Combination of Cross-Species RNA Solution Hybridization and Immunoprecipitation Aids in the Cloning of RT-PCR Products

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cDNA cloning by means of reverse transcription-polymerase chain reaction (RT-PCR) is a widely used technique. Ever since the first published method by Veres et al. (6), it has supplant conventional methods of cDNA cloning due to its ease and speed. However, there are certain constraints that are associated with this technique; e.g., it requires the knowledge of a few nucleic acid sequences of the gene of interest. These can be obtained by either microsequencing the protein itself followed by the construction of a putative cDNA sequence or, more commonly, by using the homologous cDNA sequence from other species. However, both methods use degenerate primers (4, 5), which can give rise to nonspecific priming (2). Therefore, variations of PCR have been developed to overcome this problem, e.g., touchdown PCR (3) and nested PCR (1). However, none of the techniques has been used successfully in our laboratory to clone the rabbit serotonin (5-hydroxytryptamine, 5-HT) type 3 receptor from the retina using degenerate primers based on a rat sequence (unpublished sequence data; GenBank® Accession No. U59672). Although additional manipulations of PCR methods and reagents were attempted, none of these was successful in obtaining the rabbit 5-HT3 receptor gene coding regions. Indeed, very of -

gous to the 5-HT none of which, after subsequent showed multiple bands (Figure 1A), could, in part, arise because the techniques has been used success -

ticable because of long stretches of the same genes is sufficiently high; e.g., between either RNA and DNA or DNA and DNA strands. Once the duplex is formed, it can be selectively retrieved by immunoprecipitation. Thus, if a gene sequence is known in one species, appropriate RNA or DNA probes can be generated and used to identify and isolate the gene in different species. Although this method is performed, it can be easily adapted for a broad range of applications by those molecular biologists interested in the evolution of specific genes, comparative studies of gene structure and some clinical applications, e.g., the simultaneous identification of multiple strains of infectious agents such as human immunodeficiency virus (HIV).

First, rat 5-HT3-digoxigenin (Dig) labeled RNA probes were generated using in vitro transcription with a DIG RNA Labeling Kit (SP6/T7) (Boehringer Mannheim, Indianapolis, IN, USA). A cloning vector with a rat 5-HT3 insert that we had previously cloned (unpublished data) was used. Next, standard RT-PCR on rat retina RNA was performed using degenerate primers with the exception that the annealing temperature was slightly decreased to make sure that the gene of interest, in our case, the rabbit 5-HT3 receptor, was present in the PCR product. The RNA isolation was performed using RNAzol™ B (Tel-Test, Friendswood, TX, USA) according to the manufacturer’s instructions. First-strand cDNA synthesis was performed using SUPERSCRIPT™ II RNase H- Reverse Transcriptase (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer’s instructions. The PCR was: 2.5 µL of 20x Enzyme Buffer (1.0 M Tris-HCl, pH 9.0, 0.4 M ammonium sulfate), 1 µL dNTP mixture (2.5 mM each of dATP, dCTP, dGTP, dTTP), 0.5 µg of each of the

primers and 1 mM MgCl2. The reaction was run in a PTC-100™ Thermal Cycler (MJ Research, Watertown, MA, USA) as follows: 94°C for 4 min (hot start), 35 cycles of 94°C for 45 s (denaturation), 50°C for 45 s (annealing) and

![Figure 1. Solution hybridization and immunoprecipitation followed by another round of PCR results in high-fidelity amplification of target genes.](image-url)
72°C for 2 min (extension), with an additional final extension time of 10 min. Multiple PCRs were performed to ensure that the putative PCR product was present in sufficient quantity. The PCR products were pooled and extracted with phenol/chloroform (1:1, vol/vol). The DNA was precipitated in 2.5 vol of absolute ethanol and 0.1 vol of 3 M sodium acetate, pH 5.3, for 1 h on dry ice. The resulting DNA pellet was resuspended in an appropriate volume of diethyl pyrocarbonate (DEPC; Sigma, St. Louis, MO, USA)-treated water.

Next, solution-hybridization was performed at 45°C for 16 h on the rabbit PCR products and the Dig-labeled rat 5-HT3 RNA probes. The final hybridization buffer consisted of 60% formamide, 23.25 mM sodium citrate, pH 6.4, 212.5 mM EDTA, pH 8.0, in a final volume of 10 ml of absolute ethanol and 0.1 vol of 3 M sodium acetate, pH 5.3, for 1 h on dry ice. The resulting DNA pellet was resuspended in an appropriate volume of double-distilled water. The DNA-RNA hybrids were separated by denaturing at 95°C for 3 min and immediately cooled on ice. Subsequently, 5 μL of RNase A (10 mg/mL) (Boehringer Mannheim) were added, and the mixture was incubated at 37°C for 30 min to ensure that the RNA probes were completely degraded, thus preventing cross contamination in the subsequent PCR step. Another round of phenol/chloroform extraction was performed to isolate the DNA from the Dig, digoxin and antibodies, all of which are soluble in phenol. The DNA remaining in the aqueous phase was precipitated and amplified by another round of PCR using the same degenerate primers. At this step, one major product of the expected size and few minor products were obtained (Figure 1B), which is better than results obtained by a conventional PCR (Figure 1A). Subsequently, standard PCR cloning was performed.

Analysis of the subclones by restriction digestion with PvuII (New England Biolabs, Beverly, MA, USA) showed that 7 out of 14 clones contained the correct size insert (Figure 1C). One of these was picked out and sequenced, which revealed that it is indeed the rabbit 5-HT3 receptor (GenBank Accession No. AF121107).

In conclusion, we have developed a novel method of using solution hybridization and immunoprecipitation coupled to PCR to isolate specific PCR products. In so doing, we have successfully amplified and cloned a rabbit 5-HT3 receptor from the retina. This technique, as described above, has broad applicability to evolutionary and comparative molecular biology since it can be used in cross-species studies of single genes. Moreover, variations of this technique could involve multiple nucleic acid conjugates, e.g., biotin, fluorescein or Dig for labeling RNA or DNA probes. Thereby, multiple genes or variants of the same gene could be extracted from a single sample simultaneously. Ensuing PCR analysis then would not be dominated by the major gene variants. This latter modification would be a powerful tool in both clinical molecular diagnostic work, where isolation of multiple strains of infectious organisms is important to the clinical management and in molecular phylogenetic studies of gene duplications in organisms such as zebrafish and xenopus.

REFERENCES

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