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OPTIMIZATION OF PCR TEMPLATE CONCENTRATIONS FOR *IN VITRO* TRANSCRIPTION-TRANSLATION REACTIONS

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SUMMARY

In vitro transcription-translation reactions can be used to rapidly and inexpensively synthesize small amounts of protein from cloned DNA sequences for biochemical studies. The use of PCR to amplify template sequences has many advantageous over conventional protocols. However, the amount of template needs to be carefully adjusted for optimal production of protein products.

INTRODUCTION

In vitro transcription/translation of PCR products has become an increasingly popular method for synthesizing small amounts of full length or truncated proteins for study while bypassing the laborious and time intensive cloning steps associated with traditional cellular protein expression systems. The use of PCR to generate the DNA template and/or introduce deletions or mutations in the protein has augmented the usefulness of this technique (Nishikawa et al., 1989). In a single day, a large number of templates can be amplified and translated into protein which can then be tested for functional activity. We have used this system in the functional dissection of the basic leucine zipper DNA binding protein, EmBP-1 (Guiltinan and Miller, 1992).

In the course of this work, we have used template concentrations as recommended by the manufacturer of a commercially available transcription-translation system (TnT system, Promega, Madison WI) of up to 2 ug per 50 µl reaction. This recommendation is based on plasmid templates, which are often much larger than the PCR templates we utilize. This results in a proportionate increase in the molar concentration of PCR template in the reaction. We have found that such high template molarity can severely inhibit the TnT reaction and slightly higher concentrations can result in a nearly complete inhibition of protein synthesis.

MATERIALS AND METHODS

PCR Reactions PCR reactions were performed to amplify a region of the EmBP gene spanning amino acid residues 150-308 as described (Guiltinan and Miller, 1994). The 5' primer was designed to incorporate a T7 promoter and an in-frame start codon into the final product. The 3' primer has an in frame stop codon and cloning sites 3' of the coding region. PCR reactions were performed in duplicate in a volume of 100 microliters containing 3 ng plasmid template, 20 pmoles of each primer, 240 mM dNTPs, 2-5 U Taq polymerase (Promega, Madison, WI), 2 mM $MgCl_2$, and the appropriate 10X buffer supplied with the enzyme. Samples were subjected to a 5 minute "hot start" at 95°C during which the dNTPs were added, followed by 35 cycles of denaturing at 95°C for 1 minute, annealing at 58°C for 1 minute, and extension at 72°C for 1.5 minutes; and a final 5 minute extension at 72°C. At this point, the duplicate samples were pooled and the 518 bp product purified using Wizard PCR Preps (Promega, Madison, WI) and concentrated into a final volume of 50 µl in TE (10 mM Tris, 1 mM EDTA, pH 7.6) buffer. DNA concentrations were assessed using a Gene Quant DNA Calculator (Pharmacia Biotech, Cambridge, England).

Transcription-Translation System A T7 coupled Reticulocyte Lysate System (TnT, Promega, Madison, WI) was utilized to perform the transcription/translation using the PCR product templates according to the manufacturers' instructions for 25 µl reactions. Eight microliters of undiluted PCR product, which contained 16 pmoles of DNA, were used in reaction number one. Reactions 2-7 contained 8 µl of sequential two-fold dilutions of the PCR reaction product. RNAse free water alone was used in reaction eight to serve as the unprogrammed control.

Electrophoresis Five microliters of each TnT translation product were analyzed on denaturing 10% tricine/glycine polyacrylamide gels (Schagger and Jagow, 1987). One microliter from each TnT reaction was also used in DNA-binding reactions and electrophoretic mobility shift assay (EMSA) according to Guiltinan et al., (1990). Gel bands were quantified by phosphorimagery using Molecular Dynamics Phosphorimager 445 SI and ImageQuantNT software (Molecular Dynamics, Sunnyvale, CA). A predicted full length product of 22.1 kd was observed and quantified for further analysis. In EMSA analysis, several protein-DNA complexes with differing mobility were formed consistent with our previous studies, and all of the complexes were quantified together.

RESULTS AND DISCUSSION

In order to test the effect of template concentration of the efficiency of the TnT reaction system, a two fold dilution series of the PCR template was prepared and equal volumes of each were added to TnT reactions. Reaction products were run on SDS gels to evaluate the amount of protein synthesis in each reaction (Fig. 1). As expected, the amount of protein synthesized was proportional to the amount of template added at the lower concentrations, raising to a maximum of about 1.8 pmol of template (corresponding to about 0.6 ug of DNA). However, when larger amounts of template were added, protein synthesis was inhibited. In the highest concentration tested (7.5 pmol or about 2.5 ug), protein was barely detectable. Quantitation of the protein product showed that the effect is quantitatively dramatic, resulting in a 4 fold reduction in protein product when 7.5 pmol is added (Fig. 1b).

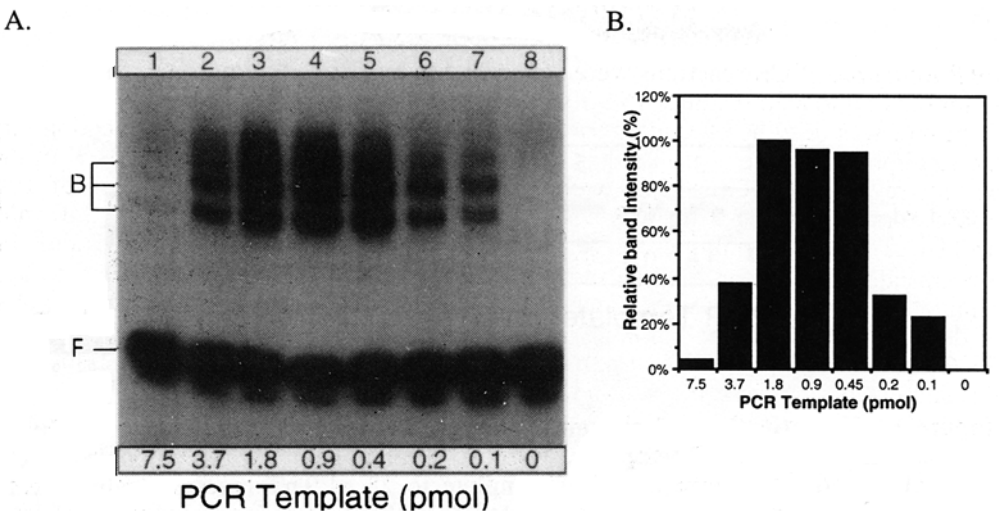

Figure 2. **Functional assay of proteins produced in TnT reactions.** Panel A. DNA binding analysis. Equal volumes of TnT reactions described in Fig. 1 were subjected to electrophoretic mobility shift assay (EMSA) as previously described, using a radiolabeled DNA fragment containing the binding site for the EmBP-1 protein. B = bound DNA-protein complexes, F = free DNA probe. Panel B. Quantitation of EMSA results. The radioactivity associated with the DNA/protein complexes in A was measured using phosphor-imagery as described above. Values are expressed as a percentage of the maximal value observed (1.8 pmol/reaction).

protein synthesized. If one doubles this concentration in a reaction, a nearly complete inhibition of protein synthesis results. It should also be noted that if one simply calculates the molarity of the template based on the manufacturers instructions and assuming a 5 kbp plasmid, only up to 0.3 pmol/25 µl reaction would be used, resulting in a sub-optimal protein production.

It is clear from these results that when considering using PCR templates for TnT reactions, a titration of template over a range between 0.2 to 7.5 pmol/25 µl reaction should first be performed to optimize the production of protein for each template. With this consideration, PCR based-coupled transcription-translation system is a powerful tool in the study of gene and protein function in vitro.

ACKNOWLEDGMENT

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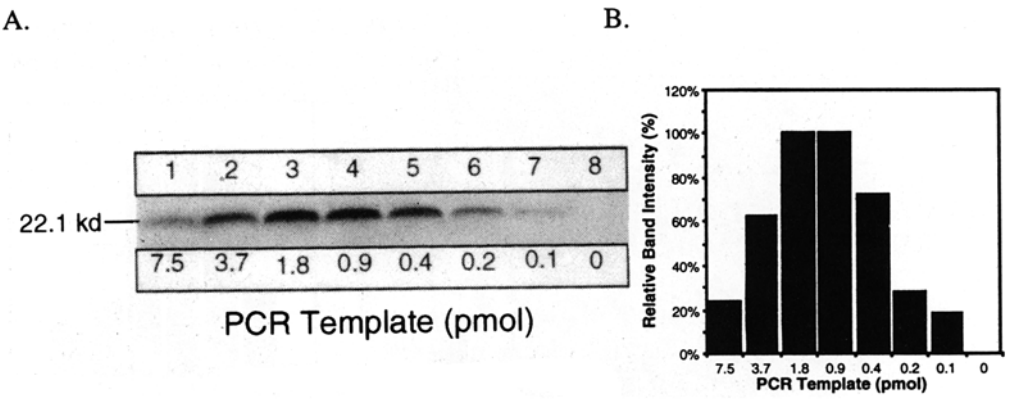

Figure 1. **Quantitation of proteins synthesized in TnT reactions containing various amounts of PCR template.** Panel A. Protein gel. Various amounts of PCR product as indicated were added as template to 25 µl TnT reactions which were incubated for 2 hours at 30°C, and separated on a 10% polyacrylamide tricine-glycine gel. After electrophoresis, the gel was dried and autoradiographed, then quantified using a phosphoimager. Panel B. Quantitation of protein bands. Total counts per band were determined using the ImageQuant software for each reaction after subtraction of background from each value. Values are expressed as a percentage of the maximal value observed (1.8 pmol/reaction). A reaction containing no template was run as a control (0 pmol lane).

To verify this result, a functional assay was performed on the protein products. DNA binding reactions were set up as described using equal volumes of the TnT reactions. Proteins were incubated with a radiolabeled DNA fragment containing the recognition site for EmBP-1. After electrophoresis, autoradiography revealed that the levels of functional EmBP-1 closely paralleled that of the protein synthesized in each reaction (Fig. 2). Quantitation of these bands shows that as in Figure 1, DNA binding activity is maximal at 1.8 pmol template and is nearly completely inhibited a 7.5 pmol (Fig. 2b).

We have shown that high concentrations of PCR template added to a TnT reaction can severely inhibit the final production of protein. This effect could be at several possible levels: inhibition of transcription or translation (or both) and could be due to; excess template forming non-productive complexes with the molecular machinery which carries out these reactions, inhibitors from the DNA template preperation, differences in codon usage bias between plants and the tRNA composition of the translation mixture, or a combination of these variables.

Following the manufacturers' instructions, if an investigator adds up to 2 ug/50 µl reaction (equivalent to 1 ug/25 µl reaction used in this study), of a 500 bp PCR fragment, this represents a final concentration of 3 pmol/25µl, 10 times higher than if a plasmid of 5 kbp were used and this can result in an approximate 50% reduction in