Reverse Transcription-Polymerase Chain Reaction Construction of Plasmid-based, Full-length cDNA Libraries from Leishmania infantum for in Vitro Expression Screening

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We describe a streamlined reverse transcription-polymerase chain reaction methodology for constructing full-length cDNA libraries of trypanosomatids on the basis of conserved sequences located at the 5' and 3' ends of transspliced mRNAs. The amplified cDNA corresponded to full-length messengers and was amenable to in vitro expression. Fractionated libraries could be rapidly constructed in a plasmid vector by the TA cloning method (Invitrogen). We believe this is useful when there are concerns over the use of restriction enzymes and phage technology as well as in cases where expression of proteins in their native conformation is desired.

Key words: kinetoplastid - cDNA libraries - spliced leader

All kinetoplastids and euglenids mRNAs and a significant proportion of them in helminths are flanked by known, conserved sequences: the spliced leader at the 5' end and a polyA tail at the 3' end (Bonen 1993, Nilsen 1995). It is therefore possible to amplify by the polymerase chain reaction (PCR), after reverse transcription (RT), only those cDNA molecules that correspond to full-length messengers, i.e. extending from the 5' spliced leader or mini-exon to the polyA+ tail. PCR amplification can also produce conveniently large quantities from small amounts of ss cDNA. Indeed, researchers have used this concept to prepare libraries or select full-length transcripts in helminths (Devaney et al. 1996, Blaxter et al. 1996, Gregory et al. 1997, Fernandez et al. 2002) and in trypanosomatids (El Sayed et al. 1995, Ajioka et al. 1995, Levick et al. 1996).

Here we describe a streamlined procedure to construct full-length cDNA libraries from small amount of Leishmania promastigote mRNA. As compared to published methods, the following changes have been made: (i) a 3' PCR primer colinear to the adaptor part of the modified oligoDT primer used for reverse transcription, (ii) the 5' end of PCR primers were chosen to favour addition of non-template A's by the Taq polymerase (Magnusson et al. 1996), (iii) size fractionation of the amplified material, (iv) direct insertion of amplicons into a linearised plasmid vector by the TA cloning method (Invitrogen), (v) in vitro expression of single or pooled clones in a combined (one-tube) eukaryotic transcription/translation system.

This has several advantages. First, the 3' PCR primer can be designed to have a melting temperature near that of the mini-exon-derived 5' primer so as to increase the annealing temperature thus reducing chances of non-specific priming; non-oligoDT 3' primers also avoid spurious priming at internal A-rich stretches (advised by Devaney et al. 1996). Secondly, direct insertion of PCR products into a plasmid vector decreases the amount of work and the risks of losing interesting clones entailed by restriction-and-ligation approaches and by λ phage derivatives technology. Thirdly, fractionation of the material is possible either before or after the RT-PCR step. Finally, given that full-length cDNA's possess signals necessary for translation, such libraries are amenable to screening by in vitro expression of pooled plasmid DNA. It is therefore possible to obtain rapidly the full-length sequence (as well as a small amount of the recombinant protein in the native conformation) of proteins for which a specific ligand is available. Finally, the methodology could be particularly relevant when searching for protective antigens by plasmid DNA immunization and challenge in a process called "expression library immunisation" (Barry et al. 1995).

RT of polyA+ RNA and PCR amplification - Poly A+ RNA was extracted from promastigotes of Leishmania infantum by standard methodology (Sambrook et al. 1989). One hundred ng was reverse transcribed using the Superscript II enzyme (life Technologies) and 100 pmole NotI-Oligo(T)18 (Pharmacia) as primer plus 40 units Human Placenta Rnase Inhibitor (Roche Molecular Biochemicals) in 20 µl final volume, followed by digestion of the template RNA with Rnase H (Life Technologies). Two µl of reverse transcription product was amplified by PCR on a DNA thermal cycler 480 (Perkin Elmer) using the Expand High Fidelity PCR System (Roche Molecular Biochemicals).

The 5' primer (5'-AACTACGCTATATAAGT ATCAGTT-3') was colinear to the 5' end of the mini-exon and the 3' primer (5'-AGAAATTCCGCAGCCGC-3') was colinear to the 3' end of the mini-exon.
**Polymerase chain reaction amplification of full-length cDNA**

**Left panel:** Four sets of amplification conditions were tested on single-stranded cDNA from unfractionated polyA+ RNA. A: 30 cycles of (94°C X 1 min, 60°C X 1 min, 72°C X 1 min); B: 25 cycles of (94°C X 1 min, 60°C X 1 min, 80°C X 5 min) with elongation time increased 10 sec per cycle starting at cycle 11; C: 25 cycles as in B but with 10 min elongation time; D: 16 cycles as in C. Right panel: Set D conditions were applied to ss cDNA obtained from fractions F23 and F25 of sucrose gradient size-fractionated polyA+ RNA.
The overestimation of relative molecular weights of lower size proteins (< 14 kDa) is a common artefact of SDS/PAGE (Hames & Rickwood 1990).

Finally, all clones possessed the native stop codon and possible chimerical or spurious protein starting at plasmid-encoded ATG’s upstream of the insert would be aborted in all reading frames by the presence of stop codons (TAA’s) at position -1, +4 and +14 relative to the first nucleotide of the mini-exon.

In conclusion, using a streamlined RT-PCR approach, µg quantities of ds cDNA ranging from 800 bp to over 4000 bp could be produced in a single reaction from one-tenth of the product of a typical reverse transcription reaction. The material could be fractionated by size and usable amounts were recovered. The cDNA was readily inserted into a plasmid vector by the TA cloning method (Invitrogen) and the ligation bias towards smaller fragments was corrected by size selection of the inserted material. Finally, sequencing and in vitro expression experiments indicated that the cloned cDNA’s indeed corresponded to functional full-length messengers.

REFERENCES


Barry MA, Lai WC, Johnston SA 1995. Protection against


