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Cell-free synthesis of tachyplesin, an antimicrobial peptide from *Tachypleus tridentatus*

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ABSTRACT

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Tachyplesin is an antimicrobial peptide isolated from the hemocytes of horseshoe crab (*Tachypleus tridentatus*). Due to the importance of producing highly active peptides that have great commercial application, we attempted to produce this protein using a continuously coupled transcription-translation in a wheat germ extract system. The system works by using a continuous flow of feeding solution containing nucleoside triphosphates and amino acids into a 1-ml reactor containing wheat germ extract, plasmid DNA, and transcription enzyme, and continuous removal of translation product through an ultrafiltration membrane fitted in the reactor. The amount of protein synthesized reached to about 80 mg after 27 h of continuous reaction. Autoradiogram of the translated product showed a distinct band at about 8.44 kDa corresponding to the calculated molecular weight of tachyplesin. Continuously coupled transcription-translation in a cell-free system offers many possible applications to create new products in biotechnology.

Keywords: cell-free protein synthesis, coupled transcription-translation, tachyplesin, *Tachypleus tridentatus*, antimicrobial peptide

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INTRODUCTION

Major advances in genetic engineering technology have made possible the design and synthesis of genes encoding any polypeptides. Proteins produced in very limited amounts in their original source can now be obtained in considerable amount through the application of techniques this area offers. However, expression of genes in living cell is subject to a number of limitations. Many gene products are insoluble and unstable and in some cases the product is toxic to the host cell (Morch *et al.*, 1986; Pollard and Clemens, 1988; Seal *et al.*, 1986). These and other limitations could be avoided using a cell-free protein synthesis system.

Development of continuous cell-free translation systems utilizing translatable mRNAs has been reported by several workers (Baranov *et al.*, 1989; Kudlicki *et al.*, 1992; Matsumoto *et al.*, 1992; Morimoto et al., 1991; Spirin *et al.*, 1988; Sullivan and Green, 1993). Translation of messenger RNA (mRNA) is becoming a useful tool for the production of proteins from cloned genes and is emerging as an important step in the regulation of specific genes in prokaryotic and eukaryotic organisms. The most commonly used systems are the rabbit reticulocyte lysate and wheat germ extract because of their ease and cheapness of preparation, high level of reinitiation of ribosomes onto mRNA and high fidelity of translation (Chung *et al.*, 1994; Pine, 1967; Tulin, 1995). When large quantities of protein are synthesized in a continuous cell-free system, the product concentration in the reactor can be kept low by constantly eluting the product from the reactor. We have further improved the method by combining transcription and translation to occur simultaneously. A coupled transcription-translation system is a novel approach to synthesize proteins because labile mRNAs do not need to be separately prepared. Coupling of transcription and translation mimics expression *in vivo* and continuous elution of the product from the reactor eliminates product inhibition and possible formation of inclusion bodies (Seal *et al.*, 1986).

We have previously reported a continuously coupled transcription-translation system for the synthesis of rice cytoplasmic aldolase, a 40 kDa protein involved in both glycolytic and gluconeogenic pathways in eukaryotic cells (Uzawa *et al.*, 1993). In this paper, we present evidence for the synthesis of tachyplesin, an antimicrobial peptide isolated from the hemocytes of the Japanese horseshoe crab (*Tachypleus tridentatus*) which has been found to exhibit strong antimicrobial activity against both gram-positive

and gram-negative bacteria and some fungi (Nakamura *et al*., 1988; Peltz *et al*., 1993). Tachyplesin consists of 17 amino acid residues having two intramolecular disulfide bonds with a COOH-terminal arginine α-amide (Mizusawa and Gottesan, 1983). The unusual chemical structure and biological function of this peptide suggest the existence of a highly active peptide with great commercial use. Expression of a cloned eukaryotic gene in a plant (wheat germ extract) system offers great promise in the production of highly valuable proteins for many applications.

MATERIALS AND METHODS

Construction of DNA template

An *Eco*RI fragment of a 598 bp tachyplesin DNA was inserted between the *Eco*RI sites of pGEM4Z vector (Promega). This expression vector contains an SP6 transcription initiation site. The resulting plasmid was subcloned in *E. coli* JM109 and the correct orientation of the insert with respect to the SP6 promoter was checked by digestion with *Xba*I followed by electrophoresis in a 0.8% agarose gel. Prior to transcription, the vector containing tachyplesin gene (*tpn-1*) was linearized by cutting with *Bam*HI located downstream of the inserted gene. The linearized template was transcribed using SP6 RNA polymerase unless otherwise stated.

Preparation of the wheat germ extract

Wheat germ used in this study was provided by Nissin Seifun, Co., Ltd. (Chiba, Japan). The extract was prepared as described elsewhere (Uzawa *et al*, 1993).

In vitro transcription

Capped mRNA transcripts were obtained as described using the linearized template (Uzawa *et al*, 1993). In experiments to determine if the wheat germ extract was able to drive transcription of the template DNA,

2 μg of the linearized template was incubated with 5 ml of the extract but without the addition of SP6 RNA polymerase in a total volume of 15 ml. The same amount of template was used for the control transcription reaction using 7 units of SP6 RNA polymerase. The reaction was added with [^{35}S] UTP (specific activity, 1000 Ci/mmol) at a concentration of 0.17 μ Ci/ml. Labeled mRNA transcripts were recovered by phenol choloform (1:1) treatment, precipitated with ethanol and centrifuged at 15,000 rpm for 10 min. The precipitate was dissolved in appropriate amount of water, treated with three volumes of a solution containing 67% formamide, 20% formaldehyde, and 13% 10X morpholinopropanesulfonic acid (MOPS) buffer, heated at 65°C for three minutes and applied to a one percent agarose gel containing 6% formaldehyde. After electrophoresis, the gel was dried and subjected to autoradiography for 24 h. Unlabelled mRNA transcript used for translation was also recovered by the same method.

Reactor

The reaction chamber (1 ml) was obtained from Tosoh (Tokyo, Japan). Feed solution was supplied to the chamber fitted with an XM-50 ultrafiltration membrane (Amicon) using the optimum flow rate of 1.5 ml/h by means of a pump (Perista SJ1211, Atto Corp., Tokyo, Japan). Two other flow rates (1.0 and 2.0 ml/h) were tested to optimize translation (data not shown). A diagram of the continuous cell-free system is illustrated in Fig. 1.

Protein synthesis

The composition of the translation buffer used in both batch and continuous translation was similar as described by Sullivan and Green (1993) except that the concentrations of some components were changed as a result of our optimization experiments (data not shown). The components and their new concentrations are as follows: potassium acetate, 125 mM, magnesium acetate, 2.5 mM and spermidine, 0.7 mM.

The optimum temperature for translation was determined under batch method. All batch experiments were performed using 5 ml of wheat germ extract and 2 μg mRNA in a total volume of 25 ml. [^{35}S] Met (specific activity, 1,175 Ci/mmol) was supplied at a concentration of 1 μ Ci/ml.

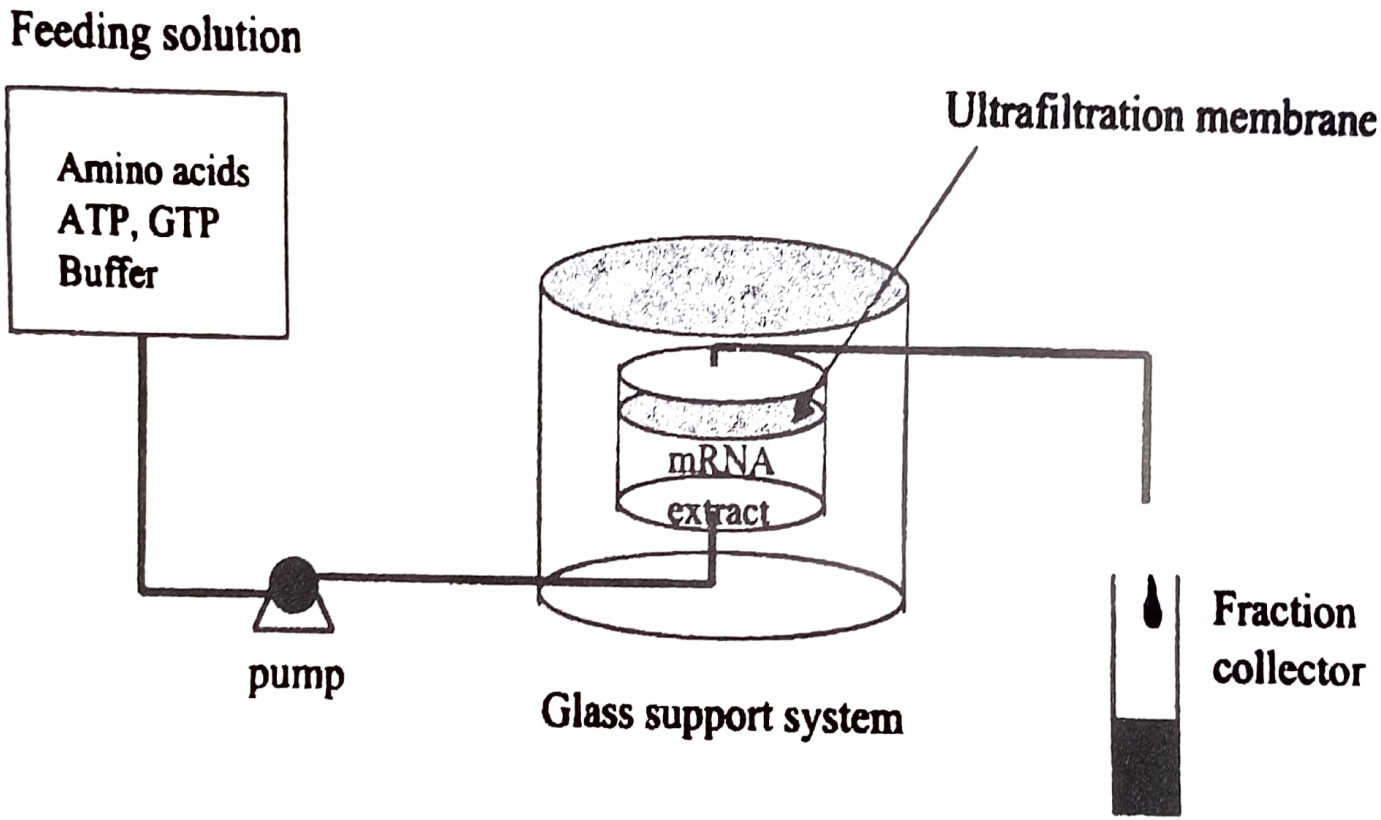

Fig. 1. Schematic set-up of a continuous cell-free coupled transcription-translation system

In continuous systems with translation only, 50 μg capped mRNA were added in the reaction chamber at the start. In the case of coupled transcription-translation system, 10 μg of linearized template DNA was added based on our result that 1 μ g of linearized template gave about 5 μg of capped mRNA. In both systems, 200 microliter of wheat germ extract was added to the 1 ml solution in the reactor. The feed solution was added with [^{35}S] Met (specific activity, 1,175 Ci/mmol) at a concentration of 1.0 μCi/ml to determine the amount of protein synthesized in the translation product.

Products of translation were precipitated with 5% trichloroacetic acid (TCA) solution, heated at 90°C for 10 min and filtered through a glass filter. The filters were washed twice with 10% TCA then once with ethanol and dried. The amount of [^{35}35] Met incorporation was measured using a liquid scintillation counter (LSC) (Aloka, Tokyo, Japan). The amount of protein synthesized was estimated from the number of moles of methionine incorporated into the protein and the calculated molecular weight of 8,436 Da for tachyplesin. Products of translation were also analyzed by SDS-PAGE using a 12.5% polyacrylamide gel followed by autoradiography.

Western blotting

Western blot analysis was carried out with polyclonal antibody raised in rabbits against tachyplesin. Products of translation were applied to SDS-PAGE using a 12.5% polyacrylamide gel and proteins in the gel were transferred to a polyvinylidene diflouride membrane (PVDF) (Japan Millipore Co., Tokyo, Japan) at 120 mA for 90 min using a BioRad Transblot cell. The rabbit antiserum and the peroxidase-labeled IgG antirabbit antibodies were diluted 1:100 and 1:1000, respectively. Detection and staining were done according to standard methods (Amersham).

RESULTS

Translation of both capped and uncapped mRNAs were performed at different temperatures of incubation. Uncapped mRNAs were hardly translated except at 37°C where a little incorporation of [^{35}S] Met in its translation product was obtained (Fig. 2). Using capped mRNA however, the level of translation increased linearly from 20°C to 37°C where the highest incorporation was observed. Increasing the temperature further to 45°C reduced translation.

In the synthesis of tachyplesin using a translation system only, separately prepared mRNA transcripts were added to the reactor for translation. In the case of a coupled transcription-translation system however, linearized template DNA together with the transcription enzyme were added to the reactor to generate mRNA transcripts for translation. Due to the complexity of the components of the wheat germ extract, we wanted to find out if it could transcribe the template DNA and produce mRNA transcripts even in the absence of SP6 RNA polymerase. Incubating the template DNA with wheat germ extract did not produce any transcripts as shown by autoradiogram in Fig. 3 (lane 1). Thus, without the addition of exogenous RNA polymerase transcription was not possible. Addition of SP6 RNA polymerase to the reactor however, yielded a distinct single band corresponding to the desired mRNA transcripts (lane 2).

Fig. 4 shows the time-course profile of protein synthesis in a continuous cell-free translation and coupled transcription-translation. A linear accumulation of protein was observed and after 27 h, the total protein reached to about 80 mg and 70 mg for the translation only system (open circles) and coupled transcription-translation system (closed circles), respectively.

Autoradiogram of the translated product (Fig. 5) showed a single distinct band in a position slightly higher than the calculated molecular weight (lanes 2 and 3 for the translation only system and coupled transcription-translation, respectively) that was not observed in the control treatment (lane 1).

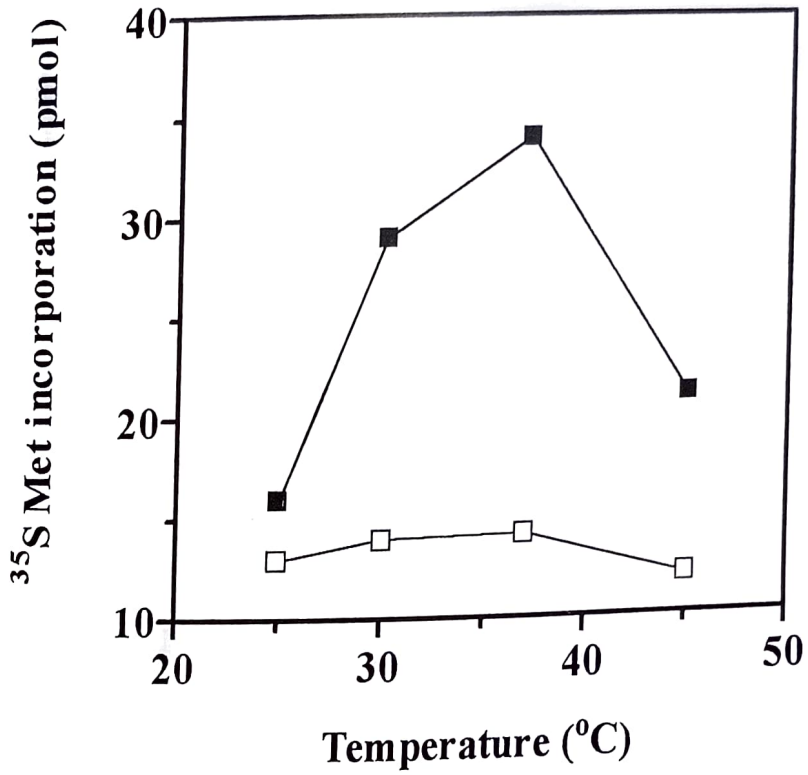

Figure 2. Effect of temperature on translation. Translations were performed as described in Materials and Methods with 2 μg capped (■) and uncapped (□) tpn-1 mRNA and 5 ml of wheat germ extract in a 25-ml reaction mixture. Incubation time was for one hour.

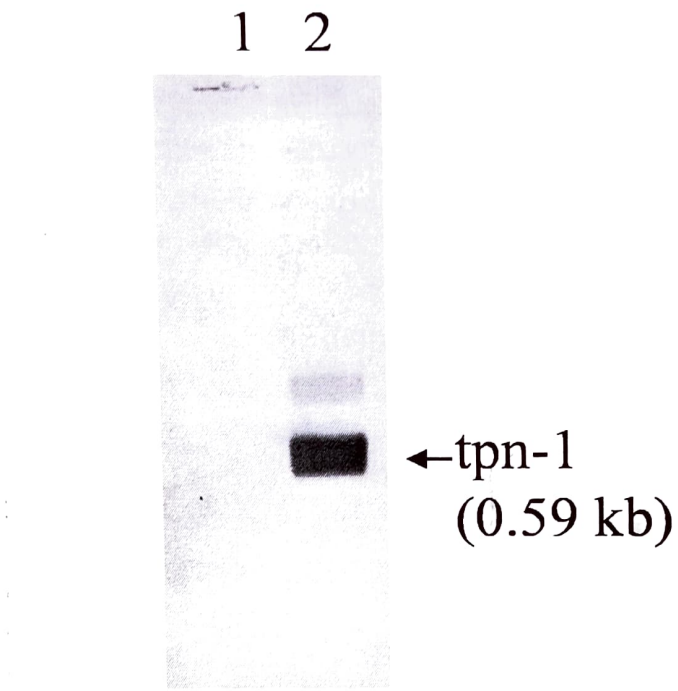

Figure 3. Autoradiogram of tpn-1 transcripts produced using wheat germ extracts alone (lane 1) and with SP6 RNA polymerase (lane 2). In each transcription reaction, 2 μg of linearized template was incubated with either 5 ml of wheat germ extract only (lane 1) or with 7 units of SP6 RNA polymerase (lane 2) in a total volume of 15 ml. Products of transcription were extracted as described in Materials and Methods and applied to a one percent agarose gel containing 6 % formaldehyde. After electrophoresis, the gel was dried and autoradiographed for 24 h.

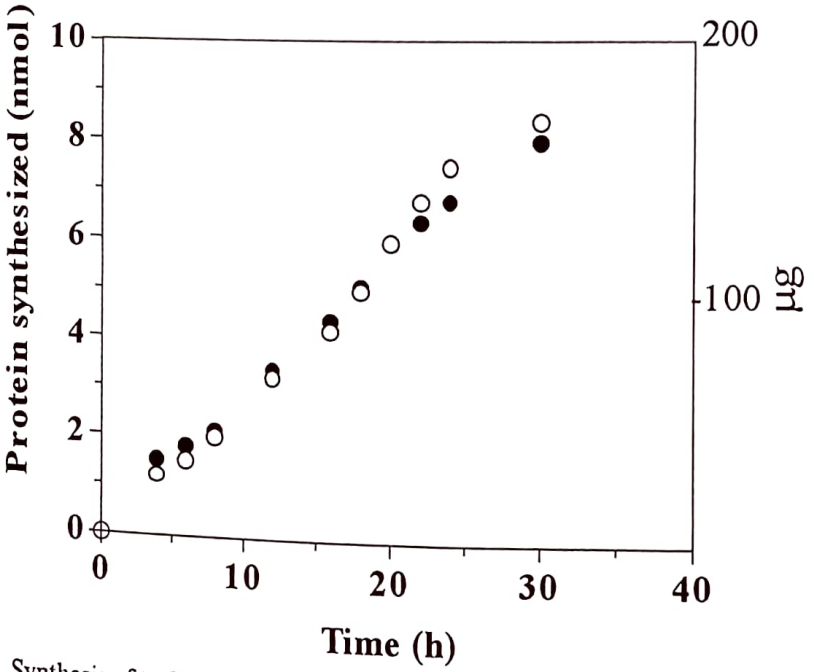

Fig. 4. Synthesis of tachyplesin in the wheat germ extract continuous cell-free system. (●) translation only, (○) coupled transcription-translation.

The rates of protein efflux in continuous systems are compared in Table 1. Protein synthesized using our system is higher among eukaryotic system and comparable to prokaryotic system using viral or *E. coli* mRNAs.

DISCUSSION

Our experience on cell-free translation using brome mosaic virus (BMV) RNA has shown that successful translation of mRNA is highly dependent on temperature of incubation (unpublished data). This is also true in the case of *tpn-1* mRNAs where the incorporation of [^{35}S] Met at different temperatures highly varied (Fig. 2, squares). Each mRNA has its own optimum temperature for translation and this should be carefully evaluated. It is worthwhile to mention that because a number of components are assembled to constitute the *in vivo* system, the concentrations of some of the components like Mg^{2+} and K^{+} should be optimized.

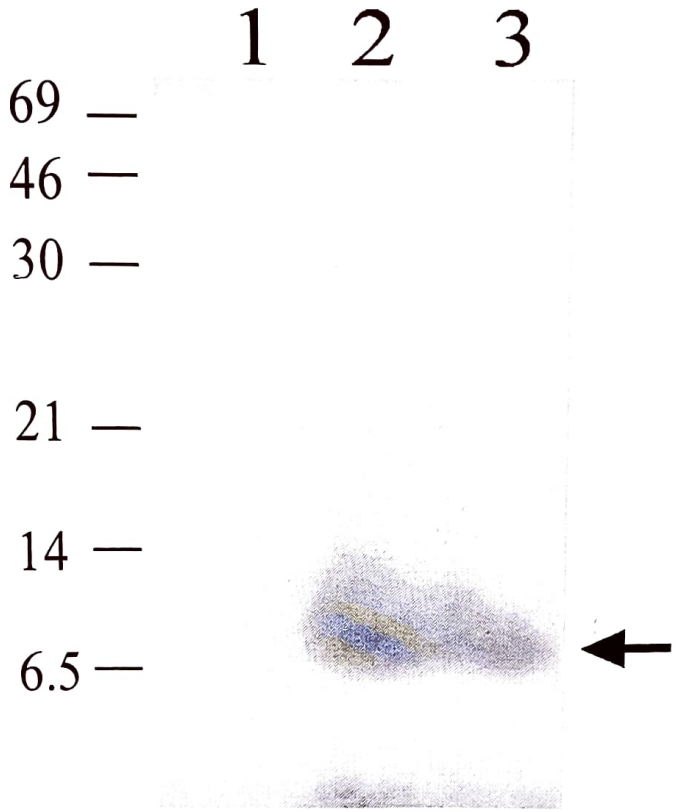

Fig. 5. Autoradiogram of tachyplesin produced by continuous cell-free translation. Ten ml of translation product collected after 20 hours were applied to a 12.5 % SDS-PAGE, elctrophoresed and processed for autoradiography. Lane 1, control continuous translation (no mRNA added); lane 2, product from continuous translation only system; lane 3, product from coupled transcription-translation. The molecular weight markers on the left are shown in kilodaltons. The arrow shows the position of tachyplesin.

We have also attempted to improve the translation capacity of the system by supplementing the translation reaction with elongation factors (EF-1 , EF-α, EF-1ββ'γ) from rice and tRNA from *E. coli* (Ejiri, 1986; Miyata et al., 1989; Yamakazi et al., 1990). Although translational initiation is thought to be the rate-limiting step in protein synthesis in most cases, new views are constantly arising about the functions of elongation factors and tRNA. Several reports have also shown that the addition of tRNA (beef liver, *E. coli*) in their cell-free translation systems (Proudfoot, 1994). This study, however, has shown that the elongation factors from rice and tRNA from *E. coli* were not necessary. The wheat germ extract itself has sufficiently amount of these factors to carry translation faithfully without further supplementation.

Without the addition of exogenous RNA polymerase to the reactor, transcription of tpn-1 mRNA by wheat germ extract was not observed. Autoradiogram data exhibited no visible band corresponding to *tpn-1* mRNA transcripts when the template DNA was incubated in the presence of the wheat germ extract under the transcription conditions used (Fig. 3, lane 1).

When SP6 RNA polymerase was added to the reactor, one distinct band corresponding to the desired mRNA transcripts was obtained. This information was essential for proper interpretation of our coupled transcription-translation results. Since the wheat germ extract alone was not able to support transcription of the template DNA and since only one appeared when SP6 RNA polymerase was added to the reactor,then majority of the mRNAs transcribed were those of the *tpn-1* DNA templates.

The time-course profile of protein synthesis (Fig. 4) shows a constantly increasing rate of synthesis in both translation only and coupled transcription-translation systems. In fact, an almost linear increase in the amount of protein synthesized was obtained.

Synthesis of tachyplesin in a cell-free system of the same composition and volume but without flow of feeding solution ceased after 1.5 h, producing about 4 mg protein (data not shown). In both continuous systems presented in this study, the synthesis rate was constant for at least 20 or 30 h and the amount of product reached nearly a hundred of mg or 20 times more than the amount obtained from the batch method. The rates of protein synthesis (Table 1) in the coupled transcription-translation system were also comparable to those reported in the literatures using viral RNA as mRNA (Chung *et al.*, 1994). Thus, this result confirmed the ability of the present system to synthesize protein from an mRNA of animal origin.

Why is prolonged translation possible in the continuous system? How does the mRNA maintain its stability during repeated translation? We believe that the permanent engagement of mRNA with ribosomes and other translation components safely protects it during prolonged incubation at a physiological temperature. Even if the ultrafiltration membrane has the pore size large enough for the penetration of small protein synthesizing factors, in particular some initiation factors and tRNA, it is possible that the translational machineries are continuously involved with each other thus creating a dynamic multi-complex

that is incapable of passing through the pores. Furthermore, there is increasing continuously involved with each other thus creating a dynamic multi-complex that is incapable of passing through the pores. Furthermore, there is increasing evidence that in the case of plant mRNA stability and translation, rather than discreet events, may be coupled. Some studies have shown that specific sequences in the mRNA are capable of binding with products of translation thus contributing to the stability of the messenger (Morimoto *et al.*, 1991; Prouty and Goldberg, 1992).

Thus, the ability to combine biochemical and genetic approaches provides basic knowledge on how specific sequences in the mRNA can be exploited for their regulatory potential. Finally, the method presented here represents a cutting-edge technology to express and prepare large number of protein samples efficiently for a number of applications.

Table 1. Comparison of rates of protein efflux from continuous system*

Extract	mRNA	Protein yield (mg protein/h)	Reference
Translation only			
E. coli	MS2 RNA	4.8	19
E. coli	Calcitonin mRNA	1.5	19
T. thermophilus	MS2 RNA	5.9	22
Wheat germ	BMV RNA	10.0	19
Wheat germ	Calcitonin mRNA	0.78	19
Wheat germ	Aldolase mRNA	6.8	21
Wheat germ	Tachyplesin mRNA	3.5	This study
Coupled transcription-translation			
E. coli	CAT	5.88	5
E. coli	DHFR	5.87	6
Wheat germ	Aldolase mRNA	5.78	21
Wheat germ	Tachyplesin mRNA	2.13	This study

* Reactions were performed at 37°C except for the *T. thermophilus* system which was conducted at 65°C.

ACKNOWLEDGMENT

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The nucleotide sequence of tpn-1 reported in this paper has been deposited in the GenBank under accession number A38345.

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