Ribozyme processed tRNA transcripts with unfriendly internal promoter for T7 RNA polymerase: production and activity

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Abstract A limitation for a universal use of T7 RNA polymerase for in vitro tRNA transcription lies in the nature of the often unfavorable 5′-terminal sequence of the gene to be transcribed. To overcome this drawback, a hammerhead ribozyme sequence was introduced between a strong T7 RNA polymerase promoter and the tDNA sequence. Transcription of this construct gives rise to a 'transzyme' molecule, the autocatalytic activity of which liberates a 5′-OH tRNA transcript starting with the proper nucleotide. The method was optimized for transcription of yeast $tRNA^{Tyr}$, starting with 5′-C_1, and operates as well for yeast $tRNA^{Asp}$ with 5′-U_1. Although the tRNAs produced by the transzyme method are not phosphorylated, they are fully active in aminoacylation with k_{cat} and K_m parameters quasi identical to those of their phosphorylated counterparts.

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Key words: Aminoacylation; Promoter; Ribozyme; Transcription; Transzyme; $tRNA^{Tyr}$

1. Introduction

Use of T7 RNA polymerase became popular for the preparation of tRNA transcripts [1] despite some intrinsic limitations linked to the nature of its promoter which encompasses the six first nucleotides to be transcribed [2]. Although the wild-type 5′-$^{+1}$GGGAGA^{+6}-3′ internal promoter, present in phage T7 genes, is the most efficient for transcription, the polymerase tolerates other internal nucleotide combinations. Efficiency of transcription depends mainly on the nature of the first nucleotide to be incorporated, namely a G-residue, and subsequently that of the following five residues (but poor transcription can also occur for molecules with seemingly favorable internal promoter sequences, as yeast $tRNA^{Val}$ starting with G_1 [3]). Since a majority of cytoplasmic tRNA sequences have a G_1 residue [4], this method has been applied successfully to prepare tRNA transcripts of many specificities and was, among others, the prerequisite for straightforward in vitro studies on their identity [5]. However, several families of natural tRNA lack a G at their 5′-end [3] and thus escaped easy investigations. Likewise, a number of potential tRNA variants in the amino acid acceptor stem one would like to study for effects on identity or other properties cannot be prepared by classical in vitro transcription.

Based on the knowledge on small catalytic RNAs [6], we have developed a method using a ribozyme to process tran-

scribed tRNA precursors. A hammerhead ribozyme gene sequence was introduced between a strong T7 RNA polymerase G-rich promoter and the first nucleotide of the tDNA sequence. Because of the presence of the specific and efficient natural G-rich promoter, it was expected that T7 RNA polymerase would transcribe this gene efficiently and produce RNA molecules constituted by the hammerhead ribozyme followed by the desired tRNA sequence terminating by -CCA_{OH}. It was anticipated that autocatalytic cleavage of the transcript will take place in 'cis' just before nucleotide +1 of the tRNA, generating full-length tRNAs with 5′-OH. As model tRNA to establish the method we have chosen yeast $tRNA^{Tyr}$ starting with 5′-CUCUCG, and thus very difficult to produce by classical transcription with T7 RNA polymerase.

In this paper, we show (i) that large amounts of $tRNA^{Tyr}$ transcripts can readily be obtained, (ii) that these transcripts are fully active, despite the absence of a 5′-phosphate as in canonical $tRNA^{Tyr}$ and (iii) that the method can be used as well to prepare $tRNA^{Asp}$ transcripts starting with 5′-U_1.

2. Materials and methods

2.1. Chemicals and enzymes

T7 RNA polymerase was purified as described in [7]. Oligonucleotides were from NAPS GmbH (Goettingen, Germany), L-[^{14}C]tyrosine (448 mCi/mmol), [α-^{32}P]ATP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) from Amersham (Les Ulis, France). Restriction enzymes (*Bst*NI, *Hin*dIII, *Bam*HI) and T4 polynucleotide kinase were from New England Biolabs (Beverly, MA, USA). T4 DNA ligase was purchased from Boehringer Mannheim (Meylan, France).

2.2. Purification of yeast TyrRS

The plasmid containing yeast TyrRS gene was overexpressed in BL21(DE3)(pLysS). The enzyme was purified according to a method described previously for purification of yeast ArgRS [8]. The activity of the purified TyrRS was 883 U/mg/min.

2.3. Cloning and artificial gene production

$tRNA^{Tyr}$ gene was cloned from eight overlapping oligonucleotides, corresponding to T7 RNA polymerase promoter directly connected to the sequence of yeast $tRNA^{Tyr}$, as described in [9]. Transzyme^{Tyr} (for terminology, see Section 3.1) was cloned from 10 overlapping oligonucleotides, using the same protocol as for $tRNA^{Tyr}$. Sequences of the oligonucleotides used for $tRNA^{Tyr}$ cloning are given below. Numbers 1–4 correspond to the plus-strand and 5–8 to the minus-strand. For transzyme^{Tyr}, oligonucleotides 1 and 2 were replaced by oligonucleotides 9, 10 and 11, and 5 by 12 and 13 in order to construct the precursor.

No. 1: 5′-AGCTTAATACGACTCACTATACTCTC-3′; no. 2: 5′-GGTAGCCAAGTTGGTTTAAGGCGC-3′; no. 3: 5′-AAGACTG-TAAATCTTGAGATCGGGCGT-3′; no. 4: 5′-TCGACTCGCCCC-CGGGAGACCAG-3′; no. 5: 5′-GGCTACCGAGAGTATAGTGA-GTCGTATTA-3′; no. 6: 5′-TTACAGTCTTGCGCCTTAAACCA-ACTT-3′; no. 7: 5′-GAGTCGAACGCCCGATCTCAAGAT-3′; no. 8: 5′-GATCCTGGTCTCCCGGGGGGC-3′; no. 9: 5′-AGCTT-AATACGACTCACTATAGGGAGAAGAG-3′; no. 10: 5′-CTGAT-GAGTCCGTGAGGACGAAACGGTACCCGGTACCGTC-3′; no.

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Abbreviations: AspRS, aspartate-tRNA synthetase; TyrRS, tyrosyl-tRNA synthetase

11: 5′-CTCTCGGTAGCCAAGTTGGTTTAAGGCGC-3′; no. 12: 5′-CGGACTCATCAGCTCTTCTCCCTATAGTGAGTCGTATTA-3′; no. 13: 5′-GGCTACCGAGAGACGGTACCGGGTACCGTT-TCGTCCTCA-3′.

2.4. In vitro transcription

Run off transcripts of tRNA^{Tyr} were obtained by incubation for 3 h at 37°C of 50-μl mixtures containing 40 mM Tris-HCl, pH 8.1, 22 or 30 mM MgCl$_2$, 1 mM spermidine, 5 mM DTE, 0.01% Triton X-100, 4 mM of ATP, CTP, GTP and UTP, 16 mM CMP, 1 μCi [α-^{32}P]ATP (3000 Ci/mmol), 25 U T7 RNA polymerase, and 4 μg of linearized plasmid. Notice the presence of CMP in the incubation mixtures, which was added to favor synthesis of molecules starting with a 5′-monophosphate instead of a 5′-triphosphate. Run off transcripts of transzyme^{Tyr} were obtained under similar conditions without CMP.

After transzyme transcription, reaction mixtures were either incubated 1 h at 60°C or diluted 5 times and then incubated 1 h at 60°C in order to enhance autocatalytic cleavage. Reactions were stopped by phenol/ether extraction followed by ethanol precipitation. Since T7 RNA polymerase makes some errors at the 3′-end of the transcripts [10], full-length transcripts correctly ending with the 3′-CCA$_{OH}$ sequence have been purified by a 12% preparative denaturing gel (40×30×0.2 cm³). The products of 1 ml of transzyme transcription were loaded onto the gel in a 24-cm wide pocket and resolution to one nucleotide is obtained after electrophoresis at 55 mA for ≈18 h. Transcripts were eluted from gel slices using a Schleicher & Schuell (Dassel, Germany) Biotrap. Concentrations of transcripts were calculated from absorbance measurements at 260 nm, assuming that 1 absorbance unit (1 cm pathway) corresponds to 40 μg/ml.

2.5. Aminoacylation assays

Activity of the synthesized transcripts was tested by their aminoacylation capacity. Transcripts were renatured by heating 2 min at 60°C and slowly cooling to room temperature. Aminoacylation reactions were performed in 150 mM HEPES-NaOH, pH 7.5, 15 mM MgCl$_2$, 10 mM ATP, 0.5 mg/ml BSA, 20 μM DTE, 11 μM ^{14}C-labeled tyrosine (560 cpm/pmol), and appropriate amounts of tRNA transcripts and yeast TyrRS. After incubation at 30°C, aliquots were removed, spotted on Whatman 3 MM paper, and trichloroacetic acid precipitated. Incorporation of radioactive tyrosine was measured by liquid scintillation spectroscopy. Kinetic constants (K_m, k_{cat}) were derived from Lineweaver-Burk plots. Since the concentration of amino acid was subsaturating, only apparent kinetic parameters are given. They represent an average of at least two independent experiments. Errors on kinetic parameters are about 20%.

2.6. Analysis of the 5′-terminus of transcripts

The lack of a 5′-phosphate in the ribozyme processed tRNA transcripts was checked by phosphorylation of the 5′-extremity of these molecules in the presence of [γ-^{32}P]ATP, using phage T4 polynucleotide kinase as described by [11]. Transcripts classically obtained, and possessing a 5′-phosphate, were submitted to this treatment as negative control. Aliquots of the reaction mixtures were submitted to electrophoresis onto 12% denaturing polyacrylamide gel.

3. Results and discussion

3.1. Methodology

Although tRNAs are widely studied for their structure and function, some of them escape profound investigations because of difficulties to obtain their corresponding in vitro transcript. These difficulties are due to the sequence of the promoter recognized by T7 RNA polymerase which encompasses the 6 first nucleotides to be transcribed. Among the 4^6 possible internal promoter sequences, those rich in G residues at their most terminal 5′-positions are good substrates of the polymerase and conversely, those starting with non-G residues, and sometimes even if starting with G, are poor substrates.

In this work, we propose a method to overcome this dependence of transcription on the gene sequence. This method is based on transcription of an artificial gene constituted by an autocatalytic RNA, a ribozyme, preceded by the strong wild-type T7 promoter sequence and followed by the tRNA sequence (Fig. 1A). A *Bst*N1 restriction site constitutes the 3′-end of the gene and should ensure production of transcripts preferably terminating by a 3′-CCA$_{OH}$-end. These RNA molecules are named 'transzyme^{aa}' (with 'trans' for transfer, '-zyme' for ribozyme, and the superscript for the amino acid specificity). The choice of the ribozyme was dictated by size and sequence requirements. The hammerhead ribozyme [12] was the ideal candidate since its size is small and its sequence can be easily adapted to the 5′-sequence of any tRNA. Only four nucleotides, not involved in the catalytic activity, have to be complementary to the 5′-end of the tRNA sequence, as illustrated by the examples of tRNA^{Tyr} and tRNA^{Asp} (see 'Hybridization Boxes' or HB in Fig. 1B). This hybridization box may be of variable size depending on the thermodynamic stability of the base-pairs formed between the promoter and the 5′-end of the tRNA. The 5′ domains of transzymes are further expected to fold in the hammerhead 3D structure, so

Table 1
Comparative in vitro transcriptions of yeast tRNA^{Tyr} and transzyme^{Tyr} genes

Procedures	tRNA^{Tyr}		Transzyme^{Tyr}					
	1	2	3	4	5	6	7	8
Critical parameters for transcription and cleavage								
For transcription:								
MgCl$_2$ (mM)	22	30	22	30	22	30	22	30
For cleavage:								
Temperature (°C)	–	–	37	37	60	60	60	60
Dilution (x-fold)	–	–	No	No	No	No	5	5
tRNA production								
Amount of transcript (pmol/50 μl)	10	25	1353	2463	1200	2225	1523	2850
Transcription cycles[a] (number)	2.3	5.7	274	520	254	471	340	638
Autocleavage of transcript (%)	–	–	11	42	44	42	86	88
Amount of tRNA^{Tyr} (pmol/50 μl)	10	25	143	1023	530	945	1313	2510

The numerical data correspond to the experiments displayed in Fig. 1C (the eight conditions assayed have the same numbering as the lanes of the autoradiogram). The transcription conditions at 37°C are described in Section 2.4 with changes for MgCl$_2$ concentrations indicated. The changes in temperature, Mg^{2+} concentration, and dilution of the transcription mix to optimize cleavage conditions are indicated. The numerical values are for 50 μl of transcription mix.
[a]Note that the theoretical number of cycles under conditions 3, 5 and 7 should be the same (experimental mean is 290±45), as well as for conditions 4, 6 and 8 (experimental mean is 540±86).
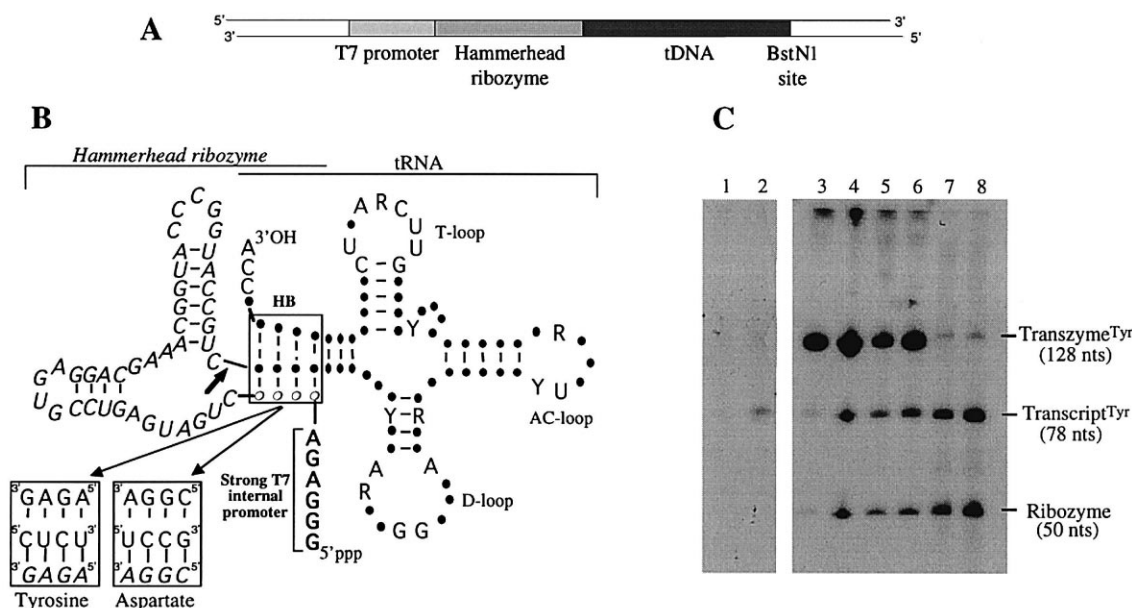
Fig. 1. Artificial transzyme[aa] gene, folding and production of its run off transcript. A: Gene construction of the transzyme[Tyr]. B: Structural organization of transzyme[aa] transcripts. The three domains of the transzyme are highlighted: (i) strong internal T7 promoter in bold, (ii) the hammerhead ribozyme in italics and empty circles for its variable region, and (iii) a consensus sequence of tRNA with conserved and semi-conserved residues indicated and variable residues given in black dots. Hybridization box (HB) is a 'mixed' domain constituted by the 4 nucleotides (nts) immediately downstream the T7 promoter (a), the 4 first nts present at the 5'-end of the tRNA sequence (b), and the 4 nts opposite to these latter in the 3'-part of the tRNA (c). Base-pairing of a and b occurs immediately after transcription of the concerned nucleotides. The correctness of the folding is verified a posteriori by the efficient cleavage at the position indicated by the arrow. After cleavage, base-pairing occurs between b and c as demonstrated by the full activity of the liberated tRNA. C: Autoradiogram of the transcription products from tRNA[Tyr] and transzyme[Tyr] synthetic genes. A volume corresponding to 2 μl transcription mix is loaded in each lane of the 12% polyacrylamide denaturing gel. Lanes 1 and 2: Transcription of tRNA[Tyr] gene at 22 and 30 mM MgCl₂, respectively. Lanes 3–8: Transcription of transzyme[Tyr] gene at 22 mM (lanes 3, 5, 7) or 30 mM MgCl₂ (lanes 4, 6, 8), followed by 1 h at 60°C (lanes 5 and 6), or by 5-fold dilution and 1 h at 60°C (lanes 7 and 8). To obtain visible bands of transcripts on lanes 1 and 2, the gel was exposed to autoradiography 4 times more than for lanes 3–8. Quantification of the bands was done using a Fujix Bio-Imaging Analyzer BAS 2000 system.

that the autocatalytic cleavage can take place in 'cis' just before nucleotide +1 of the tRNA. This generates full-length tRNAs with a 5'-OH instead of a 5'-phosphate in canonical tRNAs (or of a mixture of pppN and pN, in transcripts prepared by the conventional method).

3.2. tRNA preparation

The huge qualitative improvement of the transcription with the transzyme[Tyr] gene compared to the transcription with the tRNA[Tyr] gene is clearly seen when one compares lanes 1 and 2 with lanes 3 and 4 in Fig. 1C. Optimal transcription conditions turned out to be critically dependent on Mg²⁺ concentration (Fig. 1C and Table 1). Between 500 and 600 transcription cycles could be obtained for the transzyme. This number exceeds by 2 orders of magnitude that of the transcription of the tRNA[Tyr] gene. Note that increasing the concentration of MgCl₂ from 22 to 30 mM gave a reproducible 2-fold increase in yield.

A crucial step in the methodology is the autocatalytic cleavage of the transzyme. Under the basic conditions, this cleavage does not exceed ≈40% of the total transcription products even at 30 mM MgCl₂, known to be the optimal Mg²⁺ concentration for ribozyme cleavage [12]. Note that cleavage is more sensitive to Mg²⁺ concentration than transcription. The extent of cleavage could be improved after transcription, either by rising the temperature from 37 to 60°C (under suboptimal Mg²⁺ concentration) or by diluting 5 times and heating the reaction mixture [13] (Table 1). Under this latter

condition, up to 90% of the transzyme molecules are cleaved into tRNA[Tyr] and a RNA fragment corresponding to the ribozyme as visualized on the autoradiogram (Fig. 1C). Similarly high amounts of wild-type run off transcript of yeast tRNA[Asp] starting with the unfriendly 5'-UCCGUG sequence could be obtained.

After cleavage, incubation mixtures were subjected to electrophoresis on preparative denaturing polyacrylamide gels (12%) to resolve length heterogeneities at the 3'-end of the tRNAs. We considered the possibility to engineer a second ribozyme at the 3'-end of the tRNA gene, and this was worked out with yeast tRNA[Asp]. But in that case, generating
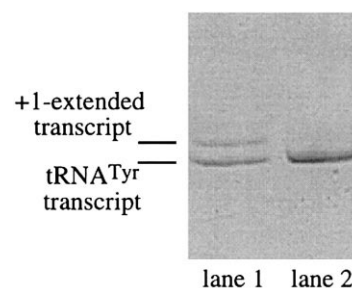

Fig. 2. Single nucleotide resolution 12% polyacrylamide denaturing gel. Lane 1: An aliquot of transcription mixture of transzyme[Tyr] and lane 2: purified tRNA[Tyr] were subjected to electrophoresis for 6 h at 70 mA. These conditions allow resolution between tRNA[Tyr] transcripts and transcripts with a +1-extension.

aminoacylatable tRNA molecules involved a number of additional steps to remove the 2′–3′ cyclic monophosphate generated by the ribozyme cleavage, so that the final yield of active molecules was lower than that obtained after resolving the length heterogeneity by electrophoresis. Therefore, we kept the most simple option with a ribozyme only at the 5′-terminus of the tRNA, especially since it is possible to resolve 3′-length-heterogeneity to the resolution of one nucleotide. For tRNA^{Tyr}, the amount of molecules with a +1-extension was less than 40% (Fig. 2).

3.3. Activity of 5′-OH tRNA transcripts

Aminoacylation capacity of *E. coli* dephosphorylated tRNAs, specific for serine, methionine, phenylalanine and histidine, have already been tested. Except tRNA^{His} [14], all tested tRNAs retain their capacity to be aminoacylated [15–17].

Here, we address the question of the importance of the 5′-phosphate group for tyrosylation and aspartylation. We first proved the existence of a 5′-OH extremity for tRNA^{Tyr} produced from the transzyme. Both tRNA^{Tyr} species obtained by classical transcription and by the transzyme method were subjected to phosphorylation. Transcripts produced by transzyme cleavage could be phosphorylated (up to 95% of the transcripts were labeled with ^{32}P), and conversely, no incorporation of radioactivity could be obtained on tRNA^{Tyr} produced by classical transcription (Fig. 3). It can be concluded that the formed tRNA has a 5′-OH extremity which can be stoichiometrically phosphorylated. Therefore, the autocatalytic cleavage of the transzyme molecule generates as expected a 5′-OH extremity.

Comparison of kinetic parameters (k_{cat} and K_m) for tyrosylation of both types of tRNA transcripts shows no difference (Fig. 4). This demonstrates that the 5′-phosphate extremity of tRNA^{Tyr} is not required for tyrosylation activity. Similar results were obtained for yeast tRNA^{Asp} (data not shown). Altogether, these results extend the earlier observations that had shown aminoacylation activity of dephosphorylated tRNA molecules. Interestingly, this property applies for tRNAs specific for class I and II synthetases whether these molecules possess modified bases or are unmodified transcripts.
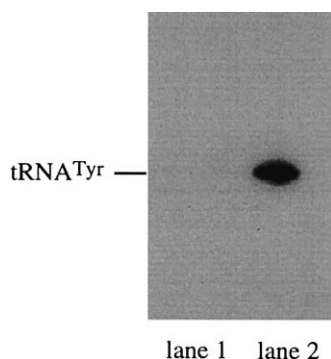

Fig. 3. 12% polyacrylamide gel of phosphorylation attempts on both tRNA^{Tyr} produced. Lane 1: tRNA^{Tyr} produced in the classical way and lane 2: tRNA^{Tyr} produced from transzyme transcription. The transcripts visualized migrate as a control (tRNA^{Tyr} not subjected to phosphorylation), revealed by staining.
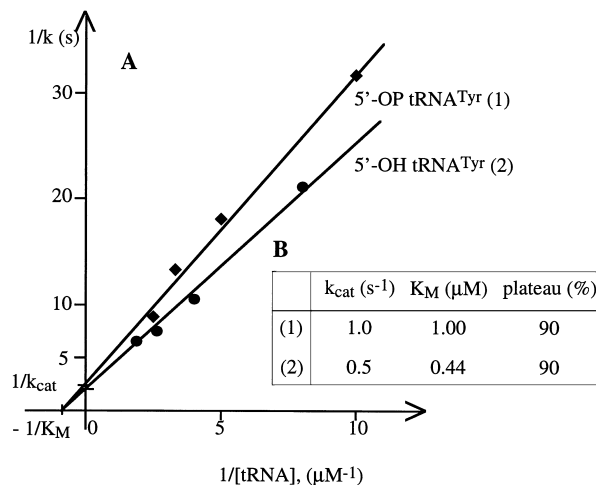

Fig. 4. Comparative aminoacylation activity of 5′-OH tRNA^{Tyr} and 5′-phosphate tRNA^{Tyr}. A: Representation of the Lineweaver-Burk curves obtained for 5′-phosphate tRNA^{Tyr} (◆) classically produced and for 5′-OH tRNA^{Tyr} (●) produced by the transzyme method. B: Aminoacylation parameters of the two sets of tRNAs. Kinetic parameters k_{cat} and K_m were deduced from the Lineweaver-Burk curves in A, and aminoacylation plateau values were obtained in the presence of identical TyrRS concentrations.

3.4. Practical considerations and perspectives

The transzyme method typically yields ≈ 0.5 mg of full-length tRNA transcript in a 1-ml transcription mixture. As high amounts of T7 RNA polymerase are easily available from an overproducing clone [2], all the required conditions are present to apply this technique to any unfavorable sequence of tRNA or tRNA-like molecule for structural or functional studies. The method will be essential for deciphering the tyrosine identity in eukaryotes and especially for preparing tRNAs from mitochondria and chloroplasts that often do not start with G_1. Equally important will be the use of the transzyme method for systematic studies of functional properties of tRNA variants with mutations in the terminal part of their amino acid accepting stems.

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