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Method for Linking a Synthesized Protein to Its mRNA-DNA Complex

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ABSTRACT

A nascent protein remains in a complex with its ribosome and mRNA if the stop codon is deleted from the mRNA. In the same manner, mRNA forms a stable complex with DNA if the transcription termination is blocked. In principle, if both mRNA translation and DNA transcription termination are prevented, the protein should stay in a complex with its mRNA and DNA. A method is designed to test these possibilities. Using an immobilized luciferase gene sequence, a functional luciferase protein is produced that remains associated through its mRNA with its DNA, confirming the feasibility of the proposed scheme. It has potential application for in vitro synthesis of proteins and protein micro-arrays.

INTRODUCTION

Protein production is the result of the two-step process of transcription and translation. During translation, the protein is in a stable complex with mRNA via the ribosome. At the end of translation when the ribosome reaches a stop codon, the newly synthesized protein is released from both the ribosome and the mRNA. Similarly, in transcription, the mRNA stays associated with the DNA through RNA polymerase and is released when the polymerase reaches a termination signal. To link the newly synthesized protein to its DNA, we prevent the termination of transcription and translation.

Translation termination is prevented by deleting the sequences that encode the stop codon from the initial DNA. Without a stop codon, the ribosome will move along the mRNA to the last codon but will not release the protein (1,2,6). Such coding has been used in experiments to obtain a stable attachment of the protein to the mRNA via the ribosome (3,4). As a further extension of the method, the protein can be covalently

linked to its mRNA to achieve a cleaner (i.e., free of ribosome) and more stable protein-mRNA linkage (8,9).

We have developed a method to prevent transcription termination. It consists of binding avidin to one end of DNA that is biotinylated downstream of the coding sequence. This method is analogous to some existing DNA-RNA linking methods such as covalently cross-linking the DNA duplex structure (12,13) or DNA adducts (7,10,11).

If both transcription and translation termination are prevented, the resulting structure is expected to be composed of the protein bound to mRNA through the ribosome, and the mRNA linked to DNA.

In our test, avidin (NeutrAvidin™; Pierce Chemical, Rockford, IL, USA) is coated on polystyrene beads, and biotinylated DNA is then immobilized on the beads. The test DNA has the luciferase gene (*luc*). We verified that the protein stays bound to the DNA through its mRNA by observing that luciferase luminescence is associated with the beads.

MATERIALS AND METHODS

We used the following buffer solutions. TE: 10 mM Tris, pH 8.5, 1 mM EDTA; TlowE: 10 mM Tris, pH 8.5, 0.1 mM EDTA; and TBSM: 10 mM Tris, pH 8.0, 50 mM NaCl and 5 mM MgCl₂.

DNA Biotinylation and Immobilization on Beads

Biotin is attached to the 5'-end of a desired DNA primer using a biotinylation kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and purified over a G-25 gel filtration column (AmiKa, Columbia, MD, USA). Using one biotinylated primer, the PCR product is biotinylated at the C-terminus of the coding region. NeutrAvidin-coated polystyrene beads of 1-μm diameter (Molecular Probes, Eugene, OR, USA) comprise the solid medium for DNA immobilization. The beads are washed once in TBSM. The DNA and beads are mixed at 1 pmol DNA/10 μg beads. The binding reaction is carried out at 37°C for 30 min. The coupling efficiency is approximately 50%. Before

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reusing the beads, they are collected by centrifugation at $14000\times g$ for 5 min.

PCR

The PCR buffer is composed of 200 mM Tris, pH 8.4, 2.5 mM $MgCl_2$ and 0.2 mM each of the four dNTPs, similar to that for RapidCycler® (Idaho Technology, Salt Lake City, UT, USA). The reaction has 0.25 μM each of the primers and less than 2 nM starting template. Either Advantage® 2 (Clontech Laboratories, Palo Alto, CA, USA) or a 1:32 mixture of *Pfu* (Promega, Madison, WI, USA) and *Taq* (Life Technologies, Rockville, MD, USA) polymerases are used. The reaction volume is 30 μL in a 0.2-mL thin-wall tube using a PCR Sprint thermocycler (Hybaid, Teddington, UK) in simulated mode. The cycle used consists of 1 s at 94°C, 1 s at the lower of the two primer melting temperatures and 1 s per 50 nucleotides of the template length at 74°C. About 25 cycles are usually needed. The PCR product is treated for 15 min at 25°C with 1 U Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs, Beverly, MA, USA). It is then purified with a PCR purification kit (Amersham Pharmacia Biotech), eluted and stored in 50 μL TlowE. If necessary, the PCR product is purified by agarose gel electrophoresis and the DNA extracted using a DNA extraction kit (Qiagen, Valencia, CA, USA).

Ligation

PCR fragments are joined together by blunt-end ligation. One fragment is biotinylated at one end and immobilized on NeutrAvidin-coated polystyrene beads. The other end is free and not phosphorylated. The second fragment is not immobilized and is phosphorylated at one end. The ligation reaction joins the free end of the immobilized DNA fragment to the phosphorylated end of the free fragment. A ligation solution of 5 μL (Clonable™ kit; Novagen, Madison, WI, USA) has 1 pmol immobilized fragment and 0.1 pmol free fragment. The reaction is incubated for 15 min at room temperature. The beads are collected by centrifugation at $14000\times g$ for 5 min. The

supernatant is discarded and the beads suspended and stored in 10 μL TE. One percent of this bead suspension can be used as the template source for any subsequent PCR.

Coupled In Vitro Transcription and Translation

TNT® T7 Coupled Wheat Germ Extract System (Promega) is used for in vitro transcription and translation of the *luc* gene and the spacer sequence. The 50 μL total reaction volume contains 0.3 pmol bead-immobilized DNA. The reaction is incubated for 50 min at 25°C with occasional shaking to keep the beads in suspension.

Luciferase Assay

Luciferase reaction mixture (Promega) containing luciferin and stabilizing agents is mixed with the transcription and translation sample. Light emission from the reaction is measured with a fluorescence polarization measurement unit, Beacon® 2000 (PanVera, Madison, WI, USA), adapted so that the excitation light is blocked and the emission filter omitted. In the sample chamber, 80 μL luciferase reaction mixture is incubated at 25°C. A 20- μL transcription and translation product is then injected into the luciferase reaction mixture. The sampling rate for luminescence measurement is about 0.14 Hz. The total light intensity, in relative lumen unit (RLU), is collected by a computer and plotted with KaleidaGraph™ software (Synergy Software, Reading, PA, USA). The instrument has a minimum threshold of approximately 0.01 RLU.

RESULTS

DNA Construction

The testing DNA has three regions: the control region, the *luc* sequence (luciferase coding sequence, 1.65 kb) and a spacer (Figure 1). The control region has a T7 promoter sequence for transcription. The sequence encoding the stop codon of the *luc* sequence was deleted by PCR. At least 120 bp are needed for the spacer, and we used 717 bp.

DNA fragments were first amplified by PCR. These fragments were joined together by directional ligation (see Materials and Methods) and further amplified by PCR. For immobilization on coated beads, the C-terminal primer (at the end of transcription) was biotinylated.

Luciferase Fusion Protein is Functional

It is known that luciferase fused with a peptide at the C-terminus is functional (6). To verify its functionality in our construct, we subjected the testing DNA to in vitro coupled transcription and translation. The transcription and translation product was mixed with luciferase assay solution, and the light output was found to be 2 RLU. The light emission demonstrates that our construct is functional.

Expressing Immobilized DNA and Binding mRNA Protein Complex to the Beads

Biotinylated DNA was captured by NeutrAvidin-coated polystyrene beads

Figure 1. DNA schematic diagram. The primer sequences are P1, 5'-GATTACGCCAAGCTCTAAT-ACGACTCACTATA-3'; lucC, 5'-CACGGCGATCTTCCGCCCTTCTTG-3'; and P2, 5'-TTTGAT-AGTTCATCCATGCC-3'.

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(1 μm diameter). The beads were washed to eliminate unbound DNA. The DNA molecules immobilized on the beads were subjected to in vitro coupled transcription and translation, followed immediately by centrifugation at $23\,000\times g$ at 4°C for 5 min to separate the beads from the supernatant.

To test if luciferase protein was bound to the beads or was free in solution, two samples were prepared. One 20- μL volume/aliquot of bead-free supernatant was collected and another 20- μL aliquot of bead-free supernatant was mixed with the beads and 80 μL luciferase reaction mixture.

The bead-containing sample was tested for light emission from luciferase catalysis (Figure 2). The level was measured at 0.3 RLU initially and gradually tapered off (typical for this kind of luciferase assay). Therefore, a functional luciferase appears associated with the immobilized DNA.

In a separate assay, the luciferase activity of bead-free supernatant was measured. The light emission registered was near the instrument noise level, and 26 out of 30 measurements were below the detection threshold (Figure 2). Therefore, luciferase activity is found to be associated with the beads and not with the supernatant.

Releasing Protein from Beads by Puromycin

In the presence of puromycin, the nascent proteins are released from the ribosomes (5). Therefore, puromycin may be used to distinguish between luciferase bound to beads via the ribosome and luciferase bound to beads via other mechanisms.

The transcription/translation conditions used in the following experiment are similar to those from the earlier one, with some modifications. The reaction volume has approximately 0.1 pmol immobilized DNA and 0.1 pmol free DNA in 40 μL . After 40 min at 23°C , the reaction mixture is divided into two equal portions in two microcentrifuge tubes.

In one tube, 1 mM puromycin (Sigma, St. Louis, MO, USA) is added, and the other tube was left untreated. Immediately after the puromycin addition, the beads are separated from the supernatant

by centrifugation at $23\,000\times g$ at 4°C for 5 min. The supernatant was then collected from each tube, and the remaining beads were resuspended in 10 μL transcription and translation solution. At this point, there were four samples: (i) puromycin-treated beads, (ii) puromycin supernatant, (iii) untreated beads and (iv) untreated supernatant. Puromycin (1 mM) was then added to all samples to prevent further translation.

Each sample was then assayed for luciferase activity (RLU). In the puromycin-treated samples, the bead-associated activity is measured to be about 1.6% ($\text{SD} = 0.2\%$, $n = 4$) of the luciferase activity in supernatant. In the untreated samples, the bead-associated luciferase activity is approximately 13% ($\text{SD} = 2\%$, $n = 4$) of the activity in the supernatant. After normalization, the ratio of puromycin-treated to untreated samples was calculated to be $(\text{RLU}_{\text{beads}}/\text{RLU}_{\text{supernatant}})_{\text{treated}}/(\text{RLU}_{\text{beads}}/\text{RLU}_{\text{supernatant}})_{\text{untreated}} = 0.13$ ($\text{SD} = 0.04$, $n = 4$).

This demonstrates that the luciferase activity associated with the beads can be released by puromycin and is bound via ribosomes to the beads. The small amount of luciferase still associated with beads after puromycin treatment can be attributed to the residual reaction solution in the bead pellet.

RNA Transcribed from Immobilized DNA Stays Bound to Beads

In vitro transcription reactions are carried out using a template DNA that is similar but shorter (0.7 kb) than in the transcription/translation experiments. The 20- μL reaction has either biotinylated DNA immobilized to 5 μg NeutrAvidin beads, 6 pmol non-biotinylated DNA mixed with the beads or beads alone without DNA. To label the RNA, 125 μM fluorescein-12-UTP (Sigma) is added to the reaction. A Riboprobe[®] System T7 transcription kit (Promega) is used according to the manufacturer's instructions; the reaction is incubated at 23°C for 30 min.

After transcription, the beads were washed in 50 μL transcription buffer, sedimented by centrifugation and resuspended in 100 μL TE. The fluorescence intensity (IT, in arbitrary units) from the washed beads was measured using the Beacon 2000 with a fluorescein filter set and an average of five readings. The fluorescence signal from beads with immobilized DNA is found to be much higher ($\text{IT} = 100.6$) than that of beads with free DNA ($\text{IT} = 11.13$). The fluorescence from beads with free DNA is mainly caused by adsorption of fluorescein-12-UTP because the beads without DNA sample

Figure 2. Luciferase reaction luminescence measurement. The full dots are the luminescence of bead-containing samples. Hollow dots refer to luminescence of bead-free samples.

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give a similar level of fluorescence (IT = 12.37). Therefore, the mRNA synthesized from immobilized DNA remains associated with the DNA.

DISCUSSION

Several *in vitro* methods have been devised to link a protein to its mRNA and DNA (2–4,8,9). The method discussed here offers a fast and simple alternative.

The design of this method is based on the following rationale: if the termination sites of both transcription and translation are blocked, the protein produced will remain in complex with its mRNA, which will in turn be associated with its DNA. Omitting the in-frame stop codon blocks the translation termination. The transcription termination is blocked by avidin-biotin binding, which also serves to immobilize the DNA to the solid support. Avidin envelops the biotinylated DNA and so

prevents the RNA polymerase from reading through at the end of the DNA. The linker between the DNA and the biotin is equivalent in length to a 9-carbon chain. The biotin binding site is, in turn, about 9 Å below the avidin surface. Given the close proximity of the end of the DNA to the avidin surface, this naïve picture is plausible. Although the details are not resolved, our results are consistent with this general scheme.

As a practical method to link the protein to its DNA through its mRNA, it is important to ensure that if the protein is released, the beads will not adsorb it. Such re-adsorption is insignificant in our method for the following reasons: (i) The transcription/translation reaction solution is rich in proteins that should function as blocking agents to minimize weak, nonspecific adsorption; (ii) the model protein luciferase is released from the beads by puromycin, which indicates that the protein is bound to the beads via its ribosome (and therefore, via its mRNA).

Also, it is desirable that the protein remains bound. Some release of protein from the DNA-RNA complex is inevitable. Compared with covalent linking methods (8,9), a non-covalent linking protocol like ours is expected to have higher levels of protein release. The method has been qualitatively tested under physiological conditions, namely, in transcription/translation solutions over time at around 25°C. If an application requires additional stability (e.g., using immobilized protein in multiple-use biosensors), the protein can be permanently immobilized by covalent cross-linking.

The present method uses common techniques and materials and DNA as the genetic material that is stable and easy to produce and handle. With these features, the method could be applicable for making protein microarrays from DNA microarrays.

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