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An *In Vitro* Assay to Screen for Translation Inhibitors

SONG, CHIN-HEE, HYOUNG-ROK PAIK, CHI NAM SEONG, AND SANG KI CHOI*

Department of Biological Sciences, College of Natural Science, Sunchon National University, Jeonnam, Korea

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Abstract Protein synthesis is the ultimate outcome of gene expression which, in turn, is regulated by several translation factors. We attempted to identify substances that can inhibit the translation process *in vitro* when the outcome protein is luciferase. To this end, we developed a sensitive cell-free protein synthesis assay using luciferase as the reporter. The synthesis of luciferase increased proportionately as mRNA was added to a 15-μl reaction medium in concentrations ranging from 5 ng to 500 ng. The maximum amount of luciferase was synthesized when the media were incubated at 25°C for 40 min. The concentration of each compound that inhibited luciferase production by 50% (IC_{50}) was calculated. Hygromycin, puromycin, and cycloheximide yielded an IC_{50} of 0.008, 0.8, and 0.7 μg/ml, respectively. A filtrate of *Streptomyces* spp. isolates inhibited protein synthesis up to 5-fold when added to the *in vitro* translation assay mixture.

Key words: *In vitro* translation, luciferase, inhibitor, *Streptomyces* spp., protein synthesis

The control of gene expression at the translational level is important in cell growth and proliferation [3, 9, 11]. Numerous *in vitro* translation assay methods have been developed to facilitate the study of this key step in gene expression. The first method was successfully developed in *Escherichia coli* [14] and was later carried out in rabbit reticulocytes [8], wheat germ [7], and *Saccharomyces cerevisiae* [13]. The translation process involves numerous components, including ribosomes, mRNA, translation factors, and aminoacyl tRNA synthetase. The initiation of translation depends on recognition of the AUG codon in mRNA by a translation initiation complex [4]. A methionyl-puromycin assay was developed as an *in vitro* assay that represented a miniature version of the initial step in translation [2]. Dipeptide met-puromycin was synthesized using this method, with AUG serving as a template in the presence of several purified components of the translation process.

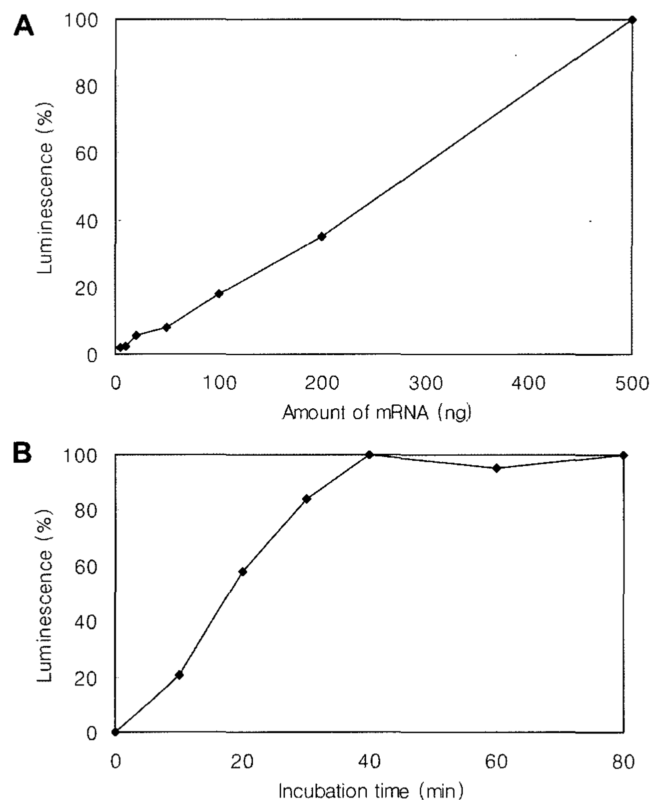

Fig. 1. Effect of the amount of luciferase mRNA and incubation time on the efficiency of the *in vitro* translation reaction.
A. Firefly luciferase mRNA was transcribed using a T7 transcription kit (Epicentre), plasmid DNA was digested by SmaI, and mRNA was purified using the RNeasy Total RNA kit (Quiagen). The concentration of mRNA was determined by spectrophotometry at an absorbance wavelength of 260 nm. The amount of mRNA indicated on the x axis was added to 15 μl of the translation mixture and used to measure the activity of newly synthesized luciferase. **B**. One-hundred ng of luciferase mRNA was added to 15 μl of the translation reaction mixture and incubated at 25°C for 80 min. The reaction was stopped with dry ice and the activity of the newly synthesized luciferase was measured.

*Corresponding author
Phone: 82-61-750-3619; Fax: 82-61-750-3608;
E-mail: sangkic@sunchon.ac.kr

Polyphenylalanine synthesis, a conventional *in vitro* translation assay, uses poly(U) as a template for the expression of phenylalanine in the presence of a high concentration of magnesium.

A cell extract containing numerous components of the translation process was prepared and should be kept intact during the extraction process to enhance translation activity [1]. Translation was monitored by radiolabeling methionine that would be taken up by nascent proteins, and then tracing the radioactivity or analyzing the results through Western blotting. Luciferase was recently used as a reporter to monitor the translation process *in vitro* [5, 6, 10]. To find compounds that can modulate the translation process, we developed an *in vitro* translation system in *S. cerevisiae*. In this report, we introduce an *in vitro* translation assay that is sensitive enough to monitor changes in protein synthesis in the presence of 8 ng/ml of hygromycin. This system has been used successfully to screen microbial culture filtrates for substances that inhibit protein synthesis. The advantage of this assay is that the numerous substances that play a role in translation and protein synthesis can be targeted by an unknown inhibitor. Thus, it provides a great chance to find inhibitors that could be developed into novel anti-infective agents.

S. cerevisiae was grown on YpD (2% bactopeptone, 1% bacto yeast extract, and 1% dextrose) to an optical density at 600 nm ($OD_{600\ nm}$) of 1.5 in 2 l at 30°C. The cells were harvested by centrifuging the supernatant at 3,000 rpm in a Supra 22 K centrifuge (Hanil Sci., Inchon, Korea). The cells were washed 5 times in buffer A (30 mM HEPES, pH 7.4, 100 mM KOAc, 2 mM MgOAc, and 2 mM dithiothreitol) containing 8.5 M mannitol. The cells were lysed with glass beads (0.5 mm, BioSpec, Bartlesville, U.S.A.), by vortexing the solution 5 times for 1 min each and cooling it on ice between cycles in a cold room. The solution was then centrifuged for 6 min at 18,000 rpm in an S-34 rotor. The supernatant was removed as soon as possible after centrifugation was complete. During its removal, the investigators were careful to avoid the lipids at the top of the supernatant and cells at the bottom. The supernatant was loaded onto a Sepharose G25 column (2×20) equilibrated with buffer A containing 0.5 mM PMSF that had been washed and divided into 0.5-ml fractions. Fractions with a value of 90 or higher at $OD_{260\ nm}$ were pooled and frozen in liquid nitrogen. They remained quite stable over 6 months while stored in a deep freezer at -80°C.

Luciferase mRNA originated from fireflies has been used as a reporter in *in vitro* translation assays. The plasmid containing the firefly luciferase gene was digested by SmaI and purified on agarose gel. DNA (1 μg) was used for *in vitro* transcription and to prepare polyadenylated luciferase mRNA (Epicentre, Madison, U.S.A.). Translation assays were performed as described previously [12], with some modification, and stored in liquid nitrogen. The extracts

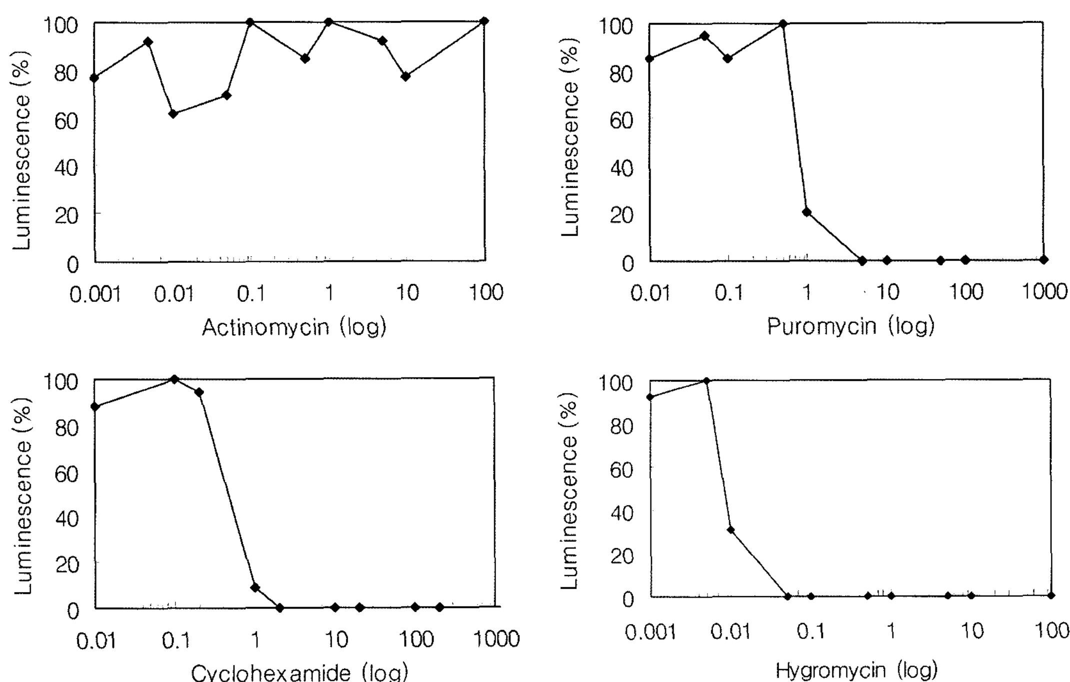

Fig. 2. Effect of several compounds known to inhibit protein synthesis on the *in vitro* translation reaction.
Each of the following compounds, which are known to inhibit either transcription or translation, was added to a tube containing a portion of the translation reaction mixture in the following amounts: actinomycin, 0.001 to 100 μg/ml; puromycin, 0.01 to 1,000 μg/ml; cyclohexamide, 0.01 to 1,000 μg/ml; and hygromycin, 0.001 to 100 μg/ml. The reaction tubes were incubated at 25°C for 40 min, and then the luciferase activity in each tube was measured.

were then thawed on ice and mixed with 2× translation buffer (40 mM HEPES, pH 7.4, 260 mM potassium acetate, 4 mM magnesium acetate, 1.5 mM ATP, 0.2 mM GTP, 3 mM dithiothreitol, 50 mg/ml creatine phosphate, 0.3 mg/ml creatine phosphate kinase, and a 0.08-mM amino acid mixture). The test sample consisted of 24% of the 15-µl reaction mixture. Translation efficiency was monitored by measuring luciferase activity using a luminometer.

We found that the luciferase concentration increased proportionately to the mRNA concentration (range: 5–500 ng) in 15 µl of the reaction mixture (Fig. 1A). Translation took place when the sample was incubated at 25°C and the translation product reached a maximum concentration at 40 min (Fig. 1B).

To determine whether this assay can be used to screen for compounds inhibiting translation, we chose several compounds that are already known to inhibit eukaryotic translation and evaluated their effect on our *in vitro* translation system. Actinomycin, which inhibits transcription, did not inhibit translation in our system until it reached a concentration of 100 µg/ml. However, puromycin, cyclohexamide, and hygromycin inhibited translation with varying degrees of efficiency, as determined by the IC_{50} of the compound. Hygromycin, puromycin, and cycloheximide revealed an IC_{50} of 0.008, 0.8, and 0.7 µg/ml, respectively (Fig. 2). These results suggest that this *in vitro* translation method is very sensitive to compounds that inhibit translation.

We evaluated the use of this method to look for new translation-inhibiting molecules. We isolated approximately 500 *Streptomyces* species primarily from soil in the Sunchon area of Korea and found 15 strains that demonstrated antifungal activity by forming a clear zone around yeast grown on a culture plate. Five of these strains were tested to determine whether they have translation-inhibiting activity. Two strains inhibited translation *in vitro*, but 3 other strains did not (Fig. 3). Distilled water (H_2O) was used in the translation assay as a positive control since culture filtrate was diluted with water up to 10^3 times. A KM11 sample had significant inhibitory activity, as indicated by its ability to reduce luciferase levels 5-fold. A GYEA medium used to grow the *Streptomyces* organisms had an inhibitory effect in protein synthesis when it comprised only 24% of the reaction mixture. When its concentration was reduced to a maximum of 2.4%, translation activity decreased by 70% compared with controls. When its concentration fell to 0.24% of the reaction mixture, it had no inhibitory effect. Therefore, the translation activity shown in Fig. 3 does not indicate any background activity in the GYEA medium.

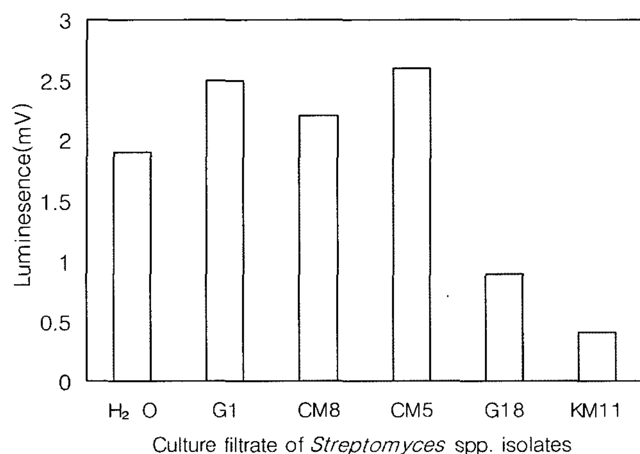

Fig. 3. Effect of microbial culture filtrates known to inhibit the growth of yeast on the *in vitro* translation reaction.
Streptomyces spp. were isolated from soil in the Sunchon area of Korea, and 5 strains were selected for their demonstrated antifungal activity *in vitro*. These strains were grown in a GYEA medium for 2 days at 30°C, and the filtrate was obtained by centrifugation at 12,000 rpm for 5 min. The filtrate was then diluted 10^3 fold, as indicated, comprising 24% of the translation reaction mixture. Luciferase activity was measured in the presence of the microbial filtrate.

We conclude that the *in vitro* translation system developed in our laboratory may be useful in the search for new antifungal molecules, as well as for understanding the molecular mechanisms involved in translation.

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