Published in final edited form as:

Exp Parasitol. 2008 June ; 119(2): 296–300.

Loss of dsRNA-based gene silencing in *Entamoeba histolytica*: implications for approaches to genetic analysis

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Abstract

The ability to regulate gene expression in the protozoan parasite *Entamoeba histolytica* is critical in determining gene function. We previously published that expression of dsRNA specific to *E. histolytica* serine threonine isoleucine rich protein (EhSTIRP) resulted in reduction of gene expression (MacFarlane, 2007). However, after approximately one year of continuous drug selection, the expression of EhSTIRP reverted to wild-type levels. We confirmed that the parasites (i) contained the appropriate dsRNA plasmid, (ii) were not contaminated with other plasmids, (iii) the drug selectable marker was functional, and (iv) sequenced the dsRNA portion of the construct. This work suggests that in *E. histolytica* long term cultivation of parasites expressing dsRNA can lead to the loss of dsRNA based silencing through the selection of “RNAi” negative parasites. Thus, users of the dsRNA silencing approach should proceed with caution and regularly confirm gene down regulation. The development and use of constructs for inducible expression of dsRNA may help alleviate this potential problem.

Keywords

ameba; parasite; RNAi; gene expression

Entamoeba histolytica is an important parasitic cause of death worldwide, causing invasive disease in 50 million people annually and resulting in 100,000 deaths (1997). The main clinical symptoms are colitis resulting in diarrhea and dysentery and systemic spread to the liver resulting in abscess formation. How the parasite causes invasive disease is not well understood and the molecular basis of amebic pathogenesis needs further investigation. The *E. histolytica* genome was recently sequenced and provided a great deal of important information about the molecular makeup of the parasite (Loftus, et al., 2005). However, in order to assign function to the large number of amebic genes without orthologs in other systems, genetic approaches to characterize gene function are essential. The *E. histolytica* genome is functionally tetraploid and integration of exogenous plasmids into the genome remains elusive to date, making gene knockout by homologous recombination unfeasible. However, a number of genetic approaches have been successfully utilized in *E. histolytica* including antisense gene

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expression (Ankri, et al., 1998), dominant negative approaches (Vines, et al., 1998) and antisense peptide nucleic acid oligomers (Stock, et al., 2001). Recently, a parasite strain in which multiple genes can be silenced, albeit by an unknown mechanism, has also been identified (Bracha, et al., 2006).

Both dsRNA and siRNA mediated gene silencing have been described in *E. histolytica* (Kaur and Lohia, 2004) (Vayssie, et al., 2004). Post transcriptional gene silencing mechanisms triggered by the presence of double stranded RNA has been described in many organisms (Mello and Conte, 2004). The most extensively studied dsRNA mediated mRNA degradation mechanism involves small interfering RNAs (siRNAs) of 20–27 nucleotides. Data from diverse species have revealed that long dsRNAs are processed into siRNAs by an RNaseIII like endonuclease called “Dicer” (Mello and Conte, 2004). Originally viewed as a means of controlling RNA viruses and mobile genetic elements, RNA interference has been associated with diverse molecular events from developmental control, heterochromatin assembly and DNA and histone methylation (Mello and Conte, 2004). Since a number of genes classically associated with the RNAi pathway are present in the *E. histolytica* genome sequence (Abed and Ankri, 2005) including two Argonaute genes and an RNA dependent RNA polymerase it has been presumed, though not proven, that the dsRNA and siRNAs were mediating gene silencing via an RNAi-like mechanism. An obvious homolog of the Dicer gene has not been identified, however *Entamoeba* does contain a gene with an RNase III domain and RNaseIII activity has been identified in *E. histolytica* lysates (Abed and Ankri, 2005). Other mechanisms by which *E. histolytica* endogenously silences genes include DNA methylation (Fisher, et al., 2004) (Bernes, et al., 2005) (Ali, et al., 2007) and histone modification (Ehrenkaufer, et al., 2007).

Using an approach in which portions of a gene are cloned in opposing orientations separated by a non-specific stuffer region and generate dsRNA, we significantly decreased expression of the *E. histolytica* serine, threonine, isoleucine, rich protein (EhSTIRP) gene family (MacFarlane, 2007). The plasmid was maintained in the parasites through constant selection with 48µg/ml of the neomycin analog G418. We confirmed the down regulation by RT-PCR, Northern blots and phenotypic analysis. EhSTIRP dsRNA(+)/silencing(+) parasites exhibited decreased cytotoxicity in Caco-2 cell assays and were less adhesive towards Chinese hamster ovary (CHO) cells (MacFarlane, 2007). Approximately one year post transfection and six months after documenting reduction of EhSTIRP we noticed that the dsRNA parasites and control parasites were no longer significantly different in the adhesion and cytotoxicity assays (data not shown). We isolated RNA from these parasites and found a return of EhSTIRP expression to levels similar to nontransfected and control levels (Figure 1A). We refer to this parasite population as dsRNA (+)/silencing negative (–) parasites.

In order to determine if spontaneous resistance to the neomycin selectable marker was arising, we probed the parasites for expression of the neomycin resistance gene (Figure 1B). We found that the control, dsRNA (+)/silencing (+) and dsRNA (+)/silencing (–) parasites all expressed equivalent levels of neomycin, indicating that the dsRNA parasites still contained a plasmid encoding the neomycin resistance gene and were still capable of expressing genes off the construct. It also should be noted that both the neomycin resistance gene and the dsRNA or control transcripts were under control of the same regulatory region, in this case the promoter and 3' regulatory regions of *EnActin*.

Previously we had noted that the control construct showed robust expression of the EhSTIRP/stuffer transcript. In contrast, the dsRNA(+)/silencing (+) parasites showed very little transcript at the expected size on a denaturing gel despite equal expression of the neomycin resistance gene (data not shown) indicating that the dsRNA transcript may be cleaved into smaller pieces for incorporation into the silencing machinery. We probed the dsRNA(+)/silencing (–)

parasites and found that there was very little expression of the dsRNA transcript in the dsRNA (+)/silencing (-) parasites (Figure 1C). This indicates that any silencing defect in these parasites may be after the cleavage of the dsRNA transcript or that the dsRNA cassette has been selectively silenced.

We then set out to confirm the presence of the appropriate construct in the dsRNA (+)/silencing (-) parasites and the absence of a contaminating construct. To do this we isolated genomic DNA from the transfected parasites and performed polymerase chain reaction using primers specific for each construct (primers are listed in Table 1). Using primers for the dsRNA construct (3' F and S-F or 3' F and S-R) (Figure 2A) we were able to amplify the appropriate sized band from the dsRNA (+)/silencing (-) parasites indicating that the construct was still present in these ameba (Figure 2B). We also confirmed that these parasites were not contaminated with the control construct as reactions performed with 3' F and 5' R did not yield a product in the dsRNA parasites but did yield a product for the control transfected parasites (Figure 2C).

Since all plasmids are maintained episomally in *E. histolytica*, we were able to isolate plasmid from the dsRNA (+)/silencing (-) parasites, transform bacteria and re-isolate the dsRNA construct. This construct was sequenced with primers from the stuffer region and did not show any errors in the expected sequence (data not shown). Additionally, in order to determine if the copy number of the dsRNA construct had decreased to a level that supported growth in 48 µg/ml of G418 but was insufficient for dsRNA production we gradually increased the G418 concentration in the dsRNA (+)/silencing (-) parasites to 80 µg/ml. Despite the increased concentration of G418 the abundance of EhSTIRP transcript remained at control levels in these parasites (data not shown).

We present here the first report of a loss of dsRNA-based silencing in *Entamoeba histolytica*. We investigated numerous mechanistic possibilities for this observation. We were able to confirm that (i) the parasites still contained the appropriate dsRNA plasmid, (ii) the parasites were not contaminated with other plasmids, (iii) the drug selectable marker, neomycin, was functional, and (iv) that the dsRNA portion of the construct was intact and did not undergo any sequence changes.

The mechanism by which dsRNA in *Entamoeba* down regulates expression of particular genes remains unknown. Additionally, the mechanism by which ameba lose the ability to down regulate genes via dsRNA is also unknown. Loss of dsRNA-mediated gene silencing has been observed in *Trypanosoma brucei* (Shi, et al., 2004). The authors attributed the phenomenon to either loss of the dsRNA cassette via recombination of the inverted repeats that flank the gene fragment of interest or selection of parasites incapable of responding to dsRNA. In respect to our system we favor the latter explanation as we have sequenced the plasmid present in our dsRNA unresponsive parasites and identified no rearrangements or alterations. The mechanism behind the loss of dsRNA based silencing in *E. histolytica* is currently unclear though we believe that any defect in silencing is post cleavage of the dsRNA hairpin. However, it is also possible that expression of the neomycin resistance gene occurs while expression of the dsRNA transcript is selectively repressed. Additionally, as the populations of transfected ameba are not clonal it is possible that a subpopulation of transfected parasites never silenced the target gene. Over time these parasites incapable of silencing genes by a dsRNA mechanism may have become the dominant subtype within the population.

We have attempted several times to repeat the gene silencing of EhSTIRP by transfecting the dsRNA cassette into *E. histolytica* parasites. However, for unknown reasons we have been unsuccessful in repeating the dsRNA based silencing. It has been observed that some *E. histolytica* lab strains appear to be capable of gene silencing while others are resistant (Anbar,

et al., 2005). We are currently investigating why the parasites no longer appear to be capable of dsRNA induced silencing.

The ability to down regulate genes in *Entamoeba* is critical as a large number of the currently annotated genes have no functional homolog in other systems. Homologous recombination has been elusive to date in *E. histolytica* and thus antisense RNA, dominant negative, dsRNA and siRNA have been used to study gene function in this important human pathogen. However, in light of this study long term down regulation of genes using dsRNA may not be desirable as the possibility of selecting for silencing resistant parasites over time apparently exists. In lieu of long term silencing of genes, drug inducible silencing may help safeguard against the loss of silencing (Ramakrishnan, et al., 1997) (Hamann, et al., 1997).

Acknowledgements

We gratefully acknowledge all members of the Singh lab for helpful comments and suggestions. US and RCM were supported by NIH grant AI-053724.

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Figure 1. Expression of EhSTIRP no longer downregulated in dsRNA (+)/silencing (-) parasites despite neomycin expression and putative cleavage of dsRNA

(A) Expression of EhSTIRP is shown by Northern blot analysis for nontransfected parasites (no trx), control transfected, dsRNA (+)/silencing (+) parasites (6 months after transfection) and dsRNA (+)/silencing (-) parasites (1 year after transfection). Actin and methylene blue stained ribosomal RNA are shown as loading controls. (B) Expression of the neomycin resistance gene (neo) is shown by Northern blot analysis for nontransfected parasites (no trx), control transfected, dsRNA (+)/silencing (+) and dsRNA (+)/silencing (-) parasites. Methylene blue stained ribosomal RNA is shown as loading control. (C) RNA from nontransfected parasites (no trx), control transfected or dsRNA(+)/silencing (-) parasites were probed for the presence the control or dsRNA cassette using the 3' gene fragment as a probe. Control transfected parasites at 48ug/ml G418 exhibited robust expression of the 5' gene fragment/stuffer region/3' gene fragment, while the dsRNA (+)/silencing (-) parasites contained very little of the 3' gene fragment/stuffer region/3' gene fragment transcript despite expression of the neomycin resistance gene (Figure 1B). Stable transfectants were obtained with pJST4-EhSTIRP dsRNA and pJST4-EhSTIRP control constructs as previously described (MacFarlane, 2007) and maintained at 48μ/ml G418. Northern blots were performed as previously described (MacFarlane, 2007). Representative images from multiple blots are shown.

Figure 2B

Figure 2C

Figure 2. Schematic of dsRNA/control constructs and PCR analysis

(A) The constructs encoding for a dsRNA hairpin or the control (non-annealing) transcript are shown. In the dsRNA construct two 395bp segments from the 3' region of the gene family (separated by a non-specific stuffer region of 500bp) were cloned in opposing orientation. The 395bp segment was amplified from the 3' end of *EhSTIRP* in a region of high similarity amongst all family members and would be predicted to down regulate the entire *EhSTIRP* gene family. The control construct contained the 395bp region from the 3' end of the gene and 350bp from the 5' end of *EhSTIRP1* and thus was incapable of forming dsRNA. Both the insert and the selectable marker *Neo* were placed under the control of the *EhActin1* 5' and 3' regulatory regions. Arrows indicate the location of the primers used for PCR based identification of the constructs. (B) PCR amplified products were run on a 1 % agarose gel. Lane 1 = PCR product

amplified from dsRNA(+)/silencing (-) gDNA using stuffer forward and 3' forward primers. Lane 2 = PCR product amplified from dsRNA(+)/silencing (-) gDNA using stuffer forward and 3' reverse primers. Lane 3 = PCR product amplified from dsRNA(+)/silencing (-) gDNA using stuffer reverse and 3' forward primers. (C) PCR amplified products were run on a 1% agarose gel. Lane 1 = PCR product amplified from dsRNA(+)/silencing (-) gDNA using 3' forward and 5' reverse primers. Lane 2 = PCR product amplified from control gDNA using 3' forward and 5' reverse primers. Lane 3 = negative control (PCR product amplified using all primers sets but no gDNA template). Polymerase chain reaction was performed as previously described (Shah, et al., 2005). Briefly, gDNA from transfected parasites was used as a template and primers corresponding to portions of the dsRNA construct or the control construct were used. The primers used in this study are listed in Table 1.

Primers used in this study are listed. 3' R = 3' portion of EhSTIRP reverse primer; 3' F = 3' portion of EhSTIRP forward primer; 5' R = 5' portion of *EhSTIRP1* reverse primer; S-F = stuffer portion forward primer; S-R = stuffer portion reverse primer; Neo F = neomycin resistance gene forward; Neo R = neomycin resistance gene reverse; Actin F = actin forward; Actin R = actin reverse.

Table 1

3' R	5'-GAACATTGCTTCAAACTCCTTTT-3'
3' F	5'-GCCATTGGTTGAAGTAGAAGG-3'
5' R	5'-ATTACCATACCAAGTAGAACATG-3'
S-F	5'-CAGACTTGGGGTGATGAGT-3'
S-R	5'-TTTTTGTGCGGTAAAGTCG-3'
Neo F	5'-AGACAATCGGCTGCTCTGAT-3'
Neo R	5'-ATACTTCTCGGCAGGAGCA-3'
Actin F	5'-GGGAGACGAAAGAGTTCAAGC-3'
Actin R	5'-GGCAAGAAATTGATCCTCCAA-3'