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Ligation-anchored PCR: A simple amplification technique with single-sided specificity

(immunoglobulin/T-cell receptor/cDNA cloning/gene trap vectors/alternative splicing)

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ABSTRACT **A simple, efficient, and sensitive technique has been developed for amplification of cDNAs encoding molecules with 5′ regions of unknown sequence. In this ligation-anchored PCR, T4 RNA ligase is used to covalently link an "anchor" oligonucleotide to first-strand cDNAs. These anchored cDNAs are then amplified by using one PCR primer specific for the anchor and another specific for a sequence within the molecule of interest. The anchor oligonucleotide has been especially designed to facilitate subsequent analysis and cloning of the resultant PCR products. This three-stage procedure does not require purification of product between steps and avoids many of the technical difficulties associated with established anchored PCR protocols. The efficacy of ligation-anchored PCR was demonstrated by amplification of a specific IgG1 cDNA; total RNA equivalent to as few as 100 cells yielded the expected PCR product.**

The anchored or single-sided PCR allows specific amplification of DNA where the 5′ sequence of the molecule of interest is unknown (1). This approach is based on homopolymer tailing of cDNA, subsequent amplification using one primer specific for the molecule of interest and a second primer containing a defined "anchor" sequence attached to a homopolymer sequence complementary to the tail, and, finally, reamplification using one primer specific for the cDNA of interest and one specific for the anchor. This technique has been very useful, but the established protocols have a number of disadvantages that have restricted their application. These protocols have often proved technically difficult, require multiple purification steps, often need relatively large amounts of starting RNA, and have the potential to generate nonspecific products due to use of homopolymer-containing primers in the PCR. We have developed an alternative approach in which an anchor of defined sequence is directly ligated to first-strand cDNA, and the resultant product is amplified by using primers specific for both the cDNA of interest and the anchor. We have used this technique to amplify a specific immunoglobulin cDNA from as little as 1 ng of total RNA. The simplicity and sensitivity of this ligation-anchored PCR (LA-PCR) suggest that it could have widespread utility.

MATERIALS AND METHODS

Preparation of total RNA and cDNA synthesis have been described (2). GSX-2 hybridoma total RNA was provided by David Tarlinton of this institute. In some experiments, residual RNA was removed after cDNA synthesis by boiling for 5 min with 150 mM NaOH and neutralizing with HCl. The sequence of the anchor oligonucleotide was TTTAGTGAGGGTTAATAAGCGGCCGCGTCGTGACTGGGAGCGC. The 5′ phosphorylation, 3′ end blocking, and T4 RNA ligase reactions were performed essentially as described (3). Briefly, the 5′ end of the anchor was phosphorylated with polynucleotide kinase, and the 3′ end was blocked with ddATP using terminal deoxynucleotidyltransferase (TdT). Ligation reactions were carried out at 22°C for 12–18 h with 10 pmol of the phosphorylated, blocked anchor in 10-μl volumes containing 50 mM Tris·HCl (pH 8), 10 mM $MgCl_2$, 10 μg of bovine serum albumin per ml, 25% (wt/vol) PEG 8000, 1 mM hexamine cobalt chloride, 20 μM ATP, 1 unit of T4 RNA ligase (New England Biolabs), and the amounts of cDNA indicated in the figure legends. Ligations were terminated by adding 32 μl of 0.5 M NaCl/10 mM Tris·HCl, pH 8/1 mM EDTA. PCR was performed with 2-μl aliquots of the terminated ligation reaction mixtures in 25-μl mixtures as described (2). Oligonucleotide primers for PCR were as follows: T3, GCGGCCGCTTATTAACCCTCACTAAA; V_H186.2 external (V_{ex}), GCTGTATCATGCTCTTCTTG; V_H186.2 internal (V_{in}), GGTGTCCACTCCCAGGTCCA; C_γ1 internal (C_{in}), CCAGGGGCCAGTGGATAGAC; C_γ1 external (C_{ex}), GGATGACTCATCCCAGGGTCACCATGGAGT (V, variable region; C, constant region) (4). The reactions were run using a water-bath thermal cycler (Bartelt Instruments, Melbourne, Australia) as follows: cycle 1, 5 min at 94°C, 60 sec at 55°C, and 90 sec at 70°C; cycles 2–40, 45 sec at 94°C, 60 sec at 55°C, and 90 sec at 70°C. PCR products were electrophoresed, transferred to membranes, and hybridized with radiolabeled cDNA probe as described (5). The probe, a PCR-generated fragment corresponding to amino acid positions 40–130 of a V186.2-utilizing IgG1 cDNA, was provided by Michelle McLean of this institute.

RESULTS

LA-PCR Protocol (Fig. 1). In this simple procedure, first-strand cDNA synthesis is carried out with avian myeloblastosis virus reverse transcriptase with oligo$(dT)_{15}$ as primer. The 3′ end of the single-stranded cDNA is then ligated to a 5′-phosphorylated, 3′-end blocked anchor oligonucleotide using T4 RNA ligase. Finally, the resultant anchored product is subjected to PCR amplification using one primer specific for a sequence within the cDNA of interest and another specific for a sequence within the anchor. The success of the LA-PCR depends on the ability of T4 RNA ligase to catalyze efficiently the covalent joining of single-stranded DNA molecules bearing 5′-phosphate and 3′-hydroxyl groups (3). The reverse-transcribed cDNA has hydroxyl groups at both termini, making only the 3′ end a potential substrate for ligation, while the anchor oligonucleotide has been blocked at the 3′ end by addition of a dideoxynucleotide leaving only the 5′-phosphorylated terminus as a potential substrate. Thus,

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Abbreviations: LA-PCR, ligation-anchored PCR; TdT, terminal deoxynucleotidyltransferase; V, variable region; C, constant region.

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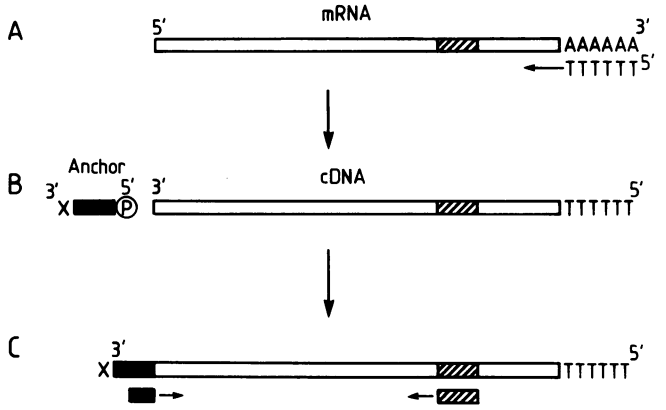

FIG. 1. Protocol for LA-PCR. (*A*) First-strand cDNA synthesis was performed with avian myeloblastosis virus reverse transcriptase and oligo(dT) as primer. (*B*) The 5'-phosphorylated, 3'-end blocked anchor oligonucleotide was ligated to first-strand cDNA with T4 RNA ligase. (*C*) PCR amplification was carried out with oligonucleotide primers specific for a known sequence within the cDNA (hatched box) and for a sequence within the anchor (solid box).

ligation of a single anchor molecule to the 3' end of the cDNA is the favored reaction. The anchor oligonucleotide has been designed to accommodate multiple uses of the amplified PCR product. Although data are presented only for amplifications carried out with the T3 primer, the anchor has been designed to allow sequential use of two nested primers as a means of increasing sensitivity and product yield and to allow direct sequencing of PCR product without prior cloning (4). The inclusion of a T3 promoter sequence allows direct *in vitro* transcription of the PCR product, and inclusion of Not I and Hha I sites facilitates cloning of the product. For optimal cloning of PCR product, sequences for convenient restriction sites can be included at the 5' end of the primer specific for the cDNA of interest.

LA-PCR Amplification of IgG1 cDNA. In experiments conducted with a cloned cDNA, LA-PCR generated a product that hybridized with cDNA-specific probe. The procedure was then tested with total RNA prepared from the GSX-2 hybridoma, which expresses an IgG1 gene using the V_H186.2 variable region (P. A. Lalor, G.J.V.N., R. D. Sanderson, and M.G.M.-W., unpublished data). The complete sequences of the V_H186.2 and C_γ regions are known (6, 7), and primers have been described that allow specific PCR amplification of IgG1 cDNAs containing these sequences (4). The expected sizes of PCR products generated by various primer combinations could therefore be predicted, and the V_H186.2/C_γ primer combinations served as controls in our LA-PCR experiments.

The combination of the anchor-specific T3 primer with the C_γ-specific primers C_{in} and C_{ex} was expected to yield PCR products of 586 and 625 base pairs (bp), respectively (Fig. 2*A*). DNA of the predicted size was detected in both cases, and both PCR products hybridized with an IgG1 cDNA probe (Fig. 2*B*). Control experiments demonstrated that generation of these specific products required the presence of both the anchor oligonucleotide and T4 RNA ligase in the ligation reaction and required the presence of both anchor-specific and cDNA-specific primers in the PCR (Fig. 3; unpublished observations). However, comparison of the sensitivity of the LA-PCR using one anchor-specific and one cDNA-specific primer (which required a starting input of 1 μg of total RNA to generate detectable PCR product) with that of conventional PCR using two cDNA-specific primers (which yielded detectable product from as little as 1 pg of total RNA) suggested that the efficiency of anchor ligation might be low (Fig. 2*B*). T4 RNA ligase can use both single-stranded DNA and single-stranded RNA as substrates, so it appeared pos-

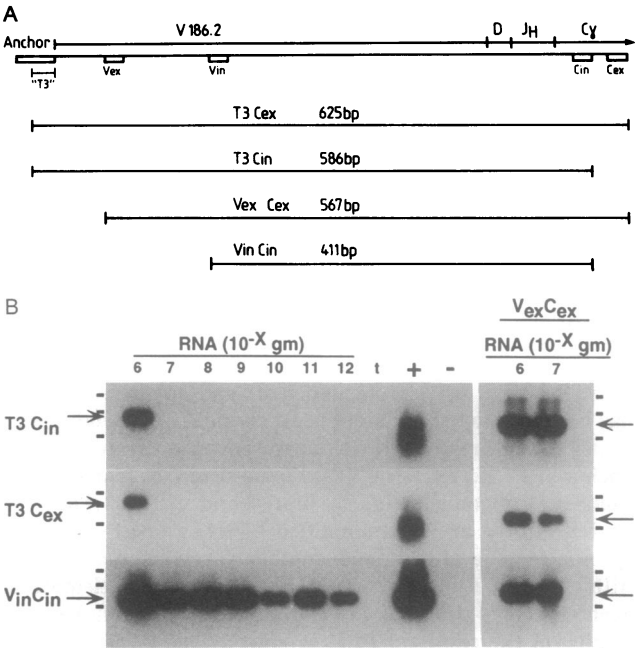

FIG. 2. LA-PCR of IgG1 cDNA. Total RNA prepared from the V_H186.2-utilizing IgG1-producing hybridoma GSX-2 was serially diluted in carrier *Escherichia coli* tRNA (2 mg/ml). Reverse transcription was performed with the amounts shown of diluted GSX-2 hybridoma total RNA, and the resultant cDNAs were used in ligation reaction mixtures containing the anchor oligonucleotide. The ligation products from each reaction were then subjected to PCR amplification with each of the indicated primer pairs. Reverse-transcription reactions containing tRNA alone (lane t) and PCRs containing no cDNA (lane −) were included as controls. An aliquot of the cDNA fragment used as probe was electrophoresed in parallel with the PCR products as a control for hybridization (lane +). (*A*) Diagram of predicted PCR products. Structure of the expected ligation product of the anchor oligonucleotide to the GSX-2 IgG1 cDNA is illustrated along with positions of the various PCR primers. Beneath this are shown the predicted sizes of PCR products generated by the indicated primer pairs. (*B*) Southern blot hybridization of PCR products. PCR products were electrophoresed, transferred to membranes, and hybridized with a radiolabeled GSX-2 IgG1 cDNA probe. Migration positions of molecular weight markers are shown, and PCR product sizes are indicated by arrows.

sible that RNA present in the ligation reactions may have acted as a competitive inhibitor of anchor–cDNA joining. Therefore, we attempted to improve the sensitivity of the LA-PCR by removing this competing substrate.

Enhanced Sensitivity of LA-PCR after NaOH Treatment of cDNA. Treatment of RNA with dilute NaOH results in hydrolysis of the nucleic acid and production of fragments bearing 5'-hydroxyl and 3'- or 2'-phosphate groups (8). These products do not serve as substrates for T4 RNA ligase, so base treatment would be expected to remove residual RNA as a potential inhibitor of anchor ligation to cDNA. When LA-PCR was performed after NaOH treatment of cDNA samples, amplification using the T3 anchor-specific primer and the C_{in} cDNA-specific primer yielded a product of the expected size that hybridized with an IgG1 cDNA probe when as little as 1 ng of GSX-2 hybridoma total RNA was used as starting material (Fig. 3). The PCR product of unexpected size detected in the T3 C_{in} amplification containing NaOH-treated cDNA derived from 1 ng of total RNA may represent single-stranded product, which has been reported to be a common by-product of anchored PCR (1).

DISCUSSION

The LA-PCR procedure described here has a number of advantages over previously described anchored PCR proto-

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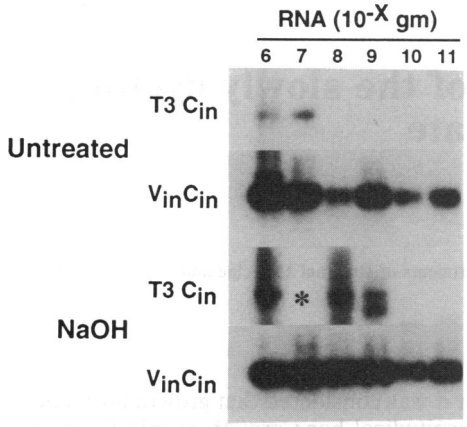

FIG. 3. Increased sensitivity of LA-PCR after base treatment of cDNA and requirement for anchor ligation. cDNA prepared from the indicated amounts of GSX-2 hybridoma total RNA was either used directly in ligation reactions or subjected to treatment with NaOH before ligation. The ligation products were used in PCRs with the indicated primers, and PCR products were analyzed by Southern blot hybridization with a GSX-2 IgG1 cDNA probe. cDNA in the indicated sample (*) was not ligated to the anchor before PCR amplification.

cols that rely on the use of TdT to add homopolymeric sequences to the 3′ end of reverse-transcribed cDNA (1, 9). Our approach avoids TdT tailing, which has often proved technically demanding, requiring assessment of tailing efficiency and optimization of reaction conditions (10). Since LA-PCR employs direct ligation of the anchor to cDNA, it eliminates the use of homopolymer-containing primers with limited specificity and the necessity of performing two rounds of PCR. The LA-PCR protocol involves only three simple enzymatic reactions and does not require purification of products between reaction steps. Experiments with oligo(dT)-coated beads as a means of coupling the first-strand cDNA to a solid phase (11), which may enable the entire set of reactions from RNA preparation through restriction enzyme digestion of PCR product to be performed in a single tube, remain to be done. Finally, the LA-PCR approach can yield specific product from as little as 1 ng of total RNA, the equivalent of 100 hybridoma cells, and with optimization of conditions may conceivably be applicable to single cells.

Although oligo(dT) was used as the primer in cDNA synthesis in these experiments, a primer specific for the molecule of interest could be used instead to increase the selectivity of the reaction. However, this reverse-transcription primer must not be complementary to the cDNA-specific primer to be used in subsequent PCR. If such a primer were used, ligation of the anchor to residual reverse-transcription primer would yield a product that could act as a competing substrate in PCR, decreasing the efficiency of cDNA amplification. Another means of increasing both specificity and sensitivity of LA-PCR is the use of nested primers in two rounds of amplification. Preliminary experiments in which LA-PCR products generated by using the T3 and C_{ex} primer pair were reamplified by using the T3 and C_{in} primer pair showed that yield of specific product was increased and indicated that sensitivity may be markedly enhanced (unpublished observations). In an alternative use of this method, amplification of molecules in which the 3′ sequence is unknown could be performed by directly ligating the anchor oligonucleotide to the 3′ terminus of single-stranded RNA, synthesizing first-strand cDNA with a primer based on the complementary anchor nucleotide sequence, and subsequent PCR amplification with one primer specific for the cDNA of interest and one specific for the anchor.

The simplicity and sensitivity of the LA-PCR approach should allow a more general use of single-sided PCR technology than is possible with established protocols. Some of its potential applications include the production of general cDNA libraries from very small amounts of starting material, analysis of T-cell receptor and immunoglobulin V regions, cloning of developmentally regulated mRNAs detected with gene trap vectors, and characterization of alternative splice products and alternative promoter usage (1, 9, 12–14). It should also be possible to use this technique to clone specific genes that share a single region of sequence similarity (e.g., a conserved functional domain such as a catalytic site), by amplification with one primer specific for the anchor and a set of degenerate primers designed to hybridize with the conserved domain. An analogous approach in which sets of primers recognize two different conserved domains has been used to clone members of the protein-tyrosine kinase family (15).

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