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Simple and rapid 5′ and 3′ extension techniques in RT-PCR

Friedhelm Struck and John Collins*
Institute of Molecular Biology, Department of Applied Genetics, GBF — National Research Center for Biotechnology, D-38124 Braunschweig, Germany

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We describe a procedure for successively extending or walking along cDNA clones beyond a known sequence. In order to obtain sequences 5′ to the constant a domain sequence (C α) of the human T cell receptor (TCR), we routinely used a nested primer set for RT-PCR. The unusual feature of the procedure described here is the use of a single spurious primer as one of two primers in the first and subsequent PCR reactions. This allows the use of previously available primers. We have demonstrated it to be effective for a wide range of primer sequences. The first PCR amplification is inefficient but in the second round the nested primer in the TCR-C α region allows specific exponential amplification of the desired sequence. We used the method routinely to isolate variable α (V α) segments for sequencing and subsequent cloning into expression vectors. Using three parallel reactions for each cDNA sample with different arbitrarily chosen spurious primers we have been able to isolate and determine the V α and joining α (J α) sequences of all of the ten human T cell clones investigated.

One μg of total RNA (1) from T cell leukemia lines, peripheral blood lymphocytes or T cell clones in 25 μl were reverse transcribed as outlined (2) using 800 ng of random hexamers and 200 U of MLV reverse transcriptase (BRL). 2.5 μl was used for PCR in 50 μl using primer C α 1 complementary to a known C α domain sequence and a randomly chosen, spurious annealing primer, for example C β, a primer derived from the constant β domain sequence of the human TCR (Figure 1). 30 cycles of 94°C — 1 min, 50°C — 1 min, 72°C — 3 min, with 25 pmol of each primer in 50 μl, were performed. No fragments were observed after the first PCR on ethidium bromide stained agarose gels (Figure 2d). 2.5 μl of the first PCR reaction was used as a template for the second PCR using a nested primer C α 2 complementary to a sequence upstream of the C α 1 sequence. This second reaction usually yielded one or two defined fragments (Figure 2a, b, c).

Final amplification products were blunt-end ligated into pCR-Script SK(+) (Stratagene). Two clones derived from each RNA sample after separate reverse transcription and PCR amplification were sequenced. The clones exhibited the expected sequence in the C α and V α regions. The initial experiments led to isolation of V α 1.3, V α 4.1, V α 11.1, V α 13.1, and V α 30.1 segments.

We tested the procedure with fifteen arbitrarily chosen primers to determine the V α sequences of ten human T cell clones. Three primers have been tested in parallel on each T cell clone cDNA in order to extend the sequence beyond the C α domain. Although at least one positive sample was obtained for each T cell clone, six out of the thirty samples did not yield any visible fragment after the second PCR. The average length of the extended sequences was 400 bp. In each case the amplification product

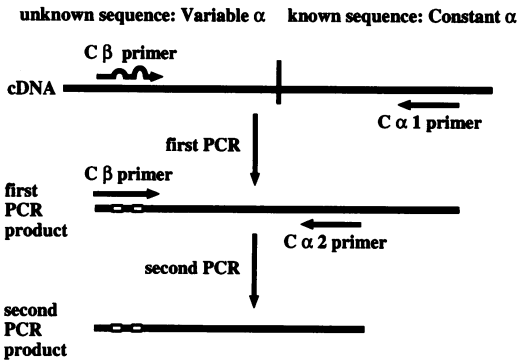

Figure 1. Diagram of nested primer RT-PCR. C β primer: 5′-ATCTCCCACA-CCCAAAAGGCCA-3′, C α 1 primer: 5′-TACACATCAGAATTCTTACTT-TG-3′, C α 2 primer: 5′-GACTTGTCACTGGATTTAGAG-3′. Loops in the arrow of the C β primer correspond to mismatches with the cDNA sequence. C α 1 and C α 2 were complementary to sequence near the 5′ end of the constant a domain sequence.

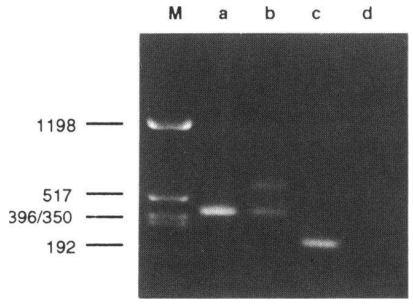

Figure 2. Agarose (1.5%) gel electrophoresis: (M) Molecular weight markers were pGEM 4/HinfI fragments. The sizes (in bp) are indicated. (d) The first, (a) the second PCR amplification of a T leukemia cell line RNA, (b) the second PCR of a peripheral blood lymphocyte RNA, (c) the second PCR of a T cell clone RNA.

* To whom correspondence should be addressed

which was visible on agarose gels contained an expressed V α−J α TCR gene insert successfully extended from the C α sequence. All fragments revealed 30 bp of the C α sequence 5′ to the nested primer (C α 2). The cDNA of five T cell clones was also amplified by using a degenerate primer derived from the V α segment in PCR, instead of the spurious primer. (5′TRCHTTWDAYWACTTAYASTGGTA-3′. R = A,G; H = A,C,T; W = A,T; D = A,G,T; Y = C,T; S = C,G). The 160 bp V α sequences in these amplified fragments were identical to the sequences of fragments from the same cDNA samples which were isolated after amplification with spurious primer as described above.

We routinely use this nested primer technique for the isolation of V α sequences 5′ to the TCR C α chain. The technique could also be applied to 3′ extensions of cDNA clones. Artifacts have not been detected because the specificity of the amplification, which is being compromised in the first round of PCR, is rescued by the nested primer in the second round. For 5′ and 3′ extensions of short sequence stretches in the range of 400 bp adjacent to known sequences as in the case of the V α domain of the T cell receptor we favour this technique to more sophisticated methodologies like RACE (3), ligation-anchored PCR (4) or similar techniques (5) in view of its simplicity and low cost. This should represent an efficient tool for routine examination of superfamily and rearranged gene expression, which can be used to successively extend beyond a known sequence, but without determination of the ends of the RNA molecule. If this latter criterium is prevalent, ligation-anchored PCR (RNA oligo ligation-mediated PCR) or RACE would be required.

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