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Cloning, expression and purification of $tRNA^{Pro}$ from bacteria *Enterococcus faecalis*

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Aim. *To elaborate the method of expression and purification of bacteria Enterococcus faecalis* $tRNA^{Pro}$ *transcript.* ***Methods***. *tRNA, co-expressed in vitro with cis-hydrolytical ribozyme, was purified by high performance liquid chromatography using anion-exchange chromatographic column.* ***Results***. *A satisfactory yield of high purity preparation was obtained. A transcript of* $tRNA^{Pro}$ *exhibits acceptor activity in aminoacylation reaction.* ***Conclusions***. *The method developed may be introduced in laboratory practice including the obtaining of other tRNAs.*

Keywords: tRNA, transcript, gene expression in vitro, high performance liquid chromatography.

Introduction. The obtaining of high purity preparations of individual tRNAs is an essential part of the investigation of both the structure and functions of these molecules and other components of protein synthesis apparatus. To date two usual ways of obtaining preparative amounts of tRNA have been used: superproduction *in vivo* (for instance, under 1pp-promoter [1]) and transcription using RNA-polymerase of T7 phage *in vitro*. In some specific cases chemical synthesis may be required] [2].

The advantages of the method, based on the use of RNA-polymerase of T7 phage, are efficiency of transcription, simple use, convenient work with the sets of mutants, and comparatively low price cost of the product. The absence of the enzymes, modifying nucleosides, in the reaction mixture *in vitro* allows obtaining a homogenous preparation, tRNA molecules of which do not differ in the number of modifications, while the differences are likely to occur in case of the superproduction *in vivo*. The homogeneity of the preparation is especially important in case of co-crystallization of tRNA and proteins. On the other hand, the disadvantage of this approach is the dependence of the efficiency of transcription initiation on the first base of synthesized RNA. Therefore, a choice of the approach depends on a specific scientific task. In our case it was determined by the requirements to homogeneity of tRNA molecules in the preparation.

Bacterial $tRNA^{Pro}$ is characterized by the presence of cytosine at the 5'-end of the molecule, which makes it complicated for T7 polymerase to initiate the transcription [3]. At the same time $tRNA^{Pro}$ transcript may serve

as an essential instrument in the investigation on bacterial proline system of aminoacylation, which is of special interest due to its philogenetic variety [4] and complexity of the specificity supporting mechanisms [5–7].

The use of co-expression of tRNA and *cis*-hydrolytical ribozyme at the 5'-end [8, 9] was found to be an efficient approach to solve the problem of 5'-end cytosine; however, the similarity of sizes of tRNA and ribozyme brings up a question of an efficient and cheap way of their separation. High performance liquid chromatography (HPLC) is used for the abovementioned purpose: either gel-filtration [10] or anion-exchange chromatography [11].

The present work is devoted to the application of novel methods of expression and purification of individual $tRNA^{Pro}$ from the bacteria *Enterococcus faecalis.* The object chosen is of great interest for both fundamental investigations on proline system of aminoacylation and possible use of prolyl-tRNA synthetase as a target molecule for the inhibition of *E. faecalis* – a dangerous pathogenic microorganism with a wide spectrum of resistance to existing antibiotics [12, 13].

Materials and Methods The cells of *Escherichia coli*, strain DH5 (F *80dlacZ M15 (lacZYA-argF)U169 deoR, recA1 endA1 hsdR17*(r_k^- m_k^+) *phoA supE44 thi-1 gyrA96 relA1*) (*Life Technologies Company,* USA); the medium components for bacterial cultures (*Difco,* USA); salts and components of buffer solutions of analytical grade; PAAG monomers (*Reanal*, Hungary); agarose (*Q-BIOgen*, USA); enzymes (*Roche*, France); DEAE-Toyopearl 650M sorbent (*Toyo Soda*, Japan); HPLC column ProSwift Monolith WAX-1S (*Dionex*, USA); radioactively labelled substances (*Amersham*, UK); and glass fiber filters (*Whatman*, UK) were used in the work.

Cloning and expression of E. faecalis $tRNA^{Pro}$ *in vitro*. The sequences of genes of *E. faecalis* $tRNA^{Pro}$, *cis*-hydrolytical ribozyme, and the sequence of T7-promoter in the form of six pairs of desoxyoligonucleotides were ligated into BamHI- and EcoRI-digested vector *pUC18* (Fig.1). The plasmid DNA was obtained in *E. coli* cells of DH5 strain, isolated accordinga to the method of Birnboim and Doly [14], then it was purified additionally using phenol, chloroform, and precipitation, and digested with *BstNI*, which ensured correct termination of the transcription of the 3'-end of the synthesized RNA. The transcription by T7 phage's RNA-polymerase was performed *in vitro* in the reaction mixture, containing 100 mM tris-HCl, pH 8.0, 30 mM $MgCl_2$, 5 mM DTT, 50 mM spermidine and ATP, GTF, UTP, CTP in the concentration of 4 mM each, using the concentration of DNA-matrix of 0.5 μM in the volumes of 1–8 ml.

Cutting the transcript and purification of tRNA. The obtained transcript was purified on DEAE-Toyopearl 650M column (the volume of the sorbent was 1 ml) in the presence of 10 mM tris-HCl, pH 8.0, 10mM $MgCl_2$. The reaction mixture, clarified by centrifugation, was 20-fold diluted, applied on the column, and eluated with 1 M NaCl after washing. After the precipitation of the obtained eluate, the detachment of *cis*-hydrolytical ribozyme was stimulated by cyclic melting of RNA and annealing in PCR-thermocycler in the buffer solution, containing 20 mM tris-HCl, pH 7.5, 30 mM $MgCl_2$, with the transcript concentration of 4 μg/μl. The programme of PCR-thermocycler included 14 cycles of heating to the temperature of 95°C during 30 sec and cooling to 60°C in 9 min. Then the transcript was melted again by heating to 70°C for 3 min and renaturated by gradual cooling in water, which got colder in the air from 70°C to room temperature. The obtained tRNA was purified from the detached ribozyme, using HPLC on ProSwift Monolith WAX-1S column (DEAE-polymetacrylate) in the volume of 0.73 ml at 57°C and the rate of solution feed of 1 ml/min. Portions of 250 μg of the transcript in the buffer solution were applied on the column (50 mM tris-HCl, pH 7.5, 4 mM $MgCl_2$, 10% isopropanol) and eluated by the gradient of NaCl concentration from 0.2 to 1 M.

Analysis of acceptor activity of $tRNA^{Pro}$. The mixture for aminoacylation reaction in the volume of 130 μl contained 100 mM tris-HCl, pH 8.0, 20 mM $MgCl_2$, 0.5 mg/ml BSA, 3 mM ATP, 3 mM proline, 20 μM ^{14}C-labelled proline (268 mCu/mmol); 1 μM $tRNA^{Pro}$ and 50 nM *E. feacalis* prolyl-tRNA synthetase. During the reaction the aliquots of 20 μl were picked out from the reaction mixture at 37°C, tRNA and aminoacyl-tRNA were precipitated by 10% TCA in the volume of 200 μL in cold, then the precipitates were applied on filters, washed with 50 ml 5% TCA, dried,

a

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PEFokA2  AGCTTGCATGCCTGCAGGTCGACTCTA
PEFokA1      ACGTACGGACGTCCAGCTGAGATCCTACCTCCTAG

PE10Fok  GGATGGAGGATCCTGGTCGGGAAGACAGGATT
PE5                 GACCAGCCCTTCTGTCCTAAGCTTGGACGC

PE9      CGAACCTGCGACCCCTTGGTCCCAAACCAAGT
PE4                TGGGGAACCAGGGTTTGGTTCACGAGATGGTTC

PE8      GCTCTACCAAGCTGAGCTACTTCCAGGACGG
PE3                 GACTCGATGAAGGGCCTGCCATGGCCCATGG

PE7      TACCGGGTACCGTTTCGTCCTCACGGACTCAT
PE2                 CAAAGCAGGAGTGCCTGAGTAGTCGCCCTTCAG

PE6      CAGCGGGAAGTCTCCCTATAGTGAGTCGTATTAG
PE1                  AGGGATATCACTCAGCATAATCTTAA
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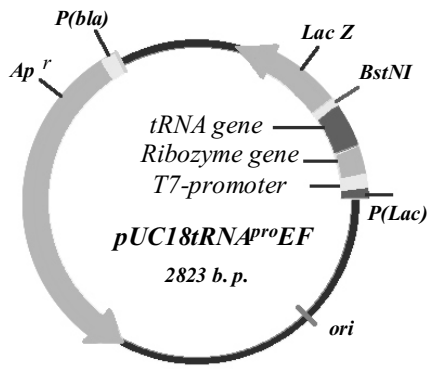
b



c

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AATTCTAATACGACTCACTATAGGGAGACTTCCCGCTGATGAGTCCGTGAGGACGAAACGGTACCCGGTA
    CATTATGCTGAGTGATATCCCTCTGAAGGGCGACTACTCAGGCACTCCTGCTTTGCCATGGGCCAT
      T7-promoter                Ribozyme gene

CCGTCCGGGAAGTAGCTCAGCTTGGTAGAGCACTTGGTTTGGGACCAAGGGGTCGCAGGTTCGAATCCTG
GGCAGGCCCTTCATCGAGTCGAACCATCTCGTGAACCAAACCCTGGTTCCCCAGCGTCCAAGCTTAGGAC
                              tRNA gene

TCTTCCCGACC*AGGATCCTCCATCCTAGAGTCGACCTGCAGGCATGCA
AGAAGGGCTGGT*CCTAGGAGGTAGGATCTCAGCTGGACGTCCGTACGTTCGA
      BstNI
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Fig.1 The gene-engineering construction for the expression of *E. faecalis* tRNA^{Pro} *in vitro*: *a* – six pairs of chemically synthesized oligonucleotides, making up a coding insert; *b* – general scheme of expressing construction, obtained on the basis of *pUC18* vector; *c* – location of functional elements in the coding insert

and the radioactivity was measured with the liquid scintillation counter.

Results and Discussion The peculiarity of *E. faecalis* tRNA^{Pro} and a number of other tRNAs is the presence of cytosine at the 5'-end of the molecule which determines a low efficiency of the transcription of its gene by RNA-polymerase of T7 phage. The solution to this problem was found to be the introduction of the sequence of the gene of *cis*-hydrolytical hammerhead ribozyme of tobacco ringspot virus [9] between T7-promoter and 5'-end of tRNA gene, which enhances the efficiency of the transcription using T7-polymerase of co-transcript of the ribozyme and tRNA. Later the catalytic activity of the ribozyme promotes its self-detachment with subsequent release of tRNA transcript.

The gene engineering construction for the expression of *E. faecalis* tRNA^{Pro} *in vitro*, developed by us (Fig.1, *b*), contains the genes of the ribozyme and tRNA under T7-promoter. The sequence of the recognition site of *BstNI* restrictase, similar to the sequence of the tRNA gene and partially localized outside the latter, was introduced for correct termination of the transcription of the 3'-end of tRNA gene (Fig.1, *c*). This is the site, along which the construction is cut in the course of its preparation for the transcription, and the place of the cut in the coding chain coincides with the end of the coding sequence of tRNA.

Fig.2 The assumed secondary structure of the tRNA co-transcript and *cis*-hydrolytical ribozyme, necessary for its self-detachment. The 5'-end of the ribozyme is complementary paired to the 5'-end of the tRNA, the secondary structure of the acceptor stem of tRNA is not formed in this folding type. The rest of the secondary structure elements of tRNA are shown in the usual form of the "shamrock". The arrow indicates the place of cutting.

The synthesis of the co-transcript starts with guanosine at the 5'-end of the ribozyme, which ensures high efficiency of T7 phage RNA polymerase. The synthesized co-transcript may have various secondary structures, as the 5'-end sequence of tRNA, adjoining the 3'-end of the ribozyme sequence, is complementary both to the 5'-end of the ribozyme, and to the 3'-end of tRNA. It is noteworthy that the self-cutting of the co-transcript due to the catalytic activity of the ribozyme is possible only in case of complementary coupling of the 5'-end of tRNA and 5'-end of the ribozyme (Fig.2). The melting-annealing cycles are required to enable all the molecules of the co-transcript to obtain the conformation, necessary for the cutting; the portion of the cut co-transcript enlarges after each cycle.

After the ribozyme is detached, it may still be connected to tRNA via interactions between complementary base pairs, that creates difficulties for their chromatographic separation if no detergents are used. The increase of temperature to 57°C was selected as a factor promoting the weakening of the complementary binding of tRNA to the ribozyme during the chromatography, that corresponds to the temperature resistance of the chromatographic column used.

The ion-exchange column WAX-1S, containing DEAE-polymetacrylate, was used for chromatographic purification of the transcript, which allowed to separate tRNA transcript from the ribozyme and the remains of

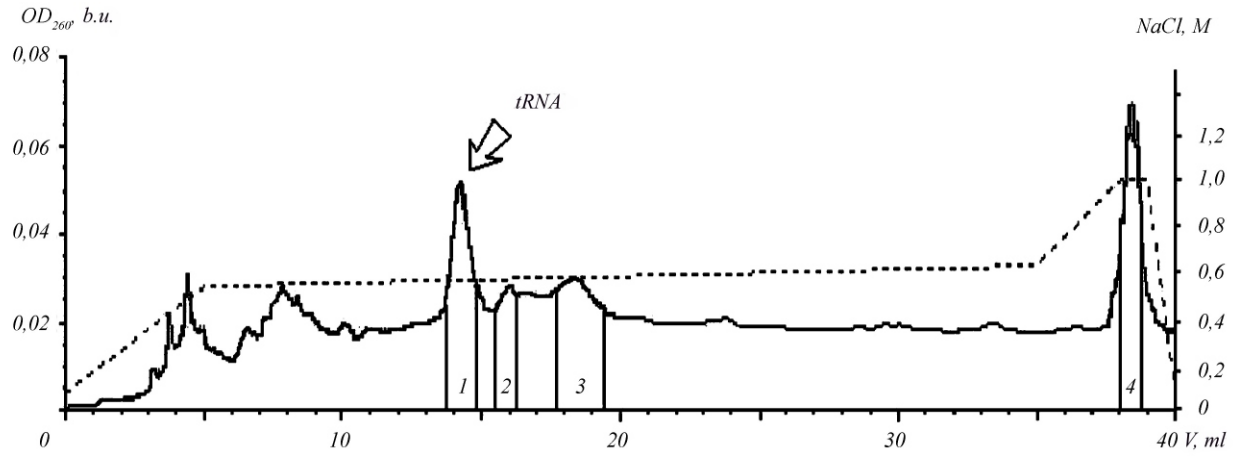

Fig.3 The chromatogramme of *E. faecalis* $tRNA^{Pro}$ co-transcript and *cis*-hydrolytical ribozyme. The composition of fractions is as follows: *1* – $tRNA^{Pro}$; *2* – $tRNA^{Pro}$ + ribozyme; *3* – ribozyme; *4* – non-identified fragments of RNA.

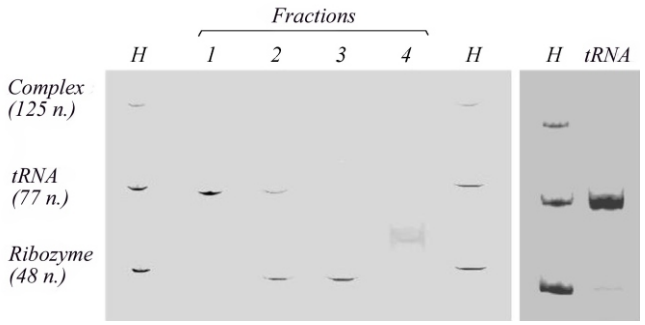

Fig.4 The electrophoregramme of fractions of the chromatogramme of *E. faecalis* tRNA^{Pro} co-transcript and *cis*-hydrolytical ribozyme: *H* – mixture, applied on the column (acting as a "witness"); *1* – tRNA^{Pro}; *2* – tRNA^{Pro} + ribozyme; *3* – ribozyme; *4* – non-identified fragments of RNA. The electrophoregramme on the right shows the purity of the preparation of tRNA transcript: the only admixture is the ribozyme in the trace quantity.

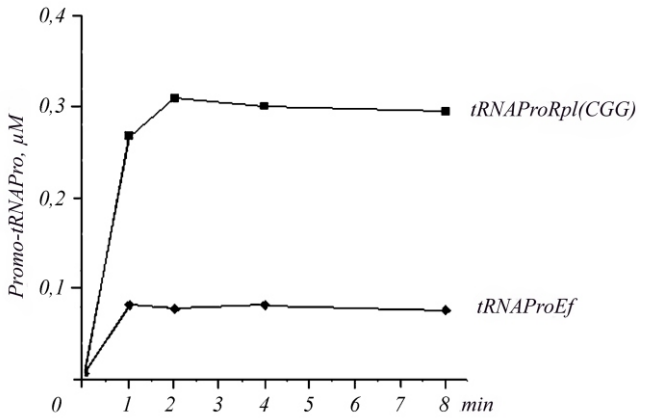

Fig.5 The acceptor activity of *E. faecalis* tRNA^{Pro} transcript and *Rhodopseudomonas palustris* bacteria tRNA^{Pro} (anticodon CGG), obtained by the method of superproduction *in vivo*

covalent tRNA–ribozyme complex in the gradient of NaCl concentration (Fig.3, 4).

The final yield of tRNA transcript from 1 mg DNA-matrix was 0.8 mg. The acceptor activity of the obtained tRNA transcript was confirmed in the test for aminoacylation (Fig.5). The level of transcript aminoacylation decreased in comparison to tRNA, obtained by the method of superproduction *in vivo*, which is the consequence of complete absence of minor nucleosides in it. The considerable influence of the modification of nucleosides of bacterial tRNA^{Pro} on its acceptor activity was proven earlier [15]. The purity of preparation, verified by electrophoresis in polyacrylamide gel, exceeded 95% (Fig.4).

Thus, we have developed a simple and reliable method of purification of tRNA transcripts consisting in co-expression with *cis*-hydrolytical ribozyme. The advantages of this method are ensured by the use of HPLC on anion-exchange sorbent of DEAE-polymetacrylate (WAX-1S column, *Dionex* company) for the purification of the preparations from *cis*-hydrolytical ribozyme, necessary for the efficient synthesis of RNA.

The method was used for the first time to accomplish the mentioned task , moreover, the method may be used for the purification of transcripts of any tRNA, which would require only slight optimization of the conditions of chromatography.

Comparing to the method of purifying tRNA transcripts in polyacrylamide gel, the obvious advantages of our method are higher rate of conducting procedures, low consumption of expensive materials, and considerable yield of the product due to low losses during the purification.

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Клонирование, экспрессия и очистка тРНК^{Pro} из бактерии *Enterococcus faecalis*

Резюме

Цель. *Разработать методику экспрессии и очистки транскрипта мРНК^{Pro} бактерии E. faecalis.* ***Методы.*** *Коэкспрессированную in vitro с цис-гидролитическим рибозимом мРНК очищали при помощи высокоэффективной жидкостной хроматографии с использованием анионообменной хроматографической колонки.* ***Результаты.*** *Получены удовлетворительные выход продукта и чистота препаратов. Транскрипт мРНК^{Pro} проявляет акцепторную активность в реакции аминоацилирования.* ***Выводы.*** *Разработанный метод может быть внедрен в лабораторную практику и стать основой для создания новых способов получения транскриптов других мРНК.*

Ключевые слова: мРНК, транскрипт, экспрессия генов in vitro, высокоэффективная жидкостная хроматография.

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Клонування, експресія та очищення тРНК^{Pro} з бактерії *Enterococcus faecalis*

Резюме

Мета. *Розробити методику експресії і очищення транскрипта мРНК^{Pro} бактерії E. faecalis.* ***Методи.*** *Коекспресовану in vitro з цис-гідролітичним рибозимом мРНК очищували за допомогою високоефективної рідинної хроматографії з використанням аніонообмінної колонки.* ***Результати.*** *Отримано задовільні вихід продукту та чистоту препаратів. Транскрипт мРНК^{Pro} виявляє акцепторну активність у реакції амінoацилювання.* ***Висновки.*** *Розроблений метод може бути впроваджений у лабораторну практику та стати основою для створення нових способів отримання транскриптів інших мРНК.*

Ключові слова: мРНК, транскрипт, експресія генів in vitro, високоефективна рідинна хроматографія.

REFERENCES

1. *Meinnel T., Mechulam Y., Fayat G.* Fast purification of a functional elongator tRNA^{Met} expressed from a synthetic gene *in vivo* // Nucl. Acids Res.–1988.–**16**, N 16.–P. 8095–8096.
2. *Sherlin L. D., Bullock T. L., Nissan T. A., Perona J. J., Lariviere F. J., Uhlenbeck O. C., Scaringe S. A.* Chemical and enzymatic synthesis of tRNAs for high-throughput crystallization // RNA.–2001.–**7**, N 11.–P. 1671–1678.
3. *Milligan J. F., Groebe D. R., Witherell G. W., Uhlenbeck O. C.* Oligoribonucleotide synthesis using T7 RNA-polymerase and synthetic DNA templates // Nucl. Acids Res.–1987.– **15**, N 21.–P. 8783–8798.
4. *Crepin T., Yaremchuk A., Tukalo M., Cusack S.* Structures of two bacterial prolyl-tRNA synthetases with and without a *cis*-editing domain // Structure.–2006.–**14**, N 10.–P. 1511–1525.
5. *An S., Musier-Forsyth K.* Cys-tRNA(Pro) editing by *Haemophilus influenzae* YbaK via a novel synthetase. YbaK tRNA ternary complex // J. Biol. Chem.–2005.–**280**, N 41.–P. 34465–34472.
6. *Boyarshin K. S., Kriklivyi I. A., Tukalo M. A.* tRNA-dependent editing of errors by prolyl-tRNA synthetase from bacteria *Enterococcus faecalis* // Ukr. Biochem. J.–2008.–**79**, N 6.–P. 40–44.
7. *Boyarshin K. S., Kriklivyi I. A., Rayevsky A. V., Himin A. A., Yaremchuk A. D., Tukalo M. A.* Putative active site of *Enterococcus faecalis* prolyl-tRNA synthetase // Biopolymers and Cell.–2009.–**25**, N 1.–P. 39–43.
8. *Taira K., Nakagawa K., Nishikawa S., Furukawa K.* Construction of a novel RNA-transcript-trimming plasmid which can be used both *in vitro* in place of run-off and (G)-free transcriptions and *in vivo* as multi-sequences transcription vectors // Nucl. Acids Res.–1991.–**19**, N 19.–P. 5125–5130.
9. *Price S. R., Ito N., Oubridge C., Avis J. M., Nagai K.* Crystallization of RNA protein complexes I. Methods for the large-scale preparation of RNA suitable for crystallographic studies // J. Mol. Biol.–1995.–**249**, N 2.–P. 398–408.
10. *Kim I., McKenna S. A., Puglisi E. V., Puglisi J. D.* Rapid purification of RNAs using fast performance liquid chromatography (FPLC) // RNA.–2007.–**13**, N 2.–P. 289–294.
11. *Shields T. S., Mollova E., Marie L. S., Hansen M. R., Pardi A.* High-performance liquid chromatography purification pf homogenous-length RNA produced by *trans* cleavage with a hammerhead ribozyme // RNA.–1999.–**5**, N 9.–P. 1259–1267.
12. *Sood S., Malhotra M., Das B. K., Kapil A.* Enterococcal infections & antimicrobial resistance // Ind. J. Med. Res.–2008.–**128**, N 2.–P. 111–121.
13. *Arias C. A., Murray B. E.* Emergence and management of drug-resistant enterococcal infections // Expert Rev. Anti-Infect. Ther.–2008.–**6**, N 5.–P. 637–655.
14. *Birnboim H. C., Doly J.* A rapid alkaline extraction procedure for screening recombinant plasmid DNA // Nucl. Acids Res.–1979.–**7**, N 6.–P. 1513–1523.
15. *Liu H., Peterson R., Kessler J., Musier-Forsyth K.* Molecular recognition of tRNA^{Pro} by *Escherichia coli* proline tRNA synthetase *in vitro* // Nucl. Acids Res.–1995.–**23**, N 1.–P. 165–169.

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