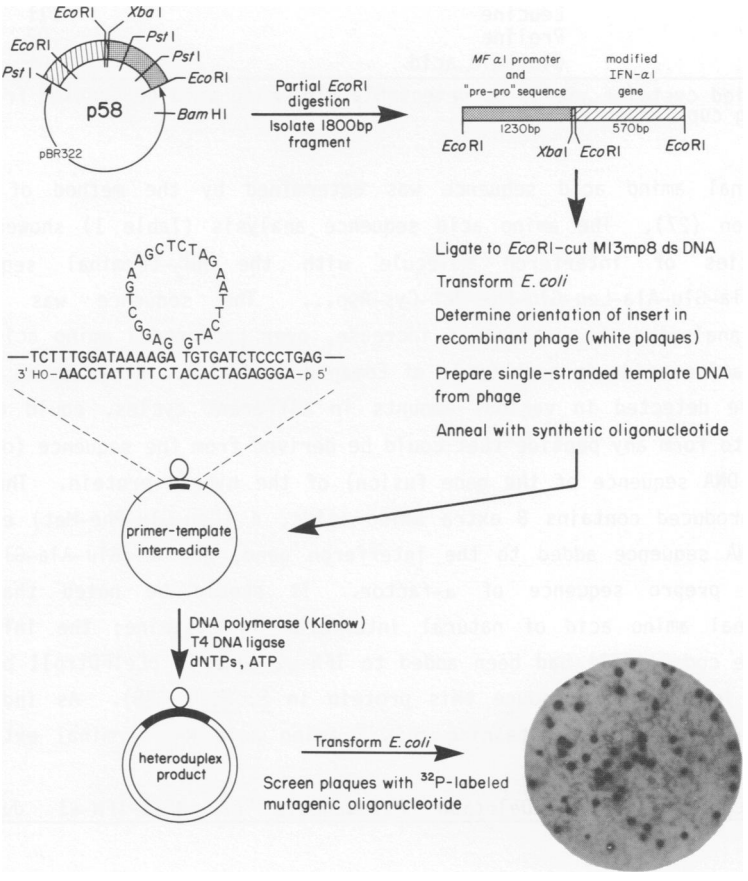

Figure 3. Scheme for oligonucleotide-directed deletion mutagenesis of MFα1-IFN-α1 junction sequences. See MATERIALS AND METHODS for details.

containing the MFα1 promoter and prepro sequence and the modified IFN-α1 gene was isolated and cloned into the EcoRI site of M13 mp8 (14). Single-stranded DNA template was prepared from the recombinant phage containing the insert in the appropriate orientation. This template was annealed with a phosphorylated oligonucleotide. The synthetic oligonucleotide is 24 bases long and is complementary to 12 bases coding for Leu-Asp-Lys-Arg near the C-terminus of the prepro sequence and to 12 bases coding for Cys-Asp-Leu-Pro, the first 4 amino acids of natural IFN-α1. The primer-template intermediate was subjected to extension and ligation reaction as described in MATERIALS AND METHODS. This mixture was used to transform E. coli JM101. The phage plaques were screened for hybridization with the ^{32}P-labeled oligonucleotide. Template DNA from 2 positive recombinant phages was prepared and sequenced using a primer complementary to IFN-α1 DNA. Double stranded DNA was prepared from one recombinant phage that contained the desired deletion (deletion of 24 nucleotides shown as a loop in Fig. 3).

Interferon Produced by Yeast Containing the Modified Junction.

The EcoRI fragment containing the modified junction, isolated from DNA described above, was used to construct an expression plasmid, p76, which, except for the 24 bp deletion, is identical to p60 (Fig. 2). Cultures of strain 20B-12 transformed with p76 and grown to an A_{660} of 10 produced $100x10^6$ units of interferon per liter of growth medium. Similarly, the cell extracts yielded interferon at a level of $100x10^6$ units per liter of culture.

Amino terminal sequence analysis, carried out as described for Table

Table 2

NH_2-Terminal Sequence Analysis of IFN-α1 from the Growth Medium of Yeast Strain 20B-12 Containing Plasmid p76.

Cycle	Residue	Yield (nanomoles)
1	Cysteine* (no modification)	--
2	Aspartic acid	.60
3	Leucine	1.20
4	Proline	.30
5	Glutamic acid	.46
6	Threonine	.09
7	Histidine	.19
8	Serine	.04
9	Leucine	.64

*Unmodified cysteine yields no detectable PTH-amino acid derivative from the spinning cup sequencer.

1, of interferon purified from the culture medium is shown in Table 2. The first 8 NH_2-terminal amino acids of the protein were Cys-Asp-Leu-Pro-Glu-Thr-His-Ser, showing that the fusion protein had been correctly processed to yield mature IFN-α1 with natural NH_2-terminus.

DISCUSSION

The studies described in this report demonstrate that the first 89 amino acids of the α-factor precursor encoded by the MFα1 gene are sufficient to direct secretion of a heterologous protein when attached to this prepro sequence. Approximately half of the interferon produced by cells containing a hybrid gene constructed by fusion of α-factor prepro and mature IFN-α1 cDNA is secreted into the growth medium. The hybrid fusion protein is recognized by the enzyme(s) involved in the processing of α-factor precursor (and other) proteins such that the secreted interferon molecules are free of most or all of α-factor prepro sequence. Emr et al. (31) reached a similar conclusion by studying a fusion between the MFα1 gene and the yeast SUC2 gene. They found that active invertase was secreted into the periplasmic space. Since the fusion studied by them retained most (14/19) of the invertase signal sequence (32,33), it is possible, though somewhat unlikely, that these residues contributed in the secretion of the hybrid protein (31). The NH_2-terminal sequence of the secreted invertase directed by the fusion protein was not determined. It has been shown (34) that the invertase signal sequence is sufficient to direct secretion of human IFN-α2 and that such a hybrid fusion protein is correctly processed to yield mature interferon.

The interferon molecules secreted by the cells containing a fusion of wild-type α-factor prepro sequence retain the last four amino acids (Glu-Ala-Glu-Ala) of α-factor prepro-leader (see Table 1). Thus the processing of the fusion protein is similar to that of the α-factor precursor protein in ste13 mutants which lack a membrane-bound dipeptidyl aminopeptidase that is specific to -X-Ala sequences (35). It is possible that the MFα1-IFN-α1 fusion protein is not recognized by this aminopeptidase. However, the observation that even the wild-type α cells carrying multiple copies of MFα1 gene produce mainly incompletely processed α-factor indicates that the dipeptidyl aminopeptidase is rate-limiting (35). Thus even if the hybrid fusion protein were recognized by the above peptidase, the bulk of the interferon molecules are expected to contain the NH_2-terminal Glu-Ala-Glu-Ala extensions derived from the α-factor prepro leader. We estimate that at least 80 percent of the interferon

molecules secreted by transformants carrying p60 and p76 had the NH_2-terminal sequence shown in Table 1 and Table 2, respectively. Our analysis would not have detected another species of interferon if several other forms of the molecule comprised up to a total of 20 percent of the secreted interferon.

Our results with the fusion made with the MFα1 deletion mutant also bear on the specificity of the enzyme(s) involved in the processing of the MFα1 precursor at Lys-Arg residues. It should be noted that all four α-factor units in the precursor encoded by MFα1 gene are immediately preceded by 6 or 8 amino acids, which invariably contain the Lys-Arg-Glu-Ala sequence (10,11). In addition, this particular sequence is also present before the two α-factor copies in the pheromone precursor encoded by the MFα2 gene (11). Yet, when the hybrid precursor protein, encoded by the plasmid p76, contained the sequence Lys-Arg-Cys-Asp at the junction of α-factor prepro and IFN-α1, interferon molecules with the sequence NH_2-Cys-Asp... (Table 2) were efficiently secreted.

Studies of secretory pathway in the yeast Saccharomyces cerevisiae have been carried out (36) using the enzymes acid phosphatase and invertase, which are secreted into the periplasmic space. The findings described here, which show that the MFα1 prepro leader can efficiently direct export of a heterologous protein, provide the opportunity to use other suitable proteins in the study of protein export in yeast.

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