In Vitro Translation of Proteins with Terminal Deletions by SP6 RNA Polymerase-mediated Transcription of PCR Products

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The PCR can be used to generate DNA substrates for RNA transcription by subcloning the cDNA of interest generated by PCR into vectors containing RNA polymerase promoters. Subcloning can be avoided by including the T7 or T3 RNA polymerase promoter in the 5' primer used for PCR amplification of the cDNA.^(1,2) The transcribed RNA can then be translated using a rabbit reticulocyte or a wheat germ lysate system.⁽³⁾ Using this approach, truncated proteins have been generated in a coupled transcription-translation system⁽⁴⁾ and a rabbit reticulocyte translation lysate.^(2,5) To generate amino- and carboxy-terminal truncations and an internal deletion near the carboxyl terminus of a mammalian mitochondrial enzyme, monoamine oxidase type B (MAO-B), we designed primers containing a SP6 RNA polymerase promoter, start or stop codon, and a Kozak sequence⁽⁶⁾ that promotes translation by eukaryotic ribosomes.

This approach could be used with any full-length cDNA generated by reverse transcription. In our case, we used a cloned cDNA covering the complete coding region for human MAO-B (520 amino acids, 1560 bp⁽²⁾) that was generated by first-strand cDNA synthesis from adult human liver mRNA (prepared by the LiCl method⁽⁷⁾) using a primer corresponding to base pairs 1654–1684 of the published sequence,⁽⁸⁾ followed by PCR amplification with primers (Biosearch Cyclone DNA synthesizer, Millipore Corporation, Bedford, MA) corresponding to base pairs 12–35 and 1654–1684. These primers had *Xba*I sites at their 5' ends for cloning into a Bluescript SK(–) vector (Stratagene, La Jolla, California) containing both T7 and T3 RNA polymerase promoters. Modified Sanger dou-

ble-stranded sequencing⁽⁹⁾ of the human MAO-B product confirmed it was identical to the published sequence.⁽⁸⁾

Five oligonucleotide primers were designed to generate a 16-amino-acid amino-terminal truncation (MAO-B-N), an 8-amino-acid carboxy-terminal truncation (MAO-B-C), or a 17-amino-acid internal deletion near the carboxyl terminus (MAO-B-I) in the MAO-B protein produced by the RNA transcript and in vitro translation. For the amino-terminal deletion (Fig. 1A), primer 1 contained the 24-nucleotide SP6 RNA polymerase promoter, including one G in the Kozak sequence, and 21 nucleotides from MAO-B starting from the second methionine located at amino acid number 17.

[For all primers, the bold sequence represents the primer generated. The underlined sequence (3'→5') corresponds to the MAO-B sequence.⁽⁷⁾ The underlined sequence (5'→3') corresponds to an ATG start codon or a TAA or TGA stop codon. The SP6 promoter sequence is highlighted by a line over the letters (5'→3'); * is placed above the first G in the Kozak sequence.⁽⁶⁾]

Primer 1 was mixed with primer 3 containing 21 nucleotides coding for the MAO-B carboxyl terminus, with the native stop codon followed by 11 nucleotides including a *Hind*III restriction site.

For the carboxy-terminal truncation (Fig. 1B), primer 2 contained the 24-nucleotide SP6 RNA polymerase promoter and 21 nucleotides of MAO-B starting from the native Kozak sequence.

[The MAO-B native Kozak sequence is the first 9 bp before the ATG in primer 2.]

We mixed primer 2 with primer 4 containing 21 nucleotides (coding for

Primer 1: 5'-TACGATTTAGGTGACACTATAGAAATGGCAGCAGCCAAACTTCTG-3'
3'-ATGCTAAATCCACTGTGATATCTTTACCGTCGTCGGTTTGAAGAC-5'

Primer 2: 5'-TACGATTTAGGTGACACTATAGAAGCGAGCGCCATGAGCAACAAA-3'
3'-ATGCTAAATCCACTGTGATATCTTTCGCTCGCGGTACTCGTTGTTT-5'

Primer 3: 5'-GGGCTACTTGTGAGAGTCTAAAAGCTTAGAGA-3'
3'-CCCGATGAACACTCTCAGATTTTCGAATCTCT-5'

Primer 4: 5'-GCTCTTGGCTTCCTGGCCCACTGAAAGCTTAGAGA-3'
3'-CGAGAACCGAAGGACCGGGTGACTTTTCGAATCTCT-5'

Primer 5: 5'-GGCCTGCTCAGGCTG AAAAGGGGGCTACTTGTGAGAGTCTAAAGA-3'
3'-CCGGACGAGTCCGAC TTTTCCCCGATGAACACTCTCAGATTTCT-5'

FIGURE 1 PCR strategy. (Dashed line) Bluescript SK(–) vector DNA; (bold solid line) MAO-B cDNA⁽⁷⁾; (solid line) PCR product DNA. (A) To generate an amino-terminal deletion (MAO-B-N), primer 1, containing SP6 RNA polymerase promoter, an artificial Kozak sequence,⁽⁶⁾ and MAO-B sequence including the second TAG codon, was mixed with primer 3, containing MAO-B sequence including the native stop codon. (B) To generate a carboxy-terminal deletion (MAO-B-C), primer 2, containing an SP6 promoter and MAO-B sequence including the native Kozak sequence and the first ATG codon, was mixed with primer 4, containing MAO-B sequence and an artificial stop codon. (C) To generate an internal deletion (MAO-B-I), primer 2 was mixed with primer 5 containing two discontinuous portions of MAO-B sequence including the native stop codon.

FIGURE 2 Autoradiograph of SDS-PAGE with ³⁵S-labeled protein translated in vitro using rabbit reticulocyte lysate. SDS-PAGE used a 0.75-mm-thick, 6.25% polyacrylamide gel followed by impregnation with Enhance (NEN-Dupont, Wilmington, Delaware) and drying. (Lane H₂O) Translation with no RNA added; (lane B) undeleted MAO-B (520 amino acids); (lane N) MAO-B deleted for 16 amino acids of the amino terminus (MAO-B-N); (lane C) MAO-B deleted for 8 amino acids of the carboxyl terminus (MAO-B-C); (lane I) MAO-B deleted for 17 internal amino acids (MAO-B-I).

amino acids 506–512), a stop codon, and 11 nucleotides including a *Hind*III restriction site.

For the 17-amino-acid internal deletion near the carboxyl terminus (Fig. 1C), we mixed primer 2 with primer 5 containing 15 nucleotides (coding for amino acids 490–494), 27 nucleotides (coding for amino acids 513–520 and the native stop codon), and 3 extra nucleotides.

[The blank space in primer 5 represents the site of the internal deletion.]

Ten nanograms of each primer, 5 μg of the MAO-B cDNA in the Bluescript vector, standard amounts⁽³⁾ of PCR

buffer, nucleotides, and HPLC water were combined to a total volume of 50 μ l. Thirty cycles of PCR were carried out at 94°C for 60 sec, 58°C for 30 sec, and 72°C for 90 sec followed by extension at 72°C for 10 min. PCR amplification produced a single band in the predicted size range of 1.6 kb. Each 50- μ l reaction generated about 20 μ g of DNA product, as estimated by the amount of UV absorption in a 1% agarose gel following ethidium bromide staining. PCR products were extracted with phenol/chloroform, precipitated with ethanol, and resuspended in water. RNA was transcribed from the PCR products in a 100- μ l reaction volume containing transcription buffer, dithiothreitol RNase inhibitor, nucleotides, and 60 units of SP6 RNA polymerase (Promega, Madison, Wisconsin).⁽¹⁰⁾ Following phenol/chloroform extraction and ethanol precipitation of RNA, protein was translated in vitro using [³⁵S] methionine and rabbit reticulocyte lysate.⁽¹¹⁾ Figure 2 illustrates appropriately sized protein products for the undeleted MAO-B (generated from RNA transcribed from the full-length cDNA clone with T7 RNA polymerase), the amino-terminal truncation (MAO-B-N), the carboxyl-terminal truncation (MAO-B-C), and the internal deletion (MAO-B-I) as visualized by SDS-PAGE⁽¹²⁾ and autoradiography.

This paper describes an uncomplicated way to generate terminal and internal deletions in proteins starting from a cDNA for the corresponding mRNA. Deletions at or near protein termini are especially useful in assessing signal sequences and stop-transfer sequences involving in intracellular targeting. The truncated MAO-B proteins generated here should help to resolve how this enzyme is targeted to mitochondria, as it lacks the typical signal sequence at the amino terminus.⁽¹³⁾ In conclusion, we directly generated RNA and deleted proteins for human MAO-B using PCR-generated DNA containing a SP6 RNA polymerase promoter.

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