Oligodeoxyribonucleotide ligation to single-stranded cDNAs: a new tool for cloning 5' ends of mRNAs and for constructing cDNA libraries by in vitro amplification

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ABSTRACT

Cloning full length cDNAs is a difficult task especially if mRNAs are not abundant or if tissue is only available in limited amounts. Current strategies are based on in vitro amplification of cDNAs after adding a homopolymeric tail at the 3' end of the ss-cDNA. Since subsequent amplification steps yield unspecific amplified DNA mostly due to non-specific annealing of the reverse primer containing a homopolymeric tail, we have devised a new strategy based on the ligation of single-stranded oligodeoxyribonucleotide to the 3' end of single-stranded cDNAs. The efficiency of the strategy was assessed by analyzing the 5' ends of the rat pineal gland tryptophan hydroxylase messenger. The 5' end of the least abundant messenger (0.005% of total mRNAs) could be cloned without selection. Sixty percent of the analyzed clones correspond to TPH. This technique revealed a 5-nt stretch not apparent using dG tailing strategy. The potentiality of the method for generating cDNAs libraries was tested with 10^4 PC12 cells. In this library, the abundance of tyrosine hydroxylase clones (0.03%) correlated well with the abundance of the corresponding messenger, showing that no major distortion was introduced into the construction of the library.

INTRODUCTION

Methods for synthesizing and cloning cDNAs have continuously evolved since the initial reports in 1976 of Maniatis et al. (1), Higuchi et al. (2), and Rougeon and Mach (3). In particular, various protocols have been established that allow efficient reverse transcription of mRNAs, as well as efficient transformation of bacteria or in vitro packaging of phages. The potentiality of these methods have recently been increased by the development of the polymerase chain reaction [PCR, (4,5)]. Indeed, combination of the former techniques with the ability of the PCR to yield clonable amounts of DNA from extremely scarce amounts of starting material offers the possibility of generating cDNA libraries from a limited number of cells, or to isolate rare fully extended cDNAs. Both achievements could open new avenues in the study of highly heterogeneous tissues such as those found in early embryos and in the nervous system.

Basically, the PCR amplification of cDNAs requires that the flanking sequences of the molecule amplified be available. A sequence can easily be imposed at the 5’ end by priming the reverse transcription with a specific primer or with an oligonucleotide tailored with a poly (dT) stretch, thus taking advantage of the poly (A) sequence that is located at the 3’ end of most mRNAs (6). Several strategies have been devised to add a determined sequence at the 3' end of the cDNAs. We (7) and others have described strategies based on the addition of a homopolymeric dG (8,9) or dA (6,10) tail using terminal deoxynucleotid transferase (TdT) (‘anchor-PCR’ (6)). However, this strategy has important limitations. Firstly, the TdT reaction is difficult to control and has a low efficiency (personal observations). Secondly, the return primers containing a homopolymeric (dC or dT) tail generate non-specific amplification, a phenomenon which can prevent the isolation of low abundance mRNAs species, and/or interfere with the relative abundance of primary clones in the library. To circumvent these drawbacks, other authors have generated double stranded cDNAs to which appropriate adaptors have been ligated at both ends (11,12). However, like conventional cDNA library protocols, the method inherently generates truncated cDNAs.

In a recent paper we showed that the combination of ‘anchor-PCR’ and of a ‘cRNA enrichment’ procedure is effective in the cloning of 5' ends of rare messengers (7) which cannot be drawn out by ‘anchor-PCR’ alone. However, the ‘cRNA enrichment’ strategy requires a few additional steps in comparison with the use of ‘anchor-PCR’. In addition, such a strategy can only be used to isolate specific sequences and not to generate complex cDNAs libraries. These considerations led us to devise a novel way to tag the 3' ends of ss-cDNAs. Here, we describe a method, which is based on the ligation of an oligonucleotide to the 3' end of ss-cDNAs. The efficiency of the strategy in cloning 5' ends of rare messengers and in generating cDNA libraries from low amounts of RNA is studied. Based on our previous work (7), the present study indicates that this novel strategy overcomes most drawbacks of other cDNA 3' end strategies.

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MATERIALS AND METHODS

Oligonucleotides

All oligonucleotides have been synthesized by Genset (France). We have adopted the following nomenclature to designate the oligonucleotide used in this study: (i) all the oligonucleotides used to clone the 5' ends of the messengers have the suffix 5', (ii) the oligonucleotides used in the 'nested' PCR amplification experiments carry an additional number which refers to the round of nested amplification in which the oligonucleotide is used, (iii) the same rules are followed to designate the oligonucleotides used at the 3' end of the messengers in the generation of the cDNA libraries. Oligonucleotides Bα, Cα, Eα, and Fα used in the cloning of 5' ends of tryptophan hydroxylase messengers have been defined in Ref. 7.

The oligonucleotide BM 5' (Fig. 1A, ID) was synthesized with a 5'-phosphate extremity and 500 ng were tailed with 2',3'-dideoxy-adenosine-triphosphate (ddATP) in 25 μl (100 mM Sodium cacodylate, 1 mM CoCl₂, 30 mM Tris-HCl (pH = 7.5), 0.05 μg/μl bovine serum albumin, 0.1 mM DTT, 100 mM ddATP, [α-32P]-ddATP 2.5 μCi of the Amersham 3000 Ci/mM solution) with 25 units of terminal transferase (Boehringer Manheim) for 1 h at 37°C. The samples were then incubated for 10 min at 75°C to stop the reaction. The modified oligonucleotide was then purified as follows: the samples were electrophoresed on a polyacrylamide gel. After autoradiography the band was excised, transferred to SPIN-X cartridge (Costar) and 200 μl of water was added. To disrupt the acrylamide, the cartridge was chilled in dry ice for 15 min, thawed, and then spun 5 min at 12000 rpm. The eluat was precipitated in a solution containing 0.5 μg of Dextran T 40 (Pharmacia), 0.5 M LiCl, 75% ethanol. The precipitate was finally dissolved in 50 μl of water (approximate concentration 5 ng/μl) and stored at −20°C until use.

Preparation of total RNA and primer extension

PC12 cells (rat pheochromocytoma cells), harvested in Versene (EDTA 200 mg/l in PBS) were counted in a Thomas hemocytometer and diluted in PBS at 10⁶ cells per ml. Aliquots of 1 ml were spun 5 min at 3000 rpm, the supernatant was removed and the pellet was stored at −80°C until use.

Total RNAs were prepared as described in Ref. 9, and cDNA synthesis was carried out using the 'standard' reverse transcription conditions of Rhymer et al (13) with minor modifications adapted for the low amount of mRNA. The synthesis of the ss-cDNAs was primed using 6 pmoles of PEX (Fig. 1B) and approx. 200 ng of total RNAs for rat pineal gland or 3 pmoles of BM 3' (Fig. 1C, 1D) and approx. 10 ng of total RNAs for PC12 cells. To label cDNAs at a level allowing their detection in the following fractionation steps, reverse transcription was first extended for 40 min at 42°C with 0.05 mM dCTP and 0.5 mM dATP [α-32P]-dCTP then dCTP to 0.5 mM was added and a second extension was performed.

Oligonucleotide ligation to ss-cDNA

After extension, primers and RNAs were eliminated as described previously (7) except that cDNAs were finally resuspended in 10 μl of water.

One tenth of ss-cDNA and 0.5 μl of modified BM 5' were incubated in the optimized buffer described in Ref. 14 (50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 10 μg/ml BSA, 25% PEG, 1.0 mM hexammine cobalt chloride, and 20 μM ATP) with 10 U of T4 RNA ligase (Biolab's) at 22°C for 24 hours (PC12 cell cDNAs) or 48 hours (pineal gland cDNAs).

PCR amplification

In this paper X/Y amplification stands for PCR amplification carried out with primers X and Y.

One fifth (pineal cDNA) or all of the product of single strand ligation (PC12 cDNA) were used for amplification in 100 μl with 0.1 μM primers (BM 1-5'/Ba for pineal cDNA or BM 1-5'/BM 1-3' for PC12 cDNA), 1 U of AmpliTaq (Cetus) in the buffer recommended by the supplier except that the pH was 8.9 instead of 8.3. Each cycle consisted of denaturation for 30 sec at 93°C , annealing at 51°C (PCR with pineal gland cDNA) or at 55°C (PCR with PC12 cDNA) for 30 sec, and extension for 1 min at 72°C.

In the case of pineal gland cDNA, after 45 cycles of amplification 1 μl of the amplified products was used in a nested amplification with internal primers chosen within BM 5' (BM 2-5', Fig. 1C, 1D) and within the sequence of TPH (Ca, Fig. 1B). Thirty cycles were carried out, in 50 μl, as described above.

In the case of PC12 cDNA, a first amplification experiment was carried out with primers BM 1-3' and BM 1-5' (Fig. 1C, 1D). After 25 cycles the products were loaded on a Sephacryl S 400 (Pharmacia) spin column to eliminate the short amplification products (mostly primer dimers). One hundredth of the eluate was further amplified using BM 2-3' and BM 2-5' primers, in 50 μl. One microliter of the product of this experiment was used in a final amplification with BM 3-3' and BM 3-5' primers, in 50 μl. These latter amplification products were fractionated through a Sephacryl S 400 spin column.

Analysis of amplification products

Amplification products were electrophoresed on 1% agarose gel. After electrophoresis, gels were blotted in alkaline conditions on Hybond N* (Amersham) membranes. When the blots were hybridized with oligonucleotides, prehybridization was performed for 30 min at 42°C in 6xSSC, 0.1% SDS, 10 mM EDTA, 25 mM phosphate buffer (pH 7), 1% Dehart and 250 μg/ml denatured herring sperm DNA. Hybridization was, then, carried out for half an hour, with 2-3 x10⁶ cpm of 32P-kinased oligonucleotide. The blots were washed from 6xSSC to 1xSSC with 0.1% SDS, at 42°C. The cDNA probes were labeled using the multiprime DNA labeling system (Amersham). Prehybridization (4h) and hybridization (overnight) were carried out at 65°C in 10xDenhart, 3xSSC, 25 mM phosphate buffer (pH 7), 10% dextran sulfate, 250 μg/ml denatured herring sperm DNA. Washes were performed at 65°C from 3xSSC to 0.1xSSC with 0.1% SDS. Membranes were autoradiographed on Amersham MP films.

Cloning, screening and sequencing of the amplification products

Prior to cloning the cDNAs corresponding to the 5' ends of the TPH mRNAs, a limited amplification was performed with appropriate kinased primers in order to preserve material. To eliminate unincorporated primers, the products were loaded on a 2 ml AcA34 column as described above. The fractions containing amplified cDNAs were pooled and ethanol-precipitated. The DNA were then blunt-ended with T4 DNA polymerase (15) and cloned in M13 mp8 Smal I cut phospahatase vector (Amersham) according to conventional protocols.
Figure 1: Respective position, designation and sequences of the oligonucleotides used in this study. (A) The modified 46 nt oligonucleotide BM 5' is ligated to the single-stranded cDNA. Following ligation, nested amplifications were carried out with the anticomplementary oligonucleotides BM 1-5', BM 2-5', BM 3-5' which overlap over 9 to 10 bases. The sequences of these oligonucleotides are given in D. (B) Oligonucleotides relative to the 5' ends of tryptophan hydroxylase mRNAs; thick line: 5' end of coding region; thin line: 5' untranslated region. Oligonucleotides are described in Ref. (7) except for PEX whose sequence is: GGTTT-CCTGGAAGATTTTCAGCGC. Sense and antisense oligonucleotides are represented above and below, respectively. (C) Priming of cDNA synthesis from PC12 cells RNAs was done with BM 3', a 40 bases oligonucleotide which has a 14 base dT tail at its 3' end. The nested amplifications were carried out with three overlapping oligonucleotides which are parts of BM 3': BM 1-3', BM 2-3', BM 3-3'. The sequences of these oligonucleotides are given in D. (D) Sequence of the oligonucleotides designed above.

PC12 amplified cDNAs were cloned in Eco RI phosphatased lambda ZAP vectors (Stratagene), following the supplier's recommendations, after addition of the appropriate linkers.

The average size of the inserts contained in the library was determined by PCR using M13 primer flanking the Multiple Cloning Site.

The recombinant phages were screened after adsorption on Hybond-N+ membranes according to the supplier's recommendations. The filters were hybridized with 32P labeled oligonucleotides or cDNA probes, under the conditions described for Southern blots. DNA sequencing was performed with the Multiwell microtitre plate DNA sequencing system-T7 DNA polymerase (Amersham), following the supplier's recommendations.

RESULTS

The strategy that is used in this study to generate cDNA sequences that are readily amplifiable in vitro is illustrated on Fig. 2. The ss-cDNA is primed taking advantage of the poly A tail (oligonucleotide BM 3') or of a known sequence within the RNA (oligonucleotide PEX, Fig. 1B). A modified oligonucleotide, BM 5' (Fig. 1A, 1D), is then ligated to the 3' end of the ss-cDNA in the presence of T4 RNA ligase, which has also been shown to ligate short single stranded DNA fragments (16,14). The strategy is referred to as 'SLIC' for single strand ligation to ss-cDNA.

Three precautions are needed to target the ligation of the BM 5' oligonucleotide (Fig. 1A, 1D) specifically to the 3' end of the ss-cDNA and to avoid self ligations and/or circularizations.

Cloning of the 5' ends of rare messengers

To assess the efficiency of the SLIC strategy we analyzed the 5' ends of the rat tryptophan hydroxylase (TPH) mRNA which we had previously studied using a strategy based on dG tailing (7). TPH catalyzes the rate-limiting step in the synthesis of serotonin. In the rat, the corresponding mRNA display a diversity both at 3' (17) and 5' (7) non-coding regions. Two populations of TPH mRNAs with distinct 5' ends, designated TPH-α and TPH-β, have been characterized by S1 mapping experiments and molecular cloning (7). TPH-α which is included in TPH-β (Fig. 1B), is the most abundant species and it accounts for about 0.5% of total mRNAs in the pineal gland. TPH-β is about one hundred fold less abundant than TPH-α. These characteristics are most appropriate to test strategies aimed at cloning 5' ends of mRNAs.
Total RNAs were extracted from one pineal gland and primer extension was carried out with PEX oligonucleotide (Fig. 1B) using one unit (approx. 200 ng) of the RNAs. After removal of PEX, the single-stranded cDNA was ligated to the modified oligonucleotide BM 5' with the T4 RNA ligase, as described in Material and Methods. A 45 cycle amplification was then performed with the two oligonucleotides BM 1-5' and Ba (Fig. 1B and 1D).

At this stage, the presence of TPH-α and TPH-β was tested by Southern blotting, using oligonucleotides Es (Fig. 1B) whose sequence is contained in both TPH-α and TPH-β, as well as the oligonucleotide Fs which is specific to TPH-β. As shown in Fig. 3A, a fairly intense signal was obtained with Es, demonstrating that the RNA ligase had anchored the BM 5' oligodeoxyribonucleotide to the ss-cDNAs. No signal was obtained with the TPH-β probe which led us to perform a further set of 30 cycles of amplifications with primer Ca and BM 2-5'. A specific TPH-β signal could then be detected, as shown in Fig. 3B. In our previous cloning (7), a special strategy based on a cDNA enrichment procedure was required to obtain these sequences.

To preserve material a sample of the products of the two previous amplifications, BM 1-5'/Ba and BM 2-5'/Ca, were subjected to an additional 20 cycles of amplification and cloned in M13 mp8 phagemid, generating about 5 x 10^6 recombinant clones (in order to visualize all the amplified material), no amplified cDNAs were detected (data not shown).

Another one was shorter than TPH-α by one nucleotide, and its 5' terminal nucleotide was a T instead of an A. The seven remaining clones contained the full length TPH sequence. It is not clear whether this addition is related to the presence of the cap structure or whether it had been added during single strand ligation.

Four clones recognized by the TPH-β specific probe were also sequenced and contained the 5' nucleotide sequence (TGCCC) in addition to the TPH-β sequence previously determined (7).

It should be noted that our sequencing experiments also revealed at the 5' end of a few clones the presence of concatemers of the oligonucleotide, a phenomenon most likely due to incomplete removal of the hydroxyl group from the oligonucleotide BM 5'.

Construction of a cDNA library from a limited number of cells

In a second set of experiments we tested the potential of the SLIC method to generate a cDNA library from a limited number of cells. To allow us to control that the library would be representative, we used a PC12 cell line where it has been shown that the tyrosine hydroxylase (TH) messenger represents about 0.05% of the total mRNAs (Faucon Biguet, personal communication).

Total RNA was prepared from a pellet containing 10^6 cells. Half of the material (about 10 ng) was used to synthesize single-stranded cDNAs primed with BM 3' (Fig. 1B, 1D). Following removal of the primer, the ss-cDNA was ligated with the modified oligonucleotide BM 5' (Fig. 1A, 1D). These cDNAs were PCR amplified, using BM 1-5' and BM 1-3' (Fig. 1C, 1D). When this PCR product was analyzed by Southern blotting, using primer BM 3'-3' as a probe (in order to visualize all the amplified material), no amplified cDNAs were detected (data not shown). This result indicates that the cDNAs synthesized from the very

hybridized with Es probes, of which approximately 1% hybridized with Fs.

Nine of the clones hybridizing with Es but not with Fs were sequenced. Only one of them was unrelated to the TPH sequence. Another one was shorter than TPH-α by one nucleotide, and its 5' terminal nucleotide was a T instead of an A. The seven remaining clones contained the full length TPH sequence. However, in four instances an additional base had been added. It is not clear whether this addition is related to the presence of the cap structure or whether it had been added during single strand ligation.

Four clones recognized by the TPH-β specific probe were also sequenced and contained the 5' nucleotide sequence (TGCCC) in addition to the TPH-β sequence previously determined (7).

It should be noted that our sequencing experiments also revealed at the 5' end of a few clones the presence of concatemers of the oligonucleotide, a phenomenon most likely due to incomplete removal of the hydroxyl group from the oligonucleotide BM 5'.
The ligation was performed with the T4 RNA ligase, an enzyme which was first identified for its ability to catalyze the cyclization of single-stranded RNA chains in the presence of ATP (18). In this reaction, the enzyme catalyzes the formation of a phosphodiester bond between a 5’ donor phosphate (5’-phosphate end) and a 3’-acceptor phosphate (3’-hydroxyl end). The enzyme has also been described to ligate oligodeoxyribonucleotides to RNA or DNA acceptors (19). More recently Tessier et al. (14) reinvestigating the ligation of oligodeoxyribonucleotides, pointed out that the yield of ligation decreased from 67% to 40% when the length of acceptor oligonucleotide increased from 25 to 40 nt. In addition, reaction times are ten to twenty times longer for ligation of DNA than for ligation of RNA. Because of these limitations, to our knowledge, the use of the enzyme to ligate oligodeoxyribonucleotide to long DNA molecules has received little attention. With the development of in vitro amplification of DNA and the possibility of working with small amounts of starting material, allowing a large excess of enzyme to be used, the limitation of the reaction no longer precludes its usefulness.

The possibility of adding an oligonucleotide to the 3’ end of cDNA circumvents the difficulties encountered with current strategies designed to amplify 5’ ends of cDNAs. The tailing method, which relies upon the terminal deoxyribonucleotide transferase’s ability to add a homopolymeric tail to the 3’ end of the cDNA, is limited by the high level of unspecific amplifications yielded by primers containing the anticomplementary homopolymeric tail. To increase the specificity of the method, we have recently developed a cRNA enrichment procedure (7). This procedure was necessary to clone TPH-β 5’ ends which could not be amplified to a detectable level by ‘anchor PCR’ alone. In the present study, the SLIC protocol allowed us to readily amplify both TPH α and β which reflects the higher efficiency of this method when compared to ‘anchor-PCR’. More importantly, 5 additional bases (TGCCC) were found in all analyzed TPH-β clones. The 4 proximal bases are contained within the recently published genomic sequence of mouse TPH (20) which exhibits near 100% homology with the previous known rat TPH sequence. This observation provides evidence that these bases correspond to genuine rat TPH-β 5’ end bases.

The finding of 5 bases, which were not included in the previous cloned TPH cDNA (7), suggests an inherent limitation to ‘anchor-PCR’. Since in this method cDNAs are 3’-tailed with a homopolymeric (dA) or (dG) stretch, the return primers contain a homopolymeric (dT) or (dC) tail, respectively. Those sequences, and especially dC tails, are likely to hybridize not only to dA or dG tails but also to dA or dG-rich sequences, respectively. Such a phenomenon may have accounted in our previous cloning experiments (7) for the absence of the 3’A-CGGG2 sequence at the 3’ end of the fully extended TPH-β cDNAs, although the length of the return primer has been purposely reduced to 6 residues in order to limit non specific hybridization. Therefore, the use of dG tailing strategies not only yield high levels of background but may also generate double stranded cDNAs shorter than their corresponding single stranded cDNAs.

In addition to the study of TPH, the SLIC protocol has recently been used in our laboratory to characterize the 5’ end of five distinct mRNAs, and extensions of over 500 bases have been obtained. The availability of thermostable reverse transcriptases which should obviate obstacles resulting from RNA secondary structures will further contribute to the analysis of 5’ ends of mRNAs.

Molecular cloning of the 5’ end of TPH mRNA demonstrates the efficiency of the SLIC method

We report the successful ligation of an oligonucleotide to the 3’ end of ss-cDNAs that provides a straightforward means of amplifying these molecules in vitro and facilitates the molecular cloning of low abundance species.

The ligation was performed with the T4 RNA ligase, an enzyme which was first identified for its ability to catalyze the cyclization of single-stranded RNA chains in the presence of ATP (18). In this reaction, the enzyme catalyzes the formation of a
The SLIC methodology could also be useful in the search of promoter sequences. Indeed, primer extension experiments could be carried out on a genomic matrix using a 5'-biotinylated oligonucleotide. Neosynthesized ss-DNA could then be collected through a streptavidin chromatography ligated to BM-5' before being subjected to nested PCR amplification.

Construction of cDNA libraries based on SLIC method

Construction of cDNA libraries has been a goal for many investigators ever since the availability of PCR-based strategies. However, as developed in the introduction, all current methods have major drawbacks. The present paper shows that the SLIC protocol offers a novel and convenient means to generate cDNA libraries, and contrary to double-stranded ligation strategies, it involves fewer enzymatic steps and theoretically allows the cloning of full length cDNAs. As exemplified by our extensive study, the SLIC protocol is also more reliable and efficient than tailing mediated strategies. The abundance of cDNA clones encoding TH in the PC 12 library that has been screened in this study suggests that this strategy does not introduce any major distortions in the abundance of clones, at least for cDNAs shorter than 2 kb. In this respect, it should be noted that the length of the extension step in amplification cycles was purposely limited to roughly 1.5 to 2 kb.

The construction of cDNA libraries from small amounts of tissue using the SLIC protocol depends firstly on the possibility of preparing RNAs from the tissues and secondly on the efficiency of both the reverse transcription and ligation reaction, since amplifications can theoretically be carried out from one molecule. Although in this study we started from 10⁴ PC 12 cells so as to be able to perform multiple assays, various protocols have been described to prepare single stranded cDNAs using only a few cells. As to the ligation reaction, experiments have been carried out with the equivalent of about 1500 cells and preliminary experiments indicate that this number can be reduced several fold. The SLIC strategy, therefore, should provide a rapid and convenient means to generate representative cDNA libraries when only limited amounts of tissue are available.

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