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Full Length Research Paper

Use of genetically induced repression of ribonucleotide reductase as target in trypanocide formulation

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In an *in vitro* experiment, ribonucleotide reductase was genetically validated as a chemotherapeutic target by engineering a plasmid vector (Recombinant DNA) for conditional expression silencing of the enzyme, by cloning the ribonucleotide reductase in a polymerase chain reaction (PCR). It was then amplified, purified and inserted into the plasmids to yield the recombinant DNA of interest, which was transfected into the parasites (clonal transfected cell lines) by electroporation. Parasite cell growth was monitored upon RNAi (Ribonucleotide reductase Interference) mediated silencing of RNR expression. The results generated showed that mRNA repression in the four groups were time dependent in parasites induced at 24hrs, 42 and 48 hours, with 48 hours cDNA showing the most significant mRNA repression. Ribonucleotide reductase was concluded to be an essential enzyme in the trypanosomes as depicted by the RNAi mediated silencing of its expression. It is also a very good drug target.

Keywords: Ribonucleotide reductase, mRNA, cDNA, Recombinant DNA, RNAi, PCR.

INTRODUCTION

Ribonucleotide reductase (RNR) is an iron-containing enzyme that is essential for DNA synthesis, as it is rate-limiting for DNA synthesis. It is a multifunctional enzyme that contains redox-active thiol groups for the transfer of electrons during the reduction reactions. In the process of reducing the rNDP to a dNDP, RNR becomes oxidized. The small R2 subunit of class I RNR contains a stable free radical tyrosine residue required for its activity. This radical is destroyed by peroxynitrite, which also inactivates the protein and induces nitration of tyrosine residues (Guittet *et al.,* 2000). In recent times, RNR has been recognized as a useful target for antiparasite (Ingram and Kinnaird, 1999), anticancer and antiviral therapies (Nuno *et al*., 2007). This enzyme plays a central role in deoxyribonucleic acid biosynthesis during cell proliferation when they are most needed. It has been suggested that it may be explored in the development or determination of potential drugs against African trypanosomiasis (Ekanem, 2001).

Ribonucleic Acid Interference (RNAi)

RNA interference (RNAi) is a system within living cells that take part in controlling which genes are active and how active they are. Two types of small RNA molecules (microRNA (miRNA) and small interfering RNA (siRNA) are central to RNA interference (Fire *et al*., 1998; Hyscience, 2006). RNAs are the direct products of genes, and these small RNAs can bind to other specific RNAs (mRNA) and either increase or decrease their activity, this they do by preventing a messenger RNA (mRNA) from producing a protein. RNA interference has an important role in defending cells against parasitic genes (viruses and transposons) as well as in directing development and gene expression in general (Fire *et al.,* 1998; Bruce *et al*., 2008). This method was used in this research to genetically repress the expression of ribonucleotide reductase in *trypanosome brucei brucei*.

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MATERIALS

Parasites

Trypanosoma brucei bloodstream (Lister 427 strain) cell culture used for the *in vitro* targeted integration of linear DNAs in the RNAi experiments and generation of stable cell lines were obtained and used in Prof. Gunzl's lab. at the Department of Genetics and Developmental Biology, University of Connecticut, Farmington, USA. All experiments were conducted within a sterile environment in the cabinet or hood of Labconco Purifier class II Biosafety Cabinet, to prevent contamination.

Primers or Oligonucleotides of interest

Primers or Oligonucleotides of interest (3' and 5' end) were purchased from IDT (Integrated DNA Technologies) Iowa, USA. The DNA sequence of the Ribonucleotide reductase (RNR) large chain subunit spanning the region from position 700 to 1200 (500 base pairs) with two complementary strands (23 base pairs) in the opposite direction was designed as the oligonucleotides of the enzyme of interest.

METHODS

DNA sequence amplification

The DNA sequence of the Ribonucleotide reductase large chain subunit spanning the region from position 700 to 1200 (500 basepairs) was amplified by PCR with two complementary oligonucleotides. The amplification products were digested and cloned into the corresponding sites of plasmid pJM325 and pT7 (Wirtz *et al.,* 1999).

In vitro cell line proliferation

Trypanosoma brucei bloodstream (strain Lister 427) cell culture, for targeted integration of linear DNAs were introduced into trypanosome cells by electroporation, and the generation of stable cell lines by selection and limiting dilution were done (Wirtz *et al;* 1999; Gunzl *et al;* 2000).

In vitro study

Generation of transgenic bloodstream form cell lines for RNAi experiment; Cloning and amplification of ribonucleotide reductase by PCR; PCR product precipitation and Purification; DNA gel elution, solubilization and purification; Digestion by restriction enzymes (HindIII/Xbal digestion and MIu1/Xbal Digestion); Ligation of each of the constructs into two different plasmids; Transforming *E. coli* with the Recombinant DNA (Mini Prep.); Digestion of both Recombinant DNA with HindIII/Xbal enzymes; Second ligated plasmid for second Recombinant DNA (Midi Prep.)

Transforming *E. coli* with the second Recombinant DNA (Midi Prep. 25-100ml); Sequencing of the Plasmid DNA by Capillary Electrophoresis; Plasmid Purification using the anion exchange chromatography principle; Transfection of bloodstream form *T. brucei* with the Recombinant DNA and finally the induction of Ribonucleotide Reductase (Gunzl *et al.,* 2006).

RESULTS AND DISCUSSION

Plate 1 shows the result of the polymerase chain reaction after cloning and purifying the ribonucleotide reductase (RNR).

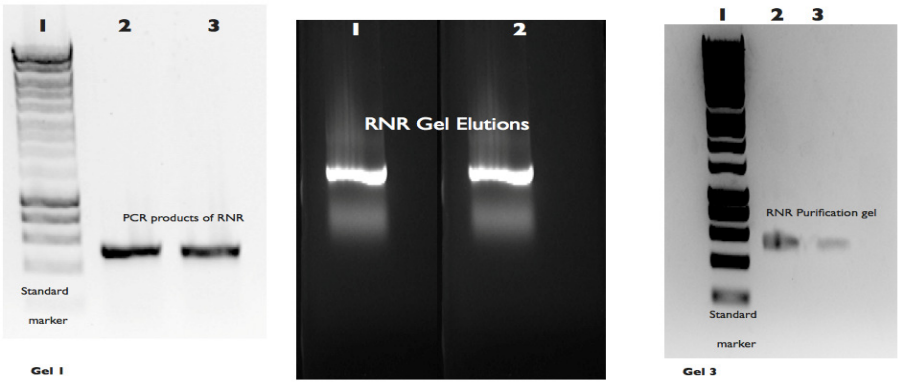
The two strands, 3' and 5' generated by digesting the enzyme, RNR with HindIII/Xbal (3' strand) and MIUI/Xbal (5' strand) are as shown in plate 2.

The two recombinant DNAs generated by transfecting the two opposing strands into *E. coli (E.coli* transformation), are as shown in Plate 3. The PCR colony of the plated clones, i.e. the recombinant DNAs generated, yielded 8 positive clones of MIUI/Xbal and a single clone of HindIII/Xbal.

The plasmids, pT7 and pJM325 (Plate 4) co-ran through the agarose gel, with their respective recombinant DNAs, MIUI/Xbal and HindIII/Xbal, generated the desired Mini prep. (the first recombinant DNA) as shown in Plate 4.

The mini preparation results showed the products generated on ligating the two plasmids to the first two recombinant DNAs by the enzyme ligase. The gel result revealed that the pJM325, was successfully ligated to the "sense strand", and the pT7, (the final plasmid carrying Both strands), successfully ligated to the "antisensestrand".

The gel result also revealed that pJM325 already contains the stuffer, a separator or DNA segments intended to stand between the sense and antisense strands to prevent their hybridization, until the hybridization is initiated (by doxycycline) in the final host, the trypanosomes. The DNA concentrations of PT7 and M/Xbal were less than those of PJM325 and X/bal (Plate 4). The gel result obtained after the second digestion of HindIII/Xbal-pJM325 complex and MIUI/Xbal-pT7 complex with HindIII/Xbal restriction enzymes is as shown in plate 5. Gel result confirmed that the sense-stuffer was successfully separated from the pJM325 and a segment of pT7 (which usually ran out of the gel due to its very small size) was cut out, to open the plasmid up for final ligation of the sense-stuffer complex (Insert) into pT7-Antisense complex (plate 6).



Gel 1

Lane 1: Standard Marker

Lane 2: First PCR product of RNR

Lane 3: Second PCR product of RNR

Gel 2

Lane 1: First RNR gel elution

Lane 2: Second RNR gel elution

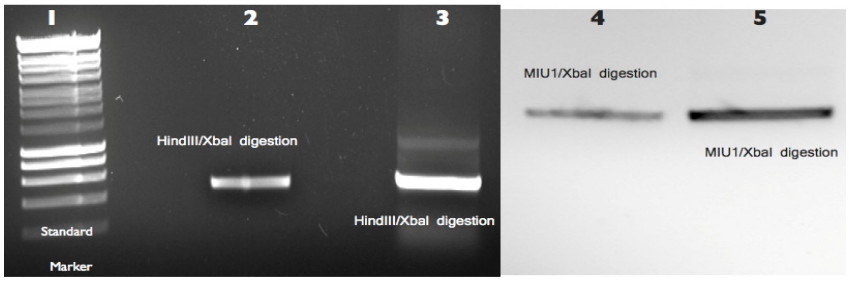
Gel 3

Lane 1: Standard Marker

Lane 2: First RNR Purification gel

Lane 3: Second RNR Purification gel

Plate 1: PCR products, gel elution and DNA Purification gels



HindIII/Xbal digestion

MIU1/Xbal digestion

Gel 1

Lane 1: Standard Marker

Lane 2: First HindIII/Xbal digestion

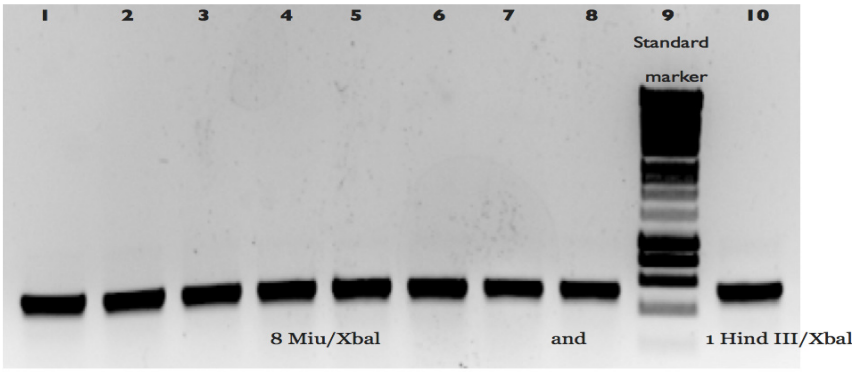
Lane 3: Second HindIII/Xbal digestion

Gel 2

Lane 4: First MIU1/Xbal digestion

Lane 5: Second MIU1/Xbal digestion

Plate 2: HindIII/Xbal and MIU1/Xbal digestion gels



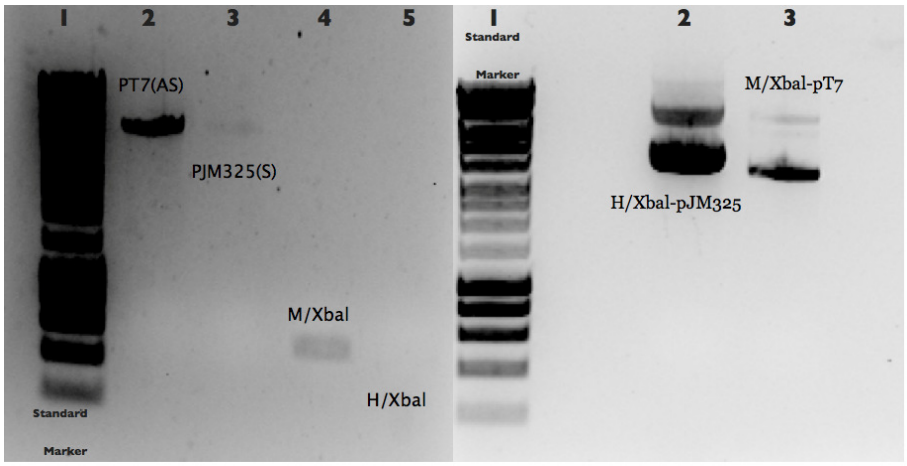
Gel 1

Lane 1-8: Eight Positive clones of Miu/Xbal

Lane 9: Standard Marker

Lane 10: One Positive clone of HindIII/Xbal

Plate 3: First Colony PCR products



Gel 1: Gel check of vectors and inserts

Lane 1: Standard Marker

Lane 2: Gel check of PT7(AS) (Vector 1)

Lane 3: Gel check of PJM325(S) (Vector 2)

Lane 4: Gel check of M/Xbal (5' strand– insert 1)

Lane 5: Gel check of H/Xbal (3' strand– insert 2)

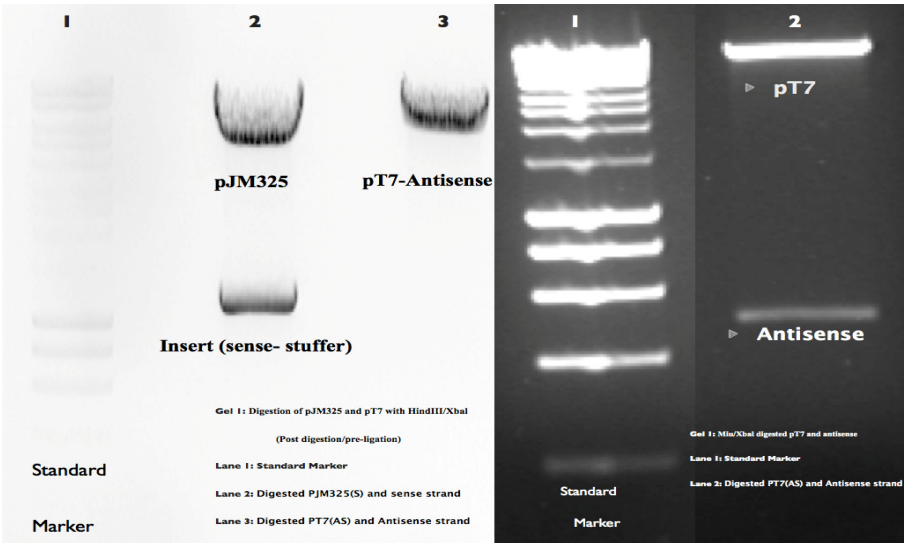
Gel 2: Minipreps / Recombinant DNA (Post ligation)

Lane 1: Standard Marker

Lane 2: Gel check of first recombinant DNA (H/Xbal-pJM325)

Lane 3: Gel check of second recombinant DNA (M/Xbal-pT7)

Plate 4: Plasmids, inserts and the Minipreps (Recombinant DNA)



pJM325, Insert (sense- stuffer) and (pT7-Antisense) Miu/Xbal digested pT7 and antisense
Post digestion/Pre ligation gels

Plate 5. Digestion of pJM325 and pT7 with HindIII/Xbal to yield pJM325, insert, and pT7 – antisense; MIu/Xbal digestion to yield pT7 and antisense

E.coli transformation with the final ligated plasmid/recombinant DNA (Midi prep.)

Plate 6 revealed the gel results generated when the "insert" cut out of the first gel was co-ran in another gel with the digested pT7, thereby aiding its comparison with the initial "mini prep" for proper DNA digest confirmation.

Plate 7 however showed the ligated "Sense-stuffer" and "pT7-Antisense" complexes (vector + insert), already ligated to form the second and last recombinant DNA, also referred to as the Midi prep (Plate 7).

The bands observed in the gel results of the second colony PCR (Plate 7) represented the complete Recombinant DNA this indicate that all

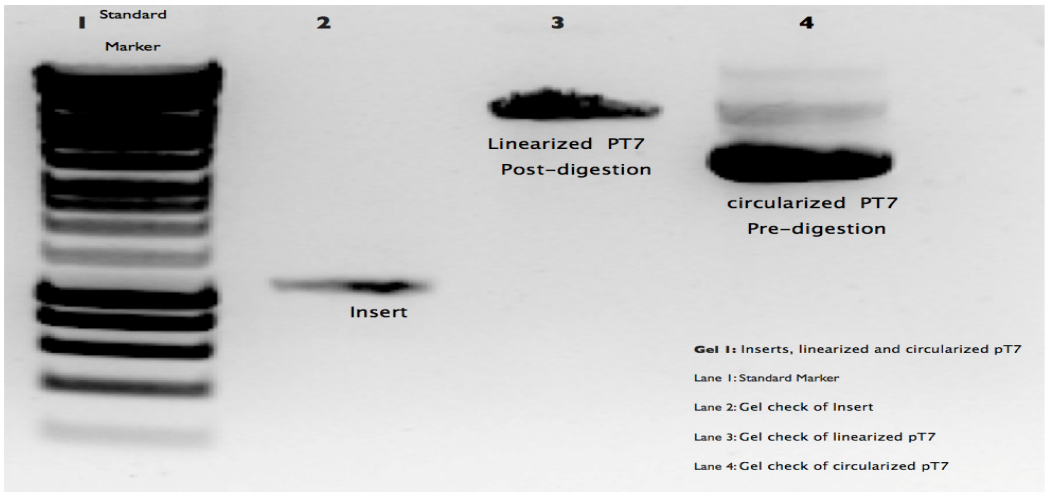


Plate 6: Insert, PT7 (Linearized plasmid, Post-digestion) and Miniprep (circular Plasmid before digestion)

Plate 7: Second and final colony PCR Plasmid Midi prep.

the clones picked from the ampicillin treated culture plates Post ligation were positive clones when compared with the only clone picked from the positive control plates, containing the vector alone, the clone picked was negative, containing no DNA, as none was visible in the gel.

The result of the sequencing of the final recombinant DNA (pT7-sense-stuffer-antisense complex), showed no possible point mutations that may interfere with the RNAi experiments. The nucleic acid sequence generated by the sequence alignment editor showed no point or segment mutation when compared with the tritrypdb sequences database.

Seq 2 and Seq 3 softwares were used to identify the beginning and the end of the final recombinant DNA. While Seq 2 identified the HindIII and MIUI sequences, which identifies the beginning of RNR as well as pT7 promoter and tet operator sequences, Seq 3 identified the T7 terminators and MIUI sequences, which identifies the end of the RNR.

After the DNA mutational check, the recombinant DNA transfected into the parasites initiated the induction of the hybridization of the "sense DNA strands" with the "antisense DNA strands" to form a stem loop at 24, 42 and 48 hours on adding doxycycline (figure 1), it showed a significant decline in the number parasites 24 hours (day 2) post induction when compared with the non-induced (the positive control) and this continued till 6 days post induction. This implies the intended repression of ribonucloetide reductase (vital for the survival of the parasite) was successful. The total mRNA, spectrophotometrically measured in the four groups post parasites's induction were; 817.4µg/ml for non-induced, 862.0µg/ml for 24 hours, 1011.1µg/ml for 42 hours and 584.4µg/ml for 48 hours. General dilution of all the mRNAs to 500 ng/ml was used to generate the

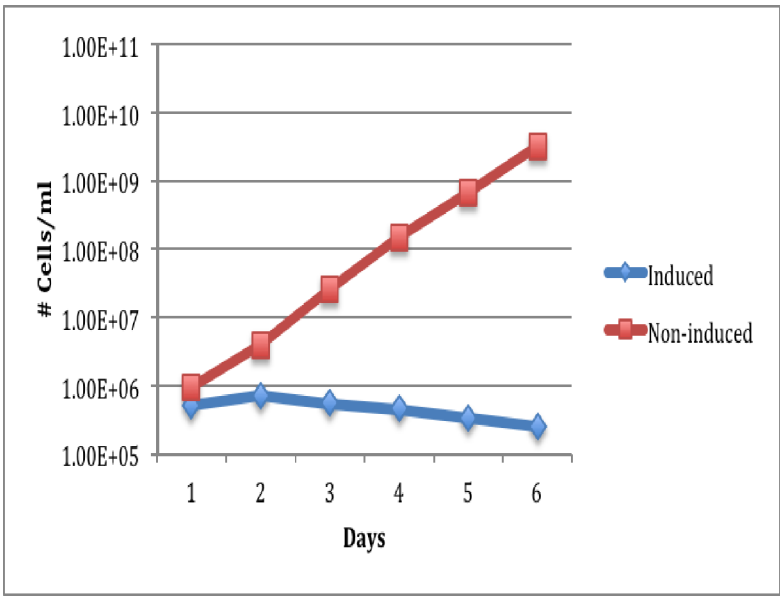


Figure 1. Parasite count post induction of hybridization in transfected parasite culture

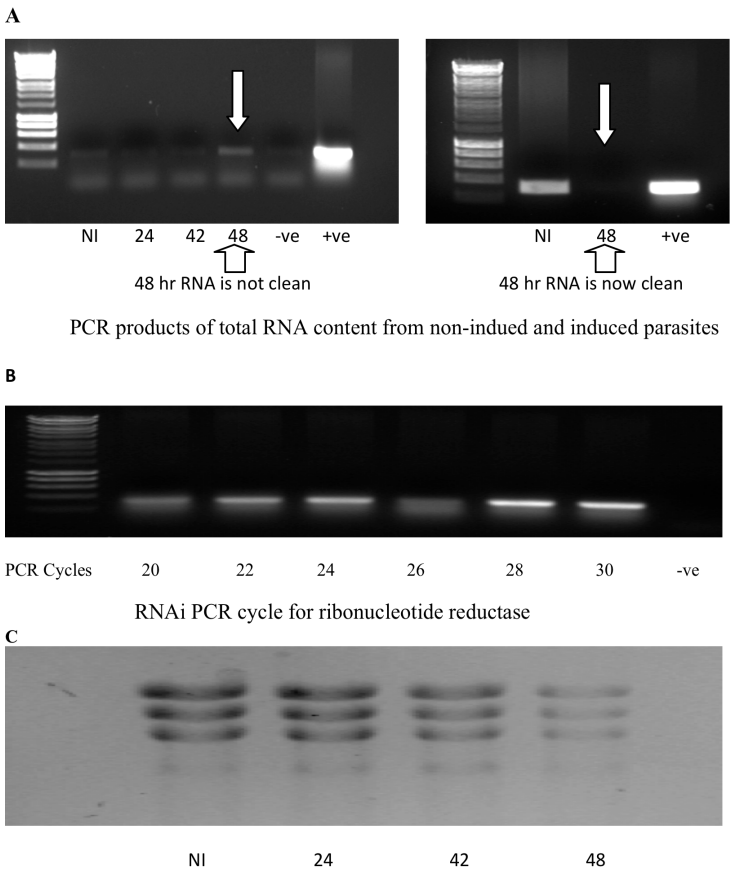


Plate 8. Total Ribosomal RNA content in non-induced and induced parasites

subsequent results shown in plate 8 and plate 9.

The total RNA Purification clean PCR gel check (plate 8A) obtained from the harvested trypanosomes was clean and free of DNA contamination in the Non-induced, 24 hr and 42 hr samples. 48 hr RNA, which was initially contaminated with DNA was DNAase treated again and

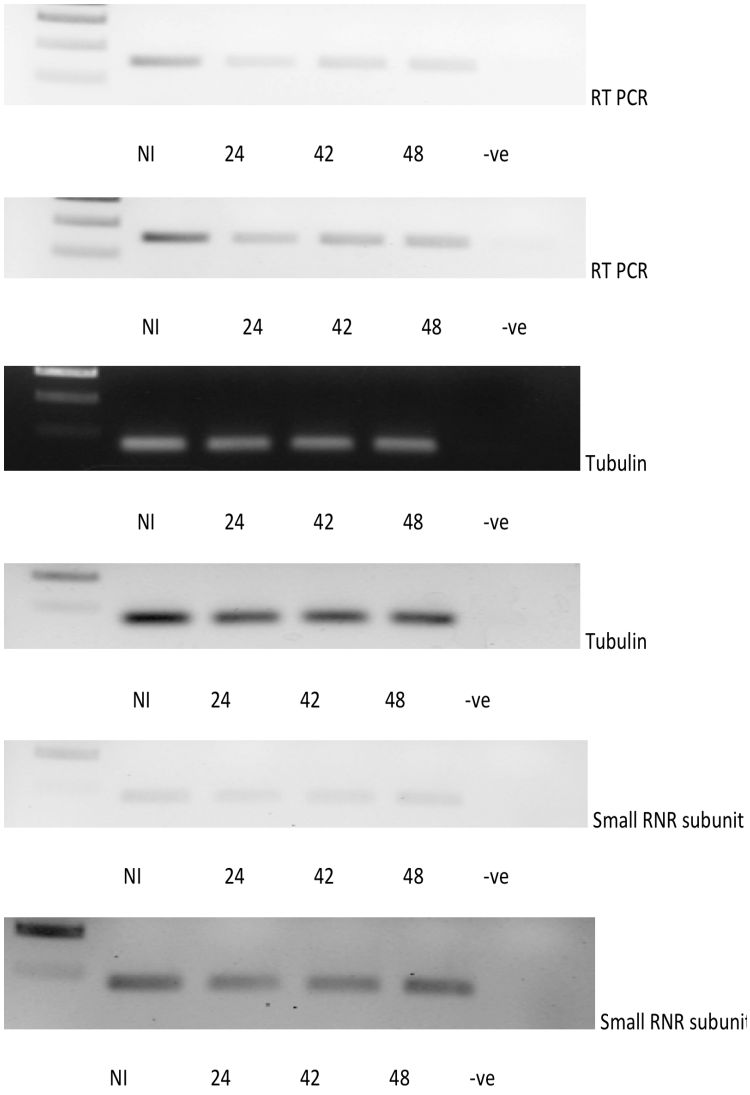


Plate 9. RT PCR, Tubulin and Small RNR subunits PCR products.

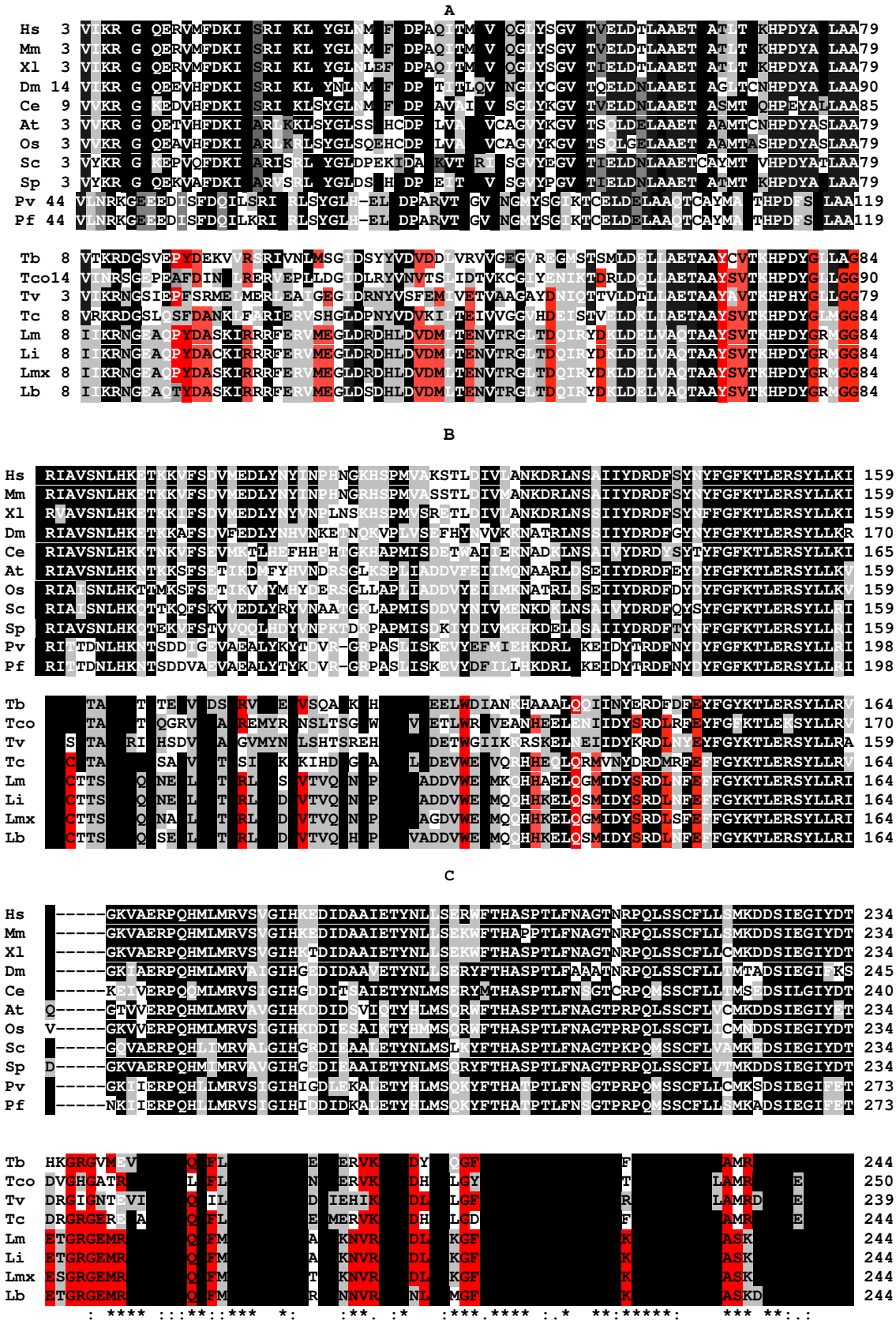
re-purified until clean (plate 8A) before it was used for the next experiment.

The RNAi PCR linear gel check (plate 8B) results showed that 20 and 26 PCR cycles were the least productive cycles for the enzyme, cycle 30 was chosen as the best PCR cycle for cloning and amplifying the large subunit of the ribonucleotide reductase, and this cycle was used throughout for the subsequent PCR reactions (i.e. RT PCR and Tubulin PCR).

The total ribosomal RNA content of the four groups, ran through the gel after diluting all to 500ng/ml is as shown in Plate 8C. The three major bands seen in all the groups' shows that the products obtained were accurate and were not contaminated with any extra bands.

Plate 9 showed the gel results of the RT PCR, Tubulin and Small RNR subunit. At various exposures of the ultra violet light to the gels, the RT PCR showed the gradual decline or repression of the trypanosomes' RNR mRNA with hybridization induction at 24, 42 and 48 hr. The non-induced showed a stronger concentration of the mRNA, as no doxycycline was added to this group to initiate RNAi (Fire *et al.,* 1998). This confirmed the silencing and the transcriptional repression of the enzyme (Cross and Gu¨nzl, 2007). With more inductions at 96 hours and above, more of the parasites are expected to be dead as a result of significant repression of RNR, an enzyme vital for their DNA synthesis.

Tubulin and small Ribonucleotide reductase (RNR) subunit were used as a check and a control against the RT PCR (plate 9). They were used as the primers (3' and 5') for the four groups which serve as the templates and the PCR result showed no significant decline in their concentrations in the 24, 42 and 48 hours samples and as such no transcriptional repression of the proteins were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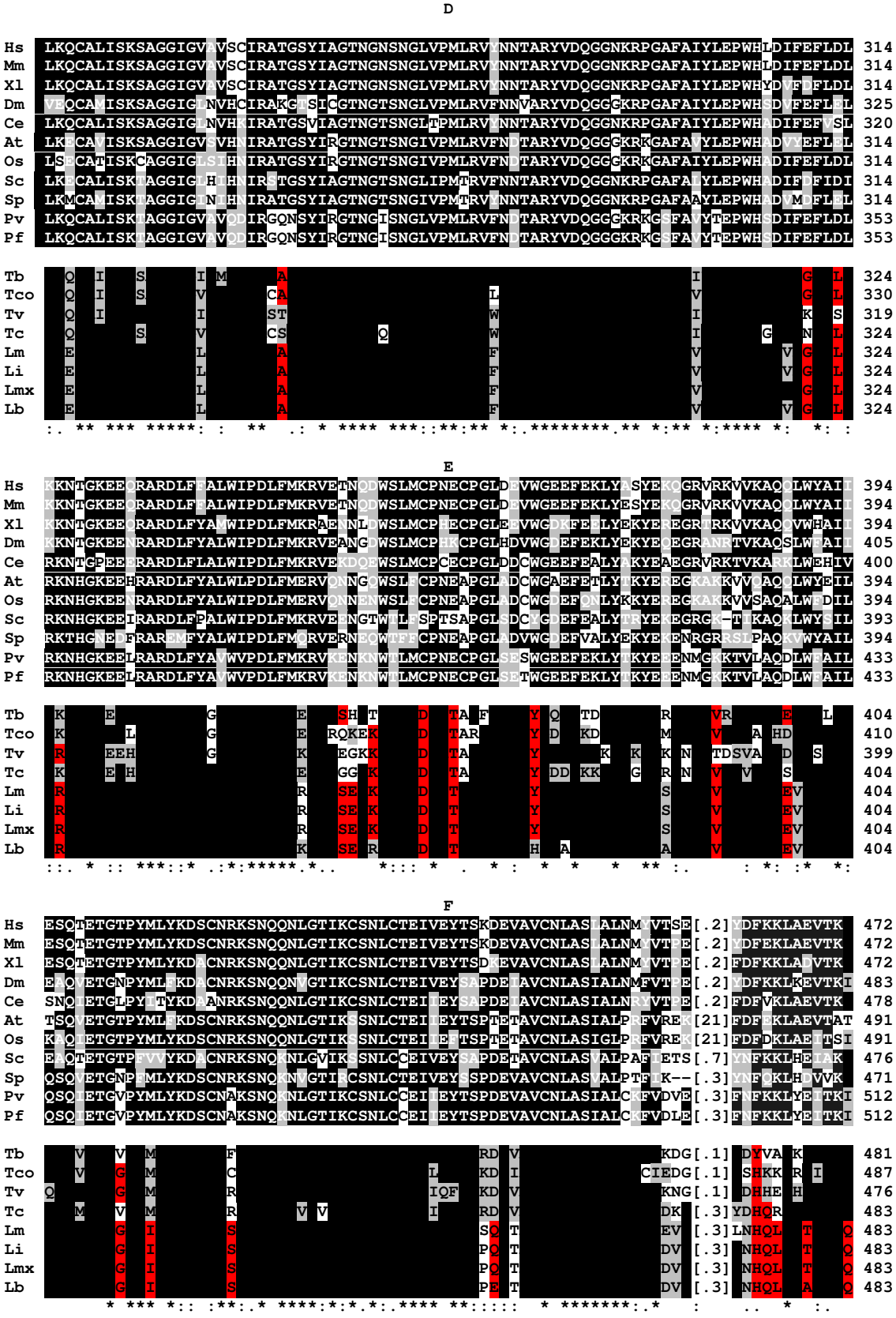
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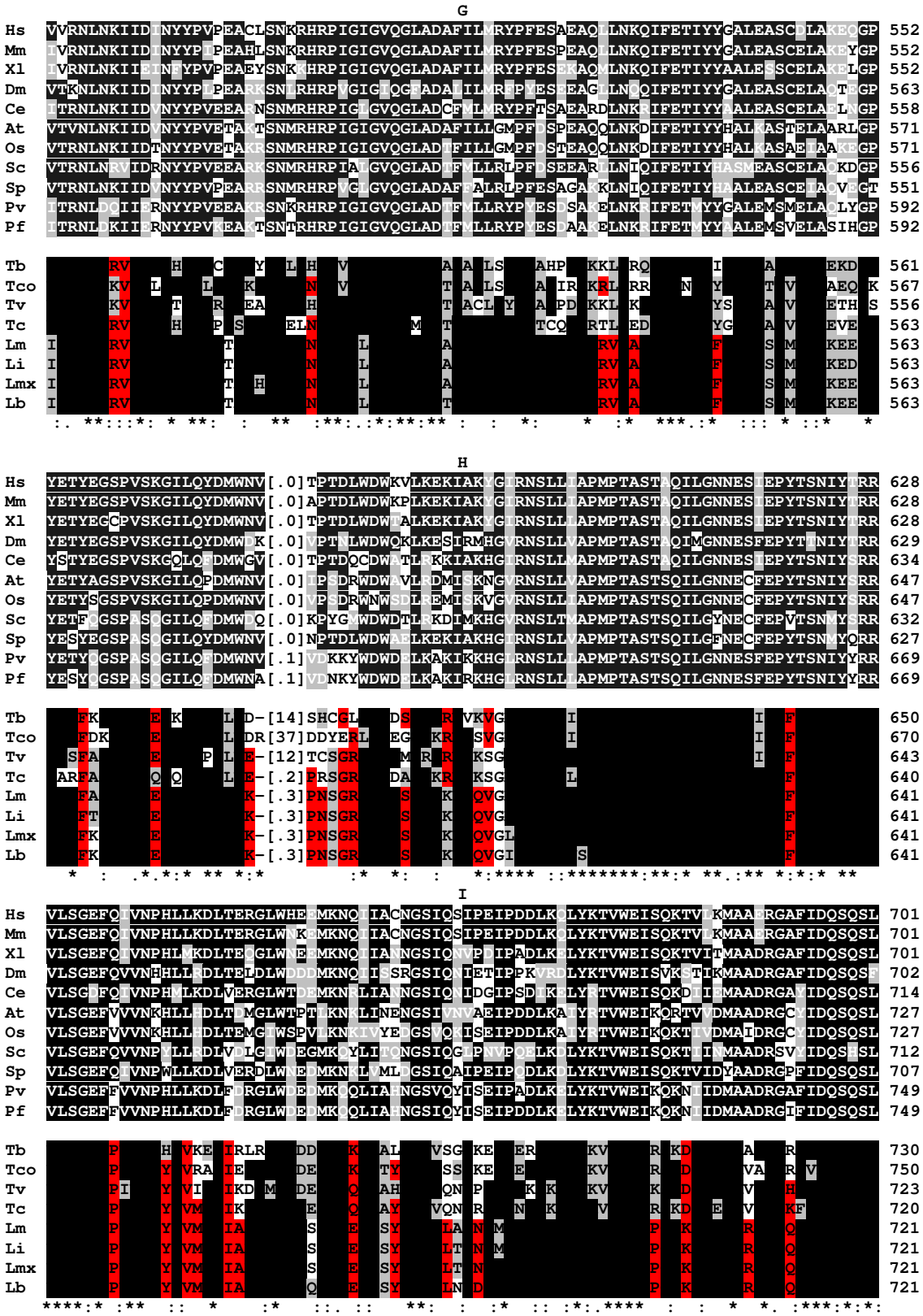
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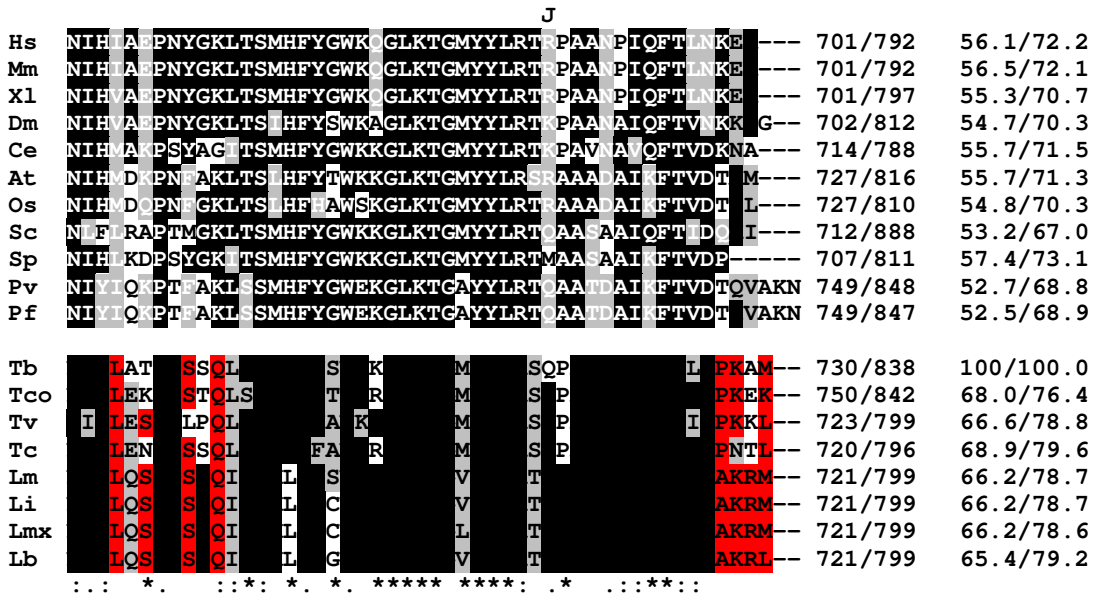

Figure 2. ClustalW sequence alignments of RNR large subunits domains A-J. (A) Alignment of RNR sequences from Homo sapiens (Hs, accession number CAA42180.1), Mus musculus (Mm, NP_033129.2), Xenopus laevis (Xl,:NP_001084312.1), Drosophila melanogaster (Dm, NP_477027.1), Caenorhabditis elegans (Ce, :CAA79574.1), Arabidopsis thaliana (At, AEC07222.1), Oryza Sativa Japonica (Os, ACC95434.1), Saccharomyces cerevisiae (Sc, AAB64606.1), Schizosaccharomyces pombe (Sp, CAA91952.1), Plasmodium Vivax (Pv, EDL46877.1), Plasmodium falciparum (Pf, PF14_0398), and from the *trypanosomatids*, T. brucei (Tb, AAB70704.1), Trypanosoma congolense (Tco, congo855a04.p1k_14), Trypanosoma vivax (Tv, TvY486_1108560), T. cruzi (Tc, EFZ23824.1), L. major (Lm, LmjF XP_001684370.1), Leishmania infantum (Li, CAM69320.1), Leishmania mexicana (Lmx, CBZ28456.1) and Leishmania braziliensis (Lbr,: CAM39626.1).
Positions with >60% of identical and conserved residues were shaded in black and gray, respectively. Red shading indicates positions in which *trypanosomatid* sequences are invariant and no conservation is present in the other sequences. Numbers of amino acids without significant similarity are specified in parentheses. Identity/similarity values specified at the end of each sequence were determined by pair-wise comparison with the *T. brucei* sequence using the EMBOSS program (http://www.ebi.ac.uk/emboss/align/).

observed (Cross and Gunzl, 2007; Thelander, 2007).

DISCUSSION

The *in vitro* PCR and RNAi experiments were set up as molecular confirmatory tests to establish the essentiality of ribonucleotide reductase (RNR) to the survival of the parasites and the possibility of being a good drug target. The results generated from plate 1 to 7 indicated a successful cloning (Dormeyer *et al.,* 1997; Hofer *et al.,* 1997) and purification of the enzyme (RNR) (Hofer *et al.,* 1997; Guittet *et al.,* 2000). Comparing the PCR amplified products (plate 1) and the digests (plate 2) with the standard marker confirmed their sizes to be accurately located at 500basepairs between 400 and 600basepairs (plate 1 and plate 2).

The Miu1/Xbal (3' strand) and HindIII/Xbal (5' strand) clones picked from the ampicillin treated agar plates were positive and of about 300 basepairs (plate 3). The ligation of both strands into pT7 and pJM325 respectively to generate the first two recombinant DNAs (Midi preps.) was successful as depicted by plate 4. HindIII/Xbal digestion gel results in Plate 5 not only establish the fact that the pT7 contain the antisense strand but also show the sense-stuffer complex was completely separated from the pJM325 for a successful final ligation as confirmed by plate 6 to form the second and final recombinant DNA (Midi prep.) (plate 7).

The pre-digestion Mini prep (first recombinant DNA) is circularized and runs faster than the linearized DNA post-digestion (pT7-Antisense complex), this is as a result of the intact supercoling, which is absent in the linearized DNA (plate 6). The insert also ran faster than both as a result of its smaller size (1000 basepairs). The final recombination into a second and final plasmid (Furukawa *et al;* 1992; LaCount *et al.,* 2000) aided the subsequent successful transfection of the desired plasmid into the parasites as well as the initiation of RNAi (Fire *et al.,* 1998). Sequencing, mutational crosscheck and transfection into the parasites were also successful (plate 7 to plate 9) (Bruce *et al*., 2008a).

24 hours post transfection and induction, the first significant decline ($P > 0.05$) in parasite count (Figure 1) observed indicate the enzyme, RNR was successfully repressed transcriptionally and this consequently affected the growth of the parasite and confirmed how vital the enzyme is to the survival of the parasites (Dormeyer *et*

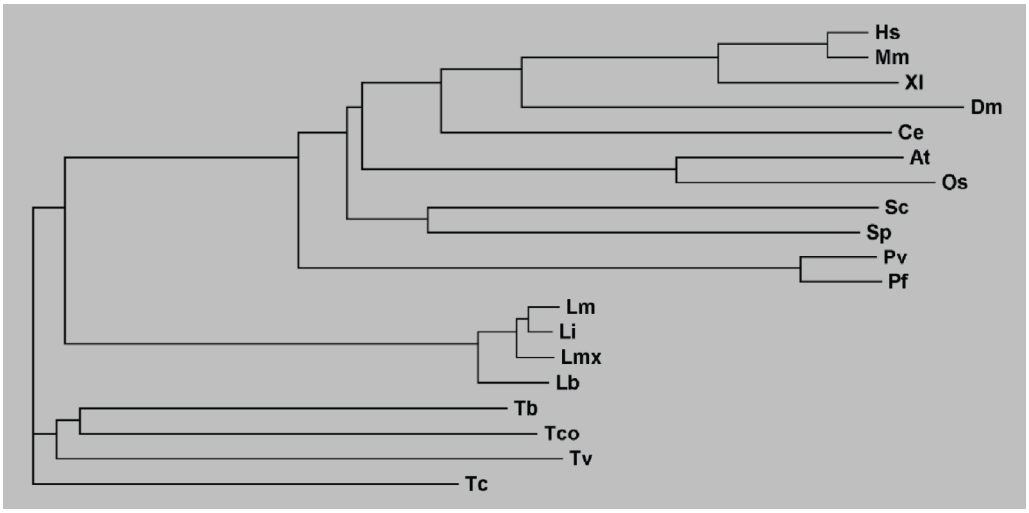

Figure 3. The RNR phylogenetic tree was constructed with the computer program ClustalX (neighbor joining method). The RNR large subunit in the trypanosomatid is highly divergent.

al., 1997; Brandenburg *et al.,* 2002).

Sufficient concentrations of mRNA (Daneholt, 2006) required for genetic validation of mRNA mediated silencing of RNR was obtained to generate the total ribosomal RNA (plate 8). The significant decline in ribosomal RNA (plate 8C) observed by the 48 hour showed and confirm the genetic silencing /repression of the enzyme (Furukawa *et al;* 1992; Dormeyer *et al.,* 1997).

The observed decline in the DNA concentration of the large subunit reverse transcriptase PCR (RT PCR) (plate 9) also confirm the repression (Hofer *et al.,* 1997; Cross and Gu¨nzl, 2007) of the enzyme and its vitality to the survival of the parasites. Tubulin and small RNA subunit's PCR showed no significant differences in their concentrations at 24, 42 and 48 hours, this is because the protein that was actually transcriptionally silenced was RNR large subunit and not tubulin nor the small RNR subunit target (Ingram and Kinnaird, 1999). This confirmed that RNR large subunit is a good drug target and is essential for the survival of the parasites.

The RNR large subunit is conserved (Figure 2) among trypanosomatids and has some similarities to other eukaryotic proteins. The clustalW analysis of RNR revealed overall identity and similarities values of (52.5 to 68.0) and (67.0 to 79.6) respectively. The RNR large subunit in the trypanosomatid is highly divergent (Figure 3) relative to the human RNR, Fungi and *plasmodium* RNR. The divergence of the *trypanosome* protein may be a target of specific inhibition. It also revealed that the functional specificity of the enzyme is conserved among trypanosomatid organisms. Ribonucleotide reductase was concluded to be an essential enzyme in the trypanosomes as depicted by the RNAi mediated silencing of its expression. It was also confirmed to be a very good drug target in the formulation of trypanocides.

ACKNOWLEDGEMENTS

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