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Multiple isoform recovery (MIR)-PCR: a simple method for the isolation of related mRNA isoforms

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ABSTRACT

We present a rapid and efficient method for the detection of related transcripts with different expression levels. This approach combines the rapid amplification of cDNA ends (RACE) method with a cDNA subtractive technique. The strategy is based on successive subtractions of prevalent isoforms resulting in enrichment of less expressed transcripts. For each subtraction, a biotinylated primer specific for the prevalent isoform is hybridized on the total cDNA and the hybrid is retained on a streptavidin affinity column. The unbound cDNA serves as a template for subsequent isoform identification. To illustrate its application we describe the isolation of three new actin cDNA isoforms in the freshwater planarian *Dugesia (S) polychroa.*

Gene expression is often characterized by different mRNA isoform synthesis. These isoforms arise either by alternative splicing or by transcription of a multigenic family. The resulting mRNAs frequently exhibit extensive regions of sequence homology and often are expressed at very different levels. In these conditions, the detection of all generated transcripts may prove difficult. This could be accomplished by library screening techniques that are time consuming and laborious, or by PCR which is rapid and sensitive, but both methods present limitations. Despite new emerging techniques allowing the enrichment of selected cDNAs (1), the problem of multiple related mRNA isoform identification remains unsolved. Indeed the less abundant mRNAs are overlooked due to the presence of the prevalent isoforms. To circumvent this problem, we present an alternative strategy, termed multiple isoform recovery (MIR)-PCR, which facilitates the isolation of cDNAs derived from closely related mRNAs when gene-specific primers are not available. We illustrate its application in the identification of actin isoform mRNAs in the lower invertebrate planarian *Dugesia (S) polychroa*. Actin is a multigenic family of proteins highly conserved during evolution (2). The presence of different actin isoforms probably reflects functional diversity despite their high degree of amino acid sequence homology (3). In this regard, it is of particular interest to identify not only the prevalent actin isoforms but also the less represented members of the family. For this purpose we have taken advantage of the variability of 5′ UTR actin mRNA sequences to design biotinylated primers specific for the prevalent actin isoforms. These biotinylated primers are added to the bulk of cDNAs and after the second cDNA strand synthesis, the biotinylated actin cDNAs are subtracted by affinity chromatography on streptavidin columns. The unretained cDNAs are then used to identify other actin isoforms. By using this approach we have succeeded in identifying three new planarian actin cDNAs. Planarians were wounded by a transversal cut (4) and the regenerating tissues, where the presence of several actin isoforms has been suggested (4), were collected. Total RNA was isolated by using the TRIzol Reagent according to the manufacturer's protocol (Gibco, Basel, Switzerland). These RNAs were subjected to the rapid amplification of cDNA ends (RACE) method (5). A sample (8 µg) of total RNA was reverse transcribed for 90 min at 37°C in 20 µl of 1× reaction buffer (50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT) containing 0.2 mM dNTPs, 200 U of reverse transcriptase (Superscript II, Gibco) and 50 pmol of a degenerated 3′ primer [P1, 5′-A(G)TAA(G)AAA-(C,G,T,)GTA(G)TGCCA] designed on the basis of an evolutionary conserved region of actin coding sequences (Fig. 1A, step A1). The enzyme was then heat inactivated for 5 min at 95°C. After reverse transcription, RNA was digested with 2 U of RNase H (Promega, Madison, WI) at 37°C for 20 min. To remove excess of dNTPs and primer, the first strand cDNA was ultrafiltered through a Centricon-30 device (Amicon, Wallisellen, Switzerland) and a poly-(dG) tail was added to 1/5 (20 µl) of the retained cDNA (Fig. 1A, step A2). This reaction was carried out in 25 µl at 37°C for 1 h using 20 U of terminal deoxynucleotidyl transferase (TdT, Pharmacia, Dübendorf, Switzerland) and 0.1 mM dGTP. The enzyme was then heat inactivated and 2 µl of the tailed cDNA used as template for a nested-PCR using a poly-$(dC)_{15}$ primer and a non-specific isoform degenerated primer (P2, 5′-TTC(T)TCCAT-GTCGTCCCAGTT) (Fig. 1A, step A3). Amplification was performed in 50 µl of 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl pH 9.0, 0.1% Triton X-100, 1.25 mM $MgCl_2$) containing 50 pmol of each primer, 8 µM dNTPs and 2.5 U Taq DNA polymerase (Promega). After 40 cycles (94°C, 30 s; 56°C, 30 s; 72°C, 30 s) the reaction product was cloned in pCR 2.1 vector (Invitrogen, Leek, Netherlands) and numerous clones were sequenced. The 5′-RACE technique evidenced a unique new actin isoform (DpAct1, GenBank accession number AF027161). This likely corresponds to the prevalent actin isoform in agreement with the assumption that the PCR products reflect relative cDNA abundance in the starting material (Fig. 2A). In

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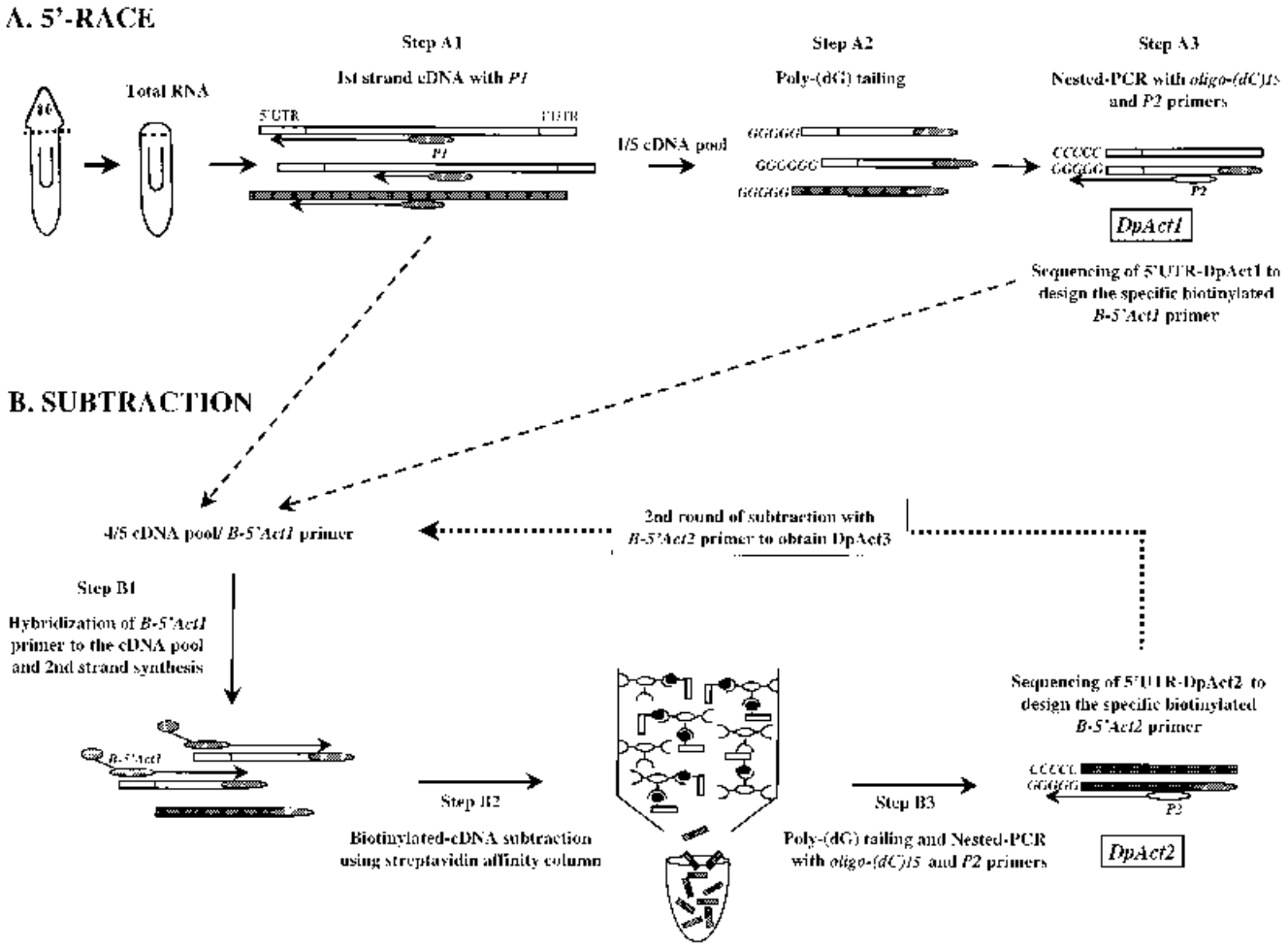

Figure 1. Schematic representation of the MIR-PCR protocol. Part of the planarian cDNAs serves as template for the RACE reaction (**A**) and another part is used for the successive subtractions (**B**).

order to detect actin transcripts present in relatively lower amount, we designed a 5′-end biotinylated oligonucleotide (B-5′Act1, Biotin-5′-GTTTAATAAAACTATTTTTCA, MWG Biotech, Ebersberg, Germany) directed against the isoform-specific 5′ UTR of DpAct1. A sample (25 pmol) of B-5′Act1 was hybridized to the 4/5 (80 µl) of the Centricon-purified cDNA pool synthesized during the RACE reaction. Annealing was performed in 100 µl for 5 min at 50°C (5°C below the calculated optimal annealing temperature) (6) in 1× PCR reaction buffer containing 0.5 µM dNTPs. The complementary strand was synthesized for 15 min at 72°C with 1 U of Taq DNA polymerase (Fig. 1B, step B1). Removal of the unincorporated B-5′Act1 was achieved by an ultrafiltration step with a Centricon-30 device. Subtraction of the biotinylated DpAct1 cDNA was done by loading the 100 µl on a streptavidin micro-affinity column (ABICAP, Abion, Jülich, Germany) previously equilibrated with 1× PCR buffer (Fig. 1B, step B2). Of the unbound cDNA, 20 µl (1/7) were poly-(dG) tailed and the same nested-PCR as described above was performed with poly-$(dC)_{15}$ and P2 primers (Fig. 1B, step B3). Cloning and sequencing identified a second new planarian actin cDNA (DpAct2, GenBank accession number AF027162). Sequence comparison between DpAct1 and DpAct2 shows 83.3% homology in the coding region and 47.8% in the 5′ UTR. Only three amino acids, located in the N-terminus of the protein fragment, are different. A second cDNA subtraction was then performed using a biotinylated primer designed on the basis of the 5′ UTR sequence of DpAct2 (B-5′Act2, Biotin-5′-AATCGATACCTTAAATAAATA, MWG Biotech). B-5′Act2 was hybridized at 56°C (2°C below the calculated optimal annealing temperature) to 6/7 (130 µl) of previously enriched cDNA pool resulting from the first subtraction. After a second round of MIR-PCR, we cloned a third new planarian actin cDNA (DpAct3, GenBank accession number AF027163). This last sequence is >95% homologous to the coding sequence of DpAct2 and >80% to the one of DpAct1. The deduced protein sequences of DpAct2 and DpAct3 are identical. The 5′ UTR sequence of DpAct3 shares 39.1% identity with DpAct1 and 47.8% with DpAct2.

The MIR-PCR protocol described here, based on the successive removal of abundantly expressed gene isoforms, results in an enrichment of less expressed isoforms allowing their identification. By this method we have identified three actin isoforms that correspond more likely to different genes than to transcripts resulting from alternative splicing. It is indeed well accepted that actin isoforms are encoded by a set of related genes probably

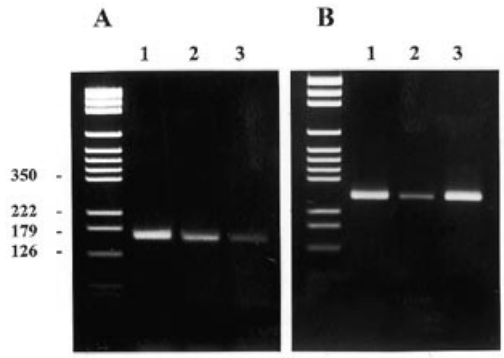

Figure 2. (**A**) RT-PCR analysis of the three planarian actin isoforms DpAct1 (lane 1), DpAct2 (lane 2) and DpAct3 (lane 3) obtained by MIR-PCR showing their relative expression levels. The two primers used either for reverse transcription (nucleotides 184–200 for DpAct1) or PCR (nucleotides 145–160 for DpAct1) were designed in actin regions where the three transcripts are 100% homologous. All the PCR conditions were the same with the exception of the isoform-specific 5′ UTR primers. (**B**) A representative PCR showing the efficiency of the subtraction is presented. The same amount of total cDNA was used as template for PCR before (lane 1) and after (lane 2) DpAct1 subtraction. An aliquot of streptavidin beads was also submitted to PCR (lane 3). These PCR were performed with P2 and B-5′Act1 primers. The size (bp) of pGEM fragments is shown on the left lanes in both (A) and (B).

derived from a common ancestor (3). Moreover, there are differences at the nucleotidic acid level in the coding region even between DpAct2 and DpAct3 that have the same protein sequence. In any event, the MIR-PCR allows multiple isoforms recovery irrespective of the origin of transcripts. We describe the isolation of three cDNAs but we expect that more isoforms can be recovered by this technique even if a limitation exists, since the subtraction is never complete. This partial subtraction does not depend on the efficiency of the chromatography but is rather due to the presence of uncomplete cDNAs, generated during the reverse transcription step, that cannot be hybridized with the 5′ UTR biotinylated primers. The MIR-PCR method may be also useful in the isolation of new mRNA isoforms when some of them are already known. These known isoforms can be substracted in one round of MIR-PCR with different 5′ UTR biotinylated primers.

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