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Expression-PCR (E-PCR): Overview and Applications

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In vitro transcription and translation are powerful tools for examining the structure–function relationships of proteins. Genes can be cloned into plasmid vectors containing the bacteriophage promoters T7, T3, and SP6 and then transcribed in the presence of an appropriate RNA polymerase. However, efficient in vitro translation of these RNA transcripts often requires the insertion of an appropriate untranslated leader sequence downstream from the promoter to provide a suitable context for ribosomal binding and initiation of protein synthesis. Standard methods for in vitro transcription and translation are limited further by their requirements for cloning, bacterial amplification, DNA extraction, and restriction enzyme digestion before the desired DNA template can be transcribed and translated.

We believe that it would be of great advantage to express functional proteins from DNA without these constraints. Because of the ease of obtaining adequate quantities of any gene segment by the use of PCR, we designed a method called expression-PCR (E-PCR)(1) to modify this DNA so that it could be expressed without having to go through the rigors of cloning. E-PCR is a rapid, simple method for the in vitro production of proteins without cloning. The resulting radiochemically pure proteins are useful for a variety of purposes, including studies on the subunit structure of proteins, epitope mapping, and protein mutagenesis.

THE DESIGN OF THE UNIVERSAL PROMOTER

The key to E-PCR was the design of a small DNA cassette that had all the functional regions needed by RNA polymerase to initiate transcription of a downstream DNA segment into an RNA molecule, which could then be translated into protein. Four functional regions were needed for this upstream segment (see Fig. 1): (1) a RNA polymerase-binding region; (2) an untranslated leader sequence; (3) a Kozak sequence; and (4) an initiation of translation codon. We incorporated these four regions into a single unit called a Universal Promoter (UP) because this unit permits the transcription of any DNA segment spliced to it.

The binding domain of T7 RNA polymerase was chosen because it is well studied and is commercially available. Nine bases were added 5′ to the T7 promoter because, although the recognition sequence of 17 bp is required, the T7 RNA polymerase also needs a number of bases 5′ of these to attach stably. Footprint analysis indicates that the sequence of the upstream fragment is not critical, but at least 5 nucleotides are needed to stabilize the protein–DNA interaction of the polymerase with the promoter site.(2) We took advantage of these base requirements to add the nucleotides of a restriction enzyme site for *Hin*dIII in the event that cloning of a final construct was desired.

In vitro transcription requires little more than the presence of a bacteriophage promoter upstream from the cloned DNA of interest; however, translating the desired gene can be more problematic. In vitro translation, unlike transcription, is often dependent on the presence, and characteristics, of an untranslated leader (UTL) sequence 5′ to the initiation codon. The efficiency of translation may be poor if the AUG initiation codon lies too close or too far from the 5′ end of the RNA,(3) resides in poor sequence context,(4) is inaccessible because of the secondary structure of the mRNA,(5) or if there are increased requirements for translation initiation factors.(6) To avoid these problems, it is often necessary to replace the normal 5′-UTL with a UTL from an efficiently translated protein. Jobling and Gehrke(7) have shown previously that replacement of a gene's native UTL with the UTL sequence derived from the coat protein of the alfalfa mosaic virus (AMV) can increase translation efficiency as much as 35-fold. For these reasons, we have

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5′ CCAAGCTTCTAATACGACTCACTATAGGGTTTTTATTTTTAATTTTCTTTCAAATACTTCCACC ATG GCA CTG 3′
T7 promoter (UTL from AMV) KS Met Ala Leu

SINGLE STRANDED UNIVERSAL PROMOTER

5′ CCAAGCTTCTAATACGACTCACTATAGGG 3′
Hind III Site T7 promoter
H3T7 PRIMER

FIGURE 1 The sequence of the single-stranded UP used in E-PCR and its 5′-specific H3T7 primer.

followed the T7 promoter site with the 33 nucleotides of the UTL of this AMV coat protein.

The eukaryotic ribosome initiates translation at the first AUG codon in a favorable context on the mRNA strand. Upstream and downstream sequence requirements have been identified by Kozak(4) and are termed "Kozak sequences." Therefore, following the UTL sequence, and upstream of the ATG, we added the sequence CCACC as a consensus Kozak sequence (KS). Immediately following the ATG, G^{+4} was added to strengthen further the consideration of the ATG as an initiator codon. This G^{+4} requirement is not absolute.

Although the above domains up to and including the ATG are needed for efficient translation, an additional extension of the 3′ end of the UP was designed to allow the UP to be installed upstream of a desired gene fragment using a process called "splicing by overlap extension (SOE)".(8) The G^{+4} was incorporated into the codon (GCA) for the amino acid alanine, a small hydrophobic amino acid, and the codon (CTG) for leucine was added so that the experimenter would have the choice of adding either [^{35}S]methionine or [^{3}H]leucine as a radioactive tag in the final translation product. The bases chosen for the codons for Ala and Leu maximized the GC content to allow a higher annealing temperature during the SOE reaction needed to link the UP to the DNA sequence of interest.

DESIGN, SYNTHESIS, AND PURIFICATION OF UP AND SPECIFIC PRIMERS

Any PCR primers can be used to amplify the DNA of interest, and many computer programs now exist that help to select optimum primer pairs. The only requirement is that the forward primer has the last 9 bases (ATGGCACTG) of the UP added to its 5′ end with the first codon (ATG) lined up in the desired open reading frame (ORF). The 73-base UP and the 29-nucleotide H3T7 (Fig. 1) primer can be made as single-stranded oligonucleotides using a standard DNA synthesizer. Purification of full-length products either by acrylamide gel or by using the "trityl on" step in the last synthesis cycle followed by an OPC column (Applied Biosystems, Foster City, CA) for purification is acceptable.

METHOD

The technique is outlined in Figure 2.

1. Amplify by a standard PCR the gene or DNA segment to be expressed. Design the 5′ primer to contain at its 5′ end a 9-nucleotide sequence identical to the 9 bases on the 3′ end of the UP. Keep amplifications to a maximum of 10–15 cycles.

   It is important to obtain a clean PCR band on the initial PCR. This may require the testing of several different thermophilic polymerase enzymes and/or buffer conditions. Use a PCR Optimizer Kit (Invitrogen, San Diego, CA, cat. K1220-01) if initial attempts do not yield acceptable results. If only a few extra bands are observed on gel analysis, then use low-melt agarose to separate and

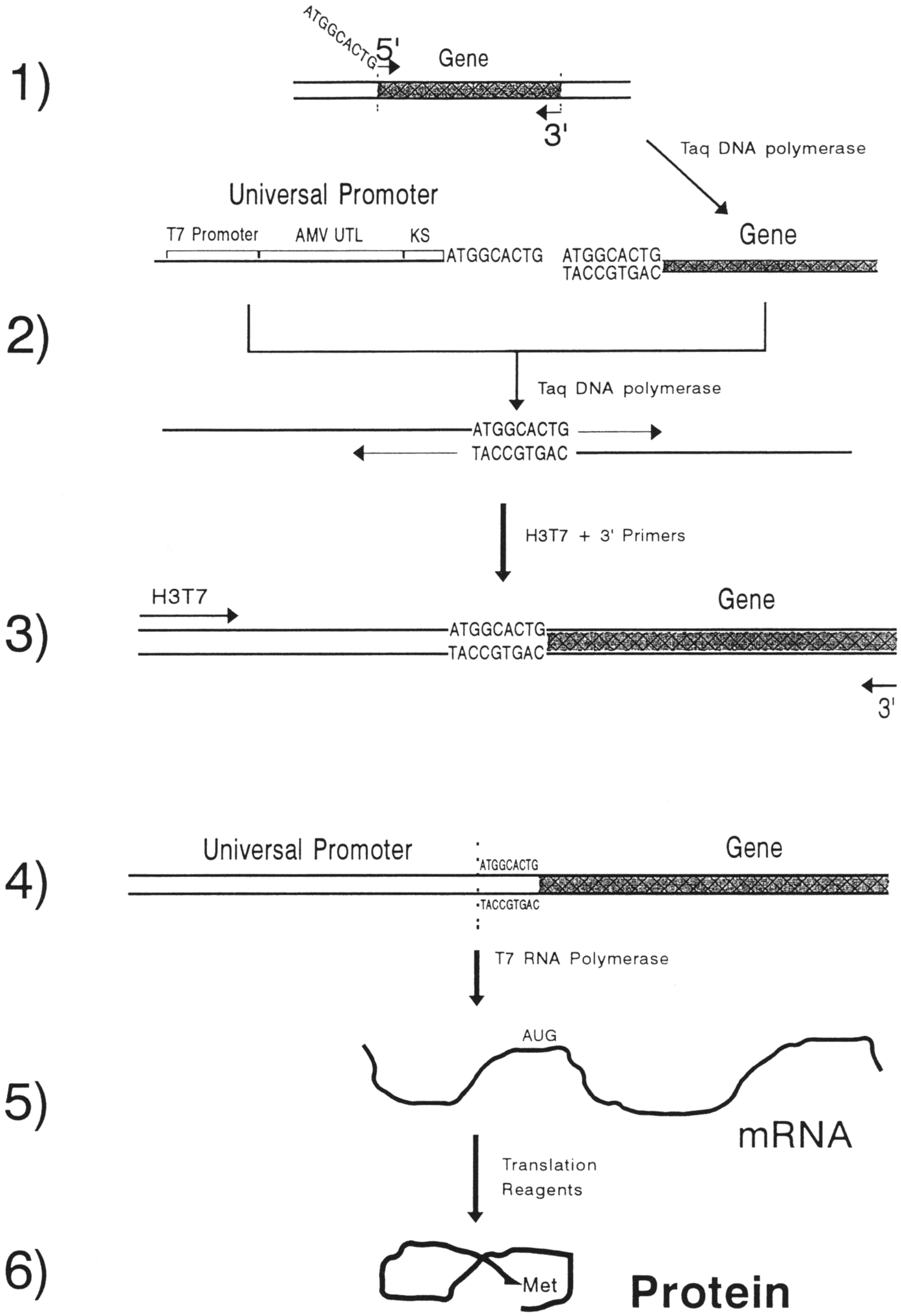

FIGURE 2 Principles of E-PCR. Outline of how a gene or gene segment can be translated into usable protein without cloning using E-PCR. See Methods section for details.

excise the DNA band of interest. The DNA can be used directly from the low-melt agarose (Fig. 2, step 1).

2. Splice the UP to the gene of interest using a two-step PCR, which includes an initial overlap extension program followed by a secondary PCR reaction. In the overlap extension program, add 1–10 ng of the primary PCR product to 30 fmoles of the single-stranded UP (higher concentrations of both can be used without adverse effects) to a 100-μl reaction containing 1× PCR buffer, 50 μM dNTPs, 1 mM magnesium chloride, 2.5 units of *Taq* DNA polymerase but no primers. Denature at 94°C for 5 min and follow by five cycles at 94° for 30 sec, 25°C for 30 sec, and 72°C for 1–6 min. Link to a soak file at 80°C (Fig. 2, step 2).

3. When the temperature has equilibrated to 80°C, add 50 pmoles of the H3T7 primer, complementary to the 5′ end of the UP, and 50 pmoles of antisense gene-specific primer. Then denature the reaction at 94°C for 5 min and follow by 20–30 cycles at 94°C for 30 sec, ~50°C for 30 sec, and 72°C for 1–6 min depending on the melting temperature of the gene-specific primer and the DNA template length. Extract the DNA products with chloroform, precipitate, and resuspend in 10 μl of RNase-free water. This is enough for at least 5 in vitro transcription reactions (Fig. 2, steps 3 and 4).
4. The gene linked to the UP is transcribed into RNA by the use of T7 RNA polymerase (Megascript, Ambion, Inc., Austin, TX, cat. 1334). Unlike cloned genes in plasmid-based systems, the DNA does not have to be CsCl banded or cut with restriction enzymes to give high yields of RNA. The mRNA is translated into protein in a separate reaction (Retic Lysate IVT Kit, Ambion, Inc., Austin, TX, cat. 1200). Alternatively, the PCR reaction containing the gene segment linked to the UP can be added directly to a transcription/translation cocktail, which allows these two reactions to be performed in a single tube (TNT Coupled Reticulocyte Lysate System, Promega Corp, Madison, WI, cat. L4610) (Fig. 2, steps 5 and 6).

USES OF E-PCR PROTEIN

Proteins made by E-PCR have been used for a variety of experiments. We have used E-PCR to map the red blood cell receptor of a *Plasmodium falciparum* protein down to 40 amino acids.(9) E-PCR has also been used to epitope-map the sites of autoantibody reactivity within the human thyrotropin receptor in patients with Grave's disease.(10) Protein that is made by E-PCR can also have functional enzymatic activity. Thornton and Rashtchian(11) have synthesized the enzyme chloramphenicol acetyltransferase (CAT) and have shown that it has the same activity as enzyme made from purified CAT mRNA synthesized using a plasmid-based system. Because the genes are transcribed and translated in vitro, problems associated with bacterial growth and expression are avoided. Carole Long's group at Hahneman University(12) have shown that a polypeptide made by E-PCR had a conformationally correct epitope as defined by a monoclonal antibody, whereas bacterially expressed protein did not fold correctly.

One of the most intriguing uses of E-PCR is for making proteins to inject into animals for the production of antibodies.(9) In this case, the synthesis of protein from mRNA is carried out without the use of radioactive methionine or leucine. Mice are injected with 100 μl of translation mixture and rabbits with 1000 μl. One potential advantage of generating antibodies against E-PCR proteins is the use of protein synthesized in a rabbit reticulocyte lysate to immunize rabbits. In this instance, only the newly synthesized protein is "foreign" to the rabbit, and thus a low complement of background antibodies is seen.

An advantage of in vitro transcription and translation is the ability to produce mutant protein by altering the DNA template. By incorporating E-PCR with the site-directed mutagenesis procedure of Higuchi et al.(13) it is possible to generate mutant polypeptides in 1 day that can than be screened for biologic activity or used as immunogens.

In summary, by using E-PCR, it is possible to move from PCR product to functional translated protein in under 8 hr. The approach offers significant advantages for reseachers performing domain mapping, epitope mapping, and site-directed mutagenesis because this system offers the potential to identify biologically important domains and constructs rapidly for further analysis.

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