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A novel 3′ extension technique using random primers in RNA-PCR

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In order to obtain sequence 3′ to a partial ~2 kb titin sequence of the >21 kb titin mRNA (1) that was too distant from the poly A tail for 3′ RACE methodologies (2), RNA-PCR (3, 4) was done using a primer containing a random hexamer at its 3′ end. Four μg of rabbit cardiac muscle total RNA (5) in 25 μL were reverse transcribed per BRL's recommendations using 100 ng RT primer (Figure 1) and 200 U MLV reverse transcriptase (BRL). After RNAse H digestion, 10 μL was used for PCR in 100 μL using primers complementary to either known titin sequence (Figure 1, TS 1) or the RT primer (Figure 1, Y primer), and 30 cycles of 93°C-45 sec, 45°C-1.5 min, 72°C-3.0 min. Although defined fragments from 100–1000 bp were observed after the first PCR (Figure 2a) fragments only, <700 bp purified by Geneclean (Bio 101) re-amplified during the second PCR using primers complementary to the RT primer (Figure 1, X primer containing a SalI site) or the known titin sequence (Figure 1, TS2 containing a NotI site). Lower or higher concentrations of RT primer resulted in no or very small amplification products, respectively. Only the random hexamer part of the RT primer initiated reverse transcription from 6-bp sequences of titin mRNA that had at least 50% G/C content.

Final amplification products (Figure 2b) were sequenced by dideoxy chain termination methods using Sequenase (US Biochemical) after digestion with NotI and SalI restriction enzymes and ligation into pBluescript (Stratagene). Two clones were sequenced because of the possibility of infidelity of the Taq polymerase. After repeating this entire procedure three times using new sets sof titin-specific primers, the titin sequence was extended 1109-bp (EMBL accession no. X59596). The second amplification step could perhaps be omitted or done asymmetrically for sequencing.

This technique should also be applicable to 5′ extensions of cDNA clones. Perhaps a modification of it could be applied to extensions of known genomic DNA in either direction.

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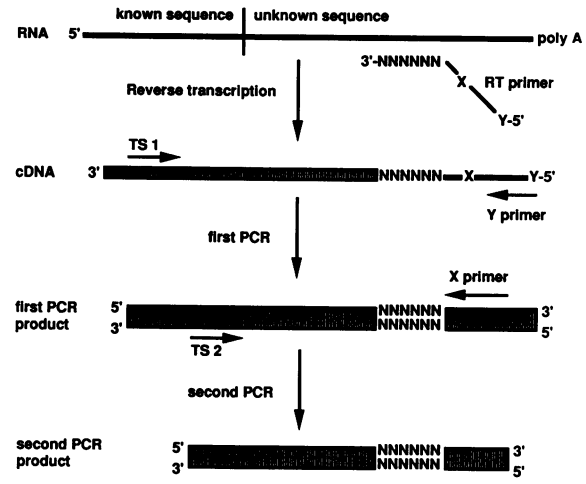

Figure 1. Diagrammatic representation of random primer initiated RNA-PCR. RT primer: 5′- CGAGGGGGATGGTCGACGGAAGCGACCNNNNNN-3′, N = A, T, G or C; Y primer: 5′-CGAGGGGGATGGTCGACGG-3′ and X primer: 5′-GATGGTCGACGGAAGCGACC-3′. For the initial 3′ extension, titin-specific primers (TS 1 and TS 2) were complementary to sequence near the 3′ end of the known titin sequence (corresponding to bp-numbers 1653 to 1674 and 1783 to 1810 in T2 clone, EMBL accession no. X17329) and thereafter titin-specific primers were complementary to the 3′ end of the newly identified sequence. The RT primer, Y primer and X primer were repeatedly used in all 3′ extensions.

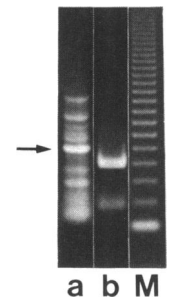

Figure 2. Horizontal agarose (1%) gel electrophoresis of the first (a) and second (b) PCR amplifications. Arrow marks the ~600-bp fragment generated from the first PCR that was excised, purified by Geneclean (Bio101) and used as a template in the second PCR. M, 123-bp ladder (BRL).