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A single-cloning-step procedure for the generation of RNAi plasmids producing long stem-loop RNA

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Abstract

RNA interference (RNAi), used as a tool, has revolutionized the studies of gene function. Long stem-loop dsRNA has been proven the most effective trigger for down-regulating target transcripts in RNAi-positive trypanosomatid parasites. Here we describe a protocol for constructing plasmids that produce long stem-loops by using a single cloning step. Inverted repeats are first obtained by self-ligation of PCR products that contain a randomized segment at one of their ends and then inserted in a plasmid vector. The random sequences create the loop (or “stuffer”) of the hairpin. This methodology was tested in *Leishmania (Viannia) braziliensis* to constitutively knock down the mRNAs for the well-studied paraflagellar rod protein 1 and 2 (*PFR1* and *PFR2*) genes and revealed that mRNA cleavage products are unusually stable in these parasites. The protocol is suitable for any plasmid (for constitutive or inducible expression) and for any organism in which long stem-loops can be used to elicit RNAi.

Keywords

RNAi; hairpin RNA; inverted repeat cloning; *Leishmania braziliensis*

African trypanosomes are among the organisms in which RNAi was first discovered [1,2] and RNAi technology for *Trypanosoma brucei* has evolved rapidly (reviewed in [3]). Research involving *T. brucei* provided the scientific community with the first example of a genome-wide RNAi library [4], and this technology has recently culminated in the development of the powerful RNAi target sequencing (RIT-Seq) method [5]. RIT-Seq is an RNAi screening approach that combines a genomic DNA library for the production of dsRNA with the high-throughput sequencing of the RNAi-inducing genomic fragments in a selected pool of viable trypanosome transformants.

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Long RNA hairpins, ranging in size from ~100 bp to more than 1,500 bp, are the most efficient trigger for RNAi in trypanosomatids [6,7]. In addition to high levels of tightly regulated inducible expression from RNA polymerase I promoters, it is possible that their superiority is due in part to very efficient formation of dsRNA by “zipping-up” of the hairpin structure, most likely leading to the accumulation of very low levels of single-stranded RNA. In contrast, sense and antisense RNA molecules generated by opposing T7 RNA polymerase promoters [8] may compete with the actual RNAi target transcript for binding and cleavage by small interfering RNA (siRNA)-programmed Argonaute. Production of a high-throughput hairpin library has been described for *Drosophila* [9], where PCR products with a mean length of 323 bp were digested at one of their ends with EcoR I and self-ligated to produce an inverted repeat lacking an intervening (“stuffer”) sequence. A total of 22,270 RNAi lines were generated that represented 12,088 (or 88%) of the predicted protein-coding genes. In this system, the hairpin-producing segments were ligated into a plasmid and propagated in a strain of *E. coli*, SURE (Stratagene), which is less prone to rearranging repeats by recombination. We have attempted several times to adapt the protocol, but encountered great difficulty in generating constructs that are stably propagated. The stability of the *Drosophila* inverted repeat constructs without a stuffer is most likely due to a specific feature of the plasmid vector used in that study. The presence of a stuffer in inverted repeats greatly improves the stability of plasmids even in commonly used strains of *E. coli* [10]. Indeed, recently a procedure was described for the generation of such inverted repeats in a Gateway®-compatible vector, pTrypRNAiGate, for inducible RNAi in *T. brucei* [11]. The method uses one cloning step followed by a recombination reaction for the production of an inverted repeat-containing plasmid. Described here is a protocol we developed for the generation of plasmids producing long stem-loop RNA. The method relies on a single cloning step, is suitable for any plasmid vector, and like the system described for *Drosophila* [9] is easily adaptable to 96-well-plate format with the potential to facilitate future development of systematic RNAi screens using stem-loop RNA in trypanosomes as well as in other organisms where RNAi can be elicited by long dsRNA.

Our goal was to assemble a gene-specific inverted repeat structure using the least possible number of steps. To this end we devised the following approach (Fig. 1). A gene fragment is amplified from genomic DNA with a forward primer containing at its 5′ end a restriction site for an enzyme (termed enzyme X, which is also used to open the plasmid vector), and a reverse primer that contains in a 5′ to 3′ direction a restriction site for a different enzyme (termed enzyme Y), followed by approximately 50 nt of randomized sequence and ending with a gene-specific region. As a result of PCR amplification, the randomized sequence plus the restriction site Y are joined to the 3′ end of the gene fragment. Following digestion with enzyme Y, the DNA is then ligated to itself in a tail to tail orientation, which generates an inverted repeat with a random sequence stuffer of approximately 100 nt. After digestion with enzyme X, the DNA is gel purified, ligated into a plasmid vector and transformed into *E. coli* DH5α. It should be noted that by this procedure each RNAi construct is endowed with a different stuffer fragment. A detailed description of the experimental procedures is provided as Supplementary information.

As a proof of principle, we decided to test this methodology in *Leishmania (Viannia) braziliensis* and *L. (V.) panamensis* in which RNAi has been recently shown to function for endogenous genes and transgenes alike [12]. Since conditional expression of dsRNA has not yet been achieved in these organisms, only non-essential genes can be downregulated by RNAi. Thus, as targets we chose the paraflagellar rod protein 1 and 2 (*PFR1* and *PFR2*) genes (LbrM.31.0160 and LbrM.16.1480, respectively), the major components of the extra-axonemal structure of the trypanosomatid flagellum that is required for cell motility, called the paraflagellar rod. Knockouts of these genes are well-described and not lethal [13,14],

and their transcripts have been successfully targeted by hairpin RNA constructs both in *Trypanosoma* [7,15] and *Leishmania* [12].

Inverted repeat structures for three chosen gene fragments, HP1, HP2 and HP1M (Fig. 2A) were assembled following the procedure outlined in Fig. 1 and described in Supplementary Methods, and cloned in the plasmid vector pSP72R_{neo}GFP [16] (Supplementary Fig. 1). The structure of the generated recombinant plasmids was confirmed by sequencing, which showed the expected gene fragment joined to each half of the stuffer fragment. In our experience, the length of one or both of the randomized regions can be shorter than the expected ~50 nt, since the random sequence may have EcoR I (or enzyme Y in Fig. 1) site(s). This does not affect the stability of the resulting plasmid constructs, because the inverted repeats still contain an intervening sequence of sufficient size to be stably propagated in *E. coli* with similar purification yields as constructs containing a full-length stuffer.

After drug selection, Western blot analysis of a stable population of *L. braziliensis* promastigotes with a monoclonal PFR antibody, which recognizes both PFR1 and PFR2 [17] (a gift from Diane McMahon-Pratt, Yale School of Public Health), revealed the specificity of PFR downregulation by the 3 different constructs (Fig. 2B). Quantification of the protein levels (signal normalized to the HSP83 loading control, relative to the wild-type cells sample [lane 1 in Fig. 2B], which was set as 100%) indicated that both HP1 and HP2 plasmids downregulated their targets (PFR1 and PFR2, respectively). HP1 knocked down PFR1 to 21% of the wild-type levels, while PFR2 was decreased only to 64% (Fig. 2B, lane 2). HP2 downregulated its intended target PFR2 to 18%, with PFR1 lowered to 83% of wild-type levels (Fig. 2B, lane 3). HP1M had a pronounced effect on both PFR1 (down to 11%) and PFR2 (down to 34%; Fig 2B, lane 4). To test whether these effects correlated with *PFR* mRNA abundance in the corresponding cell lines, we performed Northern blotting on total RNA with a number of probes against *PFR1* and *PFR2* messages. We easily detected large amounts of the synthesized hairpin RNAs (Fig. 2C, lanes 6 and 10 for HP1, lane 23 for HP2, lane 16 for HP1M) with some signal corresponding to partially processed products (weaker lower band/smear) and a range of RNAs with sizes larger than the hairpin that likely represent unprocessed readthrough molecules, in accordance with previous observations in *Leishmania* [12]. In both *L. braziliensis* (Fig. 2C, lanes 17-24) and *L. panamensis* (Supplementary Fig. 2) *PFR2* mRNA has two forms, a longer and shorter one, as a result of alternative 3'-end processing (our unpublished observation). Remarkably, we observed that the mRNA fragments resulting from RNAi cleavage were stable in *Leishmania* (*L. braziliensis* and *L. panamensis* alike, (Fig. 2C and Supplementary Fig. 2). For both HP1 and HP2, targeting the very 5' end of the mRNA produced only slightly faster migration of the corresponding cleaved *PFR1* mRNA (Fig. 2C, lanes 2 and 14) and *PFR2* messages (Fig. 2C, lane 19), since less than 300 nt are missing from the molecules, which are approximately 3.9 kb long (the difference in size is best seen for the shorter form of *PFR2* mRNA in lane 19 of Fig. 2C). The stability of both 5'- and 3'-products of mRNA cleavage by RNAi is best visualized in the HP1M samples (Fig. 2C, lanes 4, 8 and 12) since siRNAs produced from that hairpin target *PFR1* mRNA in a middle region approximately 1,100 nt downstream from the 5' end and 2,300 nt upstream from the 3' end. This phenomenon has been observed previously for RNAi in *T. brucei* [18], albeit much less pronounced, and has been recently suspected for *Leishmania* [12]. Our results are a cautionary note for measuring mRNA knockdown by RNAi in *Leishmania* by Northern blotting or RT-PCR and suggest that the appropriate design of RNAi-target regions and the methods for detection of remaining intact mRNA must take into account the unusual stability of cleaved RNA in these parasites. The slight decrease of PFR2 protein levels in the HP1 samples and PFR1 protein in the HP2 samples was not correlated with a concomitant decrease in mRNA levels, suggesting effects on PFR protein stability when any of the two are knocked down, in

agreement with previous data for *T. brucei* [7]. The pronounced reduced specificity of the HP1M construct (both PFR2 protein [Fig. 2B, lane 4] and *PFR2* mRNA [Fig. 2C, lane 20] are also downregulated, but to a lesser extent than the intended *PFR1* target) is likely the result of shared sequence identity between the *PFR1* and *PFR2* genes in the fragment chosen for creating the inverted repeat. Whereas the HP1 and HP2 sequences do not share any significant similarity, HP1M contains a region with 79% nt identity (380 out of 481 nt) with the *PFR2* gene. Within this region there are two identical 24 nt stretches, and apparently they are sufficient to cause cross-silencing. The residual PFR1 and PFR2 protein amounts in the HP1 and HP2 samples, respectively, are likely the result of individual cells in the population of selected transformants expressing different levels of the stem-loop RNA. Even a clonal cell line stably transfected with the empty plasmid vector used for inverted repeat cloning (Supplementary Fig. 1) exhibits slightly different GFP expression in individual cells (Supplementary Fig. 3). In our experience, different clonal cell lines expressing long stem-loop RNA against additional (non-*PFR*) endogenous *Leishmania* targets have different dsRNA expression levels (data not shown) and therefore we strongly recommend the use of clonal cell lines expressing the highest achievable level of dsRNA for studies focused on gene function.

We have successfully used the above-described method for hairpin-triggered RNAi in three species of RNAi-positive trypanosomatids, namely *L. braziliensis* (Fig. 2), *L. panamensis* (the results for *PFR1* and *PFR2* downregulation were very similar to the ones presented here, albeit with a slightly lower efficiency of RNAi, Supplementary Fig. 2) and *T. brucei* (inducible RNAi for several endogenous genes, E. Ullu *et al.*, unpublished). The method is appropriate for any RNAi-endowed parasitic protozoan [19], or any other organism that allows the use of long-hairpin RNA to initiate RNA interference without sequence-independent dsRNA-triggered cellular responses.

Of the currently available approaches for generating inverted-repeat-containing plasmids, several require the generation of more than one DNA fragment from the gene of interest that differ at their ends in order to construct the inverted repeat. Examples are the sequential ligation of two inserts, four-way ligation, or ligation-independent cloning [20]. This requirement renders these protocols more difficult to adapt for high-throughput applications. A major advantage for the ligation-independent cloning method is the 100% efficiency of producing the desired constructs [20].

In contrast, the Gateway® system [11] (a cloning step and a recombination step; two plasmid purification steps), the no-stuffer inverted repeat system developed for *Drosophila* [9] (a ligation step, a cloning step and a single plasmid purification step) and the variation of the latter described here (also a ligation step, a cloning step and a single plasmid purification step) all need the synthesis of only a single gene fragment. Thus, they are more suitable for generating inverted repeat RNAi plasmid libraries. The Gateway® system also offers extremely high efficiency for obtaining the correct constructs, but requires a Gateway-compatible RNAi vector for the recombination step, a specific *E. coli* strain for the propagation of the “empty” vector, system-specific reagents and two plasmid purification steps. The “no-stuffer” protocol developed for *Drosophila* appears to be suitable only for a particular plasmid vector and uses an *E. coli* strain that has minimized recombination capacity. The major advantage of the protocol described here is that it requires only commonly used reagents and is compatible with any plasmid vector suitable for constitutive or inducible expression of the generated inverted repeats. Most importantly, the protocol requires a single cloning (plasmid purification) step, which could facilitate the generation of inverted repeat libraries for systematic RNAi screens in trypanosomatids and other organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- A simple protocol for generating plasmids with “stuffer”-containing inverted repeats.
- The constructed RNAi plasmids produce long stem-loop RNA.
- Requires a single cloning step, which makes it suitable for systematic RNAi plasmid libraries.
- Appropriate for any plasmid vector and any organism in which long dsRNA can be used to elicit RNAi.
- The methodology was tested for knocking down *Leishmania (Viannia) braziliensis* mRNAs.

Figure 1.

Outline of the protocol for generating stuffer-containing inverted repeat plasmids. PCR products with a gene-specific region and a randomized segment of approximately 50 nt, $(N)_{\sim 50}$, are synthesized with the indicated primers (arrows above and below the diagram of the amplified DNA) and digested with restriction enzyme Y. Self-ligation produces the inverted repeat that contains an intervening sequence comprised of two different random fragments, A and B. The inverted repeat is cloned into the restriction enzyme X site of a plasmid vector with a species-specific upstream promoter (bent arrow denotes transcription start site). The stuffer is unique for each clone, since the synthesis scale for the initial reverse primer containing the random 50 nt sequence is lower than the one needed to cover all possible nucleotide arrangements.

Figure 2.

RNAi downregulation of *PFR* in *Leishmania braziliensis*. To obtain high expression levels of dsRNA we modified a GFP-version of the pSP72 α neo α vector [16] by inserting upstream of the neo selectable marker the *L. braziliensis* rRNA promoter, which is transcribed by RNA Pol I (plasmid vector pSP72R α neo α GFP, Supplementary Fig. 1). The inverted repeats were cloned into the Xba I (our enzyme X) site of the plasmid.

(A) From *L. panamensis* genomic DNA we PCR-amplified 3 different fragments, 2 for *PFR1* (HP1 and HP1M) and 1 for *PFR2* (HP2). The HP1 and HP2 gene-specific fragments (for hairpin against *PFR1* or *PFR2*) are 291 and 284 bp long, respectively, and both represent the very 5' end of the corresponding mRNA (5' UTR and the beginning of the coding sequence). HP1M contains a 515 bp fragment from the middle of the *PFR1* mRNA coding sequence. The regions chosen for production of hairpins are almost identical between *L. panamensis* and *L. braziliensis* with only 4 nucleotides different for the HP1, 7 for the HP2 and 2 for the HP1M sequences. *L. braziliensis* strain M2903 (MHOM/BR/75/M2903) promastigotes were transfected by electroporation with the generated RNAi plasmids in circular form (50 μ g DNA/ 10^8 cells) and after two weeks of selection with 60 μ g/ml G418, whole cell lysates and total RNA were prepared from the stable cell populations. The relative size and position of the inverted repeats (grey hairpins) and the probes used for Northern blotting (grey lines) are shown above and below the diagrams of the *L. braziliensis* *PFR1* and *PFR2* mRNAs (black arrows represents the coding sequences). *PFR2* mRNA has two forms – long (L) and short (S), both shown.

(B) Western blots for PFR (both PFR1 and PFR2 are recognized by the antibody), upper panel, and HSP83 as a loading control, lower panel. Wild-type (WT) cells or selected transformants containing the indicated constructs (HP1 and HP2 are hairpin-generating plasmids against the 5' end of *PFR1* and *PFR2* mRNA, respectively; the HP1M construct targets the middle of the CDS of *PFR1* mRNA) were collected, heated in SDS sample buffer and used for Western blotting.

(C) Northern blots of total RNA isolated from the same cell lines as in (B) for *PFR1* and *PFR2* mRNAs. Left-most upper panel is a methylene blue stained membrane prior to hybridization. Left-most lower panel is a hybridization for α -tubulin mRNA as a loading control. The indicated probes against *PFR1* and *PFR2* were either oligonucleotides (oli) end-labeled with T4 polynucleotide kinase and [γ - 32 P]ATP, or internally labeled single-stranded DNA matching the length of the gene fragment used to generate the hairpin synthesized by “asymmetric” PCR (pcr) in the presence of [α - 32 P]dCTP. Probes were either against the

middle of the CDS (M-CDS), against the 3' end of the CDS (3'CDS), against the 3' UTR (3'UTR), or the 5' end of the message (5'UTR). The positions of the hairpin (hp) RNAs, the *PFR1* mRNA and the long (L) and short (S) forms of the *PFR2* mRNA are indicated. Arrows point to the 3' (lane 4) and 5' (lanes 8 and 12) mRNA - cleavage fragments generated by RNAi from the HP1M construct.