# Cloning, sequencing and expression of ribonucleotide reductase R2 from *Trypanosoma brucei*

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**Abstract Ribonucleotide reductase (RR) is an attractive drug target molecule. The gene of the R2 protein of *Trypanosoma brucei* RR (*nrd B*) has been cloned. It encodes a protein of 337 residues which shows about 60% identity with other eukaryotic R2 proteins. All residues which bind the iron center, the tyrosyl radical or are supposed to participate in the radical transfer are conserved in the trypanosomal protein sequence. Overexpression of the gene in *E. coli* resulted in 2–5 mg pure R2 protein from 100 ml bacterial cell culture. Northern blot analysis revealed a transcript of 1.85 kb in bloodstream and procyclic forms of the parasite.**



*Key words:* Ribonucleotide reductase; Drug target; Trypanothione; (*Trypanosoma brucei*)

## 1. Introduction

Trypanosomes are the causative agents of African sleeping sickness (*Trypanosoma brucei rhodesiense*, *T. b. gambiense*), South American Chagas' disease (*T. cruzi*) and nagana cattle disease (*T. congolense*, *T. vivax*). The parasites possess a unique thiol metabolism. Instead of glutathione/glutathione reductase they have a trypanothione/trypanothione reductase system [1,2]. Since in all known eukaryotes the synthesis of deoxyribonucleotides catalyzed by ribonucleotide reductase (RR, E.C. 1.17.4.1.) is a thiol dependent process [3,4] the question raises if the unique dithiol trypanothione is involved in the parasite synthesis of DNA precursors.

Eukaryotic ribonucleotide reductases are tetrameric $\alpha_2\beta_2$ proteins [5,6]. The large α subunit (R1) is responsible for substrate and effector binding. A thiyl radical in R1 is directly involved in catalysis. It is formed by long-range radical transfer from a tyrosyl radical of the small β subunit (R2). This tyrosyl radical is generated by an oxygen-bridged di-iron center that is also localized in the small subunit [6,7]. Each subunit is inactive when assayed individually [3–7].

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*Abbreviations:* RR, ribonucleotide reductase; R1, subunit α of RR; R2, subunit β of RR; *nrd B*, gene encoding R2 of ribonucleotide reductase; *T. b., Trypanosoma brucei*; IPTG, isopropyl-β-D-thiogalactopyranoside; Ni-NTA, Ni-nitriloacetic acid

The gene sequence of the R2 protein of *T. brucei* ribonucleotide reductase has been deposited in the EMBL Nucleotide Sequence Database (accession no. Y10768).

As a first step towards the understanding of the trypanosomal synthesis of deoxyribonucleotides we describe the cloning of the gene of ribonucleotide reductase subunit R2 from *T. brucei brucei* and its overexpression in *E. coli*.

## 2. Materials and methods

### 2.1. DNA and RNA preparation

$2\times10^8$ long slender *T. brucei* cells were suspended in 10 mM Tris-HCl, 250 mM NaCl, 0.5% Nonidet P-40, pH 8.0. After 5 min incubation on ice the suspension was centrifuged and the cell pellet was homogenized in 200 μl 10 mM Tris-HCl, 10 mM NaCl, 10 mM EDTA, 0.5% SDS, pH 8.0. 50 μg proteinase K were added and the mixture was incubated for 1 h at 37°C. DNA was purified by phenol extraction.

RNA was isolated from bloodstream long slender and short stumpy as well as procyclic stages of *T. brucei* using the RNA Easy Kit (Qiagen) according to the instructions of the manufacturer.

### 2.2. Isolation of the T. brucei nrd B gene

Total RNA of long slender bloodstream *T. brucei* was reverse transcribed into single-stranded cDNA using Moloney murine leukemia virus reverse transcriptase and an oligo(T) primer [5′-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT(G/C/A)].

Primers for PCR were derived from consensus sequences of eukaryotic R2 proteins. An internal fragment of R2 was amplified from the cDNA with two oligonucleotides [5′-GCIGCIAGCGACGGCATTGTGAACGA(A/G)AA and 5′-GCGGTCCACAAACTCGATG TA(C/T)TG] (95°C, 5 min; 30 cycles: 95°C, 1 min; 45°C, 1 min; 72°C, 1 min). For cloning the 5′ end of the gene a gene specific primer [5′-AGGGATATTTT CCATAGCAATCTGGAACCC(A/G)TA(A/G)-AA] and a spliced leader specific primer (5′-TAGAACAGTTTCTGTACTATATTG) were used in a modified 5′ RACE-PCR (95°C 2 min; 25 cycles: 95°C, 30 s; 55°C, 1 min; 72°C, 1 min). The 3′ end was amplified (95°C, 2 min; 25 cycles: 94°C, 1 min; 50°C, 1 min; 72°C, 30 s) using the oligo(T) and a gene specific primer (5′-ATTGGAATGAATTCTCAG).

The complete gene was obtained from genomic DNA using a 3′ primer which contained an additional *Xho*I cleavage site (5′-GTGTCTCGAGCACCAACGATAACATCCCTA) and a 5′ primer (5′-ACAGAATTCAAACGATGCCACCCAAATCTCACAA). PCR was carried out with *Pfu* polymerase (95°C, 5 min; 30 cycles: 94°C, 1 min; 45°C, 1 min; 72°C, 3 min). The PCR products were cloned into the pBluescript SK(+) vector (Stratagene) for sequencing.

### 2.3. DNA sequencing

DNA was sequenced by the dideoxynucleotide chain-termination method using the T4 Sequencing Kit (Pharmacia Biotech). Both strands were completely sequenced.

### 2.4. Expression of T. b. brucei nrd B in E. coli

The gene of *T. b. brucei* R2 was excised from the pBluescript vector by *Xho*I digestion and subcloned into the *Sal*I site of the pQE-31 vector. This construct encodes the R2 protein with a 34 residues long N-terminal extension containing a $His_6$– Tag. Competent *E. coli* SG13009 were transformed with the pQE/*His-nrd B* plasmid. A 25 ml culture of recombinant cells in 2× YT medium supplemented with 100 μg/ml carbenicillin and 25 μg/ml kanamycin was grown at 37°C to an $OD_{600}$ of 0.8. Expression was induced with 1 mM IPTG for 4 h at 37°C. The protein was chromatographed on Ni-NTA Se-


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T. brucei  ..........  ..........  ..........  ..........  ..........
human      MLSLRVPLAP  ITDPQQLQLS  PLKGLSLVDK  ENTPPALSGT  RVLASKTARR  50
mouse      MLSVRTPLAT  IADQQQLQLS  PLKRLTLADK  ENTPPTLSST  RVLASKAARR  50
P. falcip. ..........  ..........  ..........  ..........  MADVINISRI  10
E. coli    ..........  ..........  ..........  ..........  ..........

T. brucei  ....MPPKSH  KRSRKEGEVE  EPLLTENPDR  YVIFPIKYPD  IWQ.KYKKAE  45
human      IFQEPTEPKT  KAAA.PGVED  EPLLRENPRR  FVIFPIEYHD  IWQ.MYKKAE  98
mouse      IFQDSAELES  KAPTNPSVED  EPLLRENPRR  FVVFPIEYHD  IWQ.MYKKAE  99
P. falcip. PIFSKQEREF  SDLQKGKEIN  EKILNKESDR  FTLYPILYPD  VWD.FYKKAE  59
E. coli    ......AYTT  FSQTKNDQLK  EPMFFGQPVN  VARYDQQKYD  IFEKLIEKQL  44

                                                        #
           □                                            □
T. brucei  SSIWTVEEID  LGNDMTDWEK  LDDGERHFIK  HVLAFFAASD  GIVLENLAER  95
human      ASFWTAEEVD  LSKDIQHWES  LKPEERYFIS  HVLAFFAASD  GIVNENLVER 148
mouse      ASFWTAEEVD  LSKDIQHWEA  LKPDERHFIS  HVLAFFAASD  GIVNENLVER 149
P. falcip. ASFWTAEEID  LSSDLKDFEK  LNENEKHFIK  HVLAFFAASD  GIVLENLASK 109
E. coli    SFFWRPEEVD  VSRDRIDYQA  LPEHEKHIFI  SNLKYQTLLD  SIQGRSPNVA  94

                                   #  #
                                      □   *
T. brucei  FMCEVQVPEV  RCFYGFQIAM  ENIHSETYSV  LIDTYVVDPD  EKQRLLHAIR 145
human      FSQEVQITEA  RCFYGFQIAM  ENIHSEMYSL  LIDTYIKDPK  EREFLFNAIE 198
mouse      FSQEVQVTEA  RCFYGFQIAM  ENIHSEMYSL  LIDTYIKDPK  EREYLFNAIE 199
P. falcip. FLREVQITEA  KKFYSFQIAV  ENIHSETYSL  LIDNYIKDEK  ERLNLFHAIE 159
E. coli    LLPLISIPEL  ETWVETWAFS  ETIHSRSYTH  IIRNIVNDPS  ...VVFDDIV 141

T. brucei  TIPCIEKKAK  ..........  ...WAIEWIG  .SQTSFPTRL  VAFA...... 175
human      TMPCVKKKAD  ..........  ...WALRWIG  DKEATYGERV  VAFA...... 229
mouse      TMPCVKKKAD  ..........  ...WALRWIG  DKEATYGERV  VAFA...... 230
P. falcip. NIPAVKNKAL  ..........  ...WAAKWIN  DTN.SFAERI  VANA...... 189
E. coli    TNEQIQKRAE  GISSYYDELI  EMTSYWHLLG  EGTHTVNGKT  VTVSLRELKK 191
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Fig. 1. Amino acid sequences of R2 proteins. The derived protein sequence of *T. brucei* RR subunit 2 is aligned with that of human (accession number P31350; ref. [9]), mouse (P11157; ref. [10]), *Plasmodium falciparum* (P50650; ref. [11]) and *E. coli* R2 (P00453; ref. [12]). 16 Residues (bold letters) are invariant in all R2 proteins sequenced so far (Asp139 is a Glu in the Epstein-Barr virus protein). Most of them are iron ligands (#), form the hydrophobic pocket (+) around the tyrosyl radical (*) or are proposed to be involved in the electron transfer mechanism (□).

pharose in the presence and absence of 8 M urea according to the instructions of the manufacturer (Qiagen). The denatured protein was eluted from the column with 100 mM EDTA, the native protein with 250 mM imidazol.

### 2.5. Northern blot analysis

10 μg total RNA from long slender, short stumpy and procyclic *T. brucei* cells was electrophoresed on a 1% agarose gel containing 5% formaldehyde and transferred to a GeneScreen Plus membrane (NEN/Dupont) by capillary blot with 1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0 (10×SSC). The membrane was hybridized overnight with randomly primed [α-$P^{32}$]dCTP radiolabeled complete *nrd B* in 250 mM $NaH_2PO_4$, 1 mM EDTA, 7% SDS at 68°C. After washing the membrane with 2×SSC, 0.5% SDS followed by 0.1×SSC, 0.5% SDS, signals were quantified on a Fuji Phosphorimager. Equivalent amounts of RNA were loaded as determined by methylen blue staining and hybridization with a *T. brucei* actin probe.

## 3. Results and discussion

### 3.1. Cloning and sequencing of T. brucei ribonucleotide reductase nrd B

An internal fragment of the gene (position 244–828) coding for ribonucleotide reductase subunit 2 (*nrd B*) was isolated from *T. brucei* cDNA by PCR using primers which represented highly conserved regions of eukaryotic R2 proteins. For amplifying the 3′- and 5′-ends of the gene a poly(T) primer and a spliced leader primer, respectively each in combination with a gene specific oligonucleotide were used. The spliced leader is the very 5′ end of all trypanosomal mRNAs which is added by *trans*-splicing [8].

The complete gene of *T. b. brucei* R2 was isolated from genomic DNA by PCR and sequenced. An open reading

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                          #                                        #  #
                              +    +                          +  □
T. brucei  ..........  AVEGIFFSGS FCAIFWLKKR GLMPGLTFSN ELISRDEGLH 215
human      ..........  AVEGIFFSGS FASIFWLKKR GLMPGLTFSN ELISRDEGLH 269
mouse      ..........  AVEGIFFSGS FASIFWLKKR GLMPGLTFSN ELISRDEGLH 270
P. falcip. ..........  CVEGILFSGS FCAIFWFKKQ NKLHGLTFSN ELISRDEGLH 229
E. coli    KLYLCLMSVN  ALEAIRFYVS FACSFAFAER ELMEGNAKII RLIARDEALH 241

T. brucei . TDFACLLYE KYIVNKLPR. ........DR VLEIICNAVS IEREFICDAL 255
human      .CDFACLMF. KHLVHKPSE. ........ER VREIIINAVR IEQEFLTEAL 308
mouse      .CDFACLMF. KHLVHKPAE. ........QR VREIITNAVR IEQEFLTEAL 309
P. falcip. .TDFNCLIY. SLLDNKLPE. ........QI IQNIVKEAVE VERSFICESL 268
E. coli    LTGTQHMLNL LRSGADDPEM AEIAEECKQE CYDLFVQAAQ QEKDWADYLF 291

T. brucei  P.VRLIGMNS QLMTQYIEFV ADRLLVSLGY DRHYNSK.NP FDFMD..MIS 301
human      P.VKLIGMNC TLMKQYIEFV ADRLMLELGF SKVFRVE.NP FDFME..NIS 354
mouse      P.VKLIGMNC TLMKQYIEFV ADRLMLELGF NKIFRVE.NP FDFME..NIS 355
P. falcip. P.CDLIGMNS RLMSQYIEFV ADRLLECLGC SKIFHSK.NP FNWMD..LIS 314
E. coli    RDGSMIGLNK DILCQYVEYI TNIRMQAVGL DLPFQTRSNP IPWINTWLVS 341

                   □     □
T. brucei  LQGKTNFFEK KVGEYQKAGV MSSERSSKVF SLDADF.336
human      LEGKTNFFEK RVGEYQRMGV MSS.PTENSF TLDADF.389
mouse      LEGKTNFFEK RVGEYQRMGV MSN.STENSF TLDADF.390
P. falcip. LQGKTNFFEK RVADYQKSGV MA.QRKDQVF CLNTEF.349
E. coli    DNVQVAPQEV EVSSYLVGQI DSEVDTDDLS NFQL...375
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Fig. 1. (*Continued*)

frame of 1011 bp corresponds to a deduced protein sequence of 337 amino acid residues with a calculated molecular weight of 39.060.

### 3.2. Sequence comparison of T. brucei R2 with other R2 proteins

The deduced amino acid sequence of the *T. brucei* gene shares about 60% identity with the RR subunit 2 of other eukaryotes but only 20% with the *E. coli* enzyme. The amino ends of R2 proteins vary considerably in length between different species. The *T. brucei* protein is about 50 residues shorter than mammalian and yeast enzymes as are those of plants, viruses, plasmodia and *E. coli*. The N-terminal amino acid sequence of *T. brucei* R2 is very hydrophilic. 8 out of the first 11 residues are positively charged or neutral hydrophilic.

Residues which bind the binuclear iron center interact with the tyrosyl radical or participate in the radical transfer to subunit 1 are invariant within the R2 protein family (Fig. 1). Asp85, Glu116, His119, Glu178, Glu212 and His215 correspond to the iron ligands in mouse and *E. coli* R2 [7,13,14].

Tyr123 (Tyr122 in the *E. coli* enzyme) forms the radical which is essential for catalysis [13]. Three invariant residues (Phe182, Phe186, and Ile208) create a hydrophobic pocket around the tyrosine and are supposed to stabilize the radical. Other residues in the vicinity of the tyrosine are not strictly conserved. As outlined by Kauppi et al. [14] the tyrosine environment in mouse R2 is more hydrophobic than that in the *E. coli* enzyme since two Phe and a Met replace a Tyr, Gln and Ser. In the *T. brucei* sequence, Phe80, Phe81 and Thr122 occupy the respective positions. Interestingly, in lower eukaryotes a threonine precedes the catalytic tyrosine whereas in higher organisms this residue is Met.

All residues which participate in the radical transfer to the active site in subunit R1, namely Trp49, Asp85, His119, Asp211, Glu310, and Tyr316 [5,15,16] are conserved. These findings suggest that the mechanism of ribonucleotide reduc-

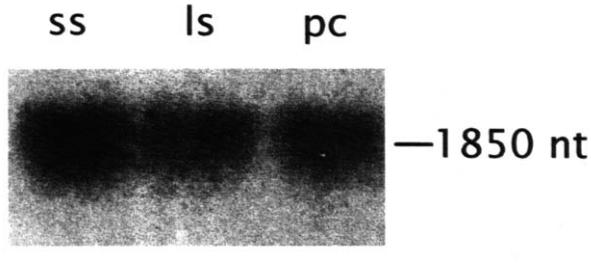


Fig. 2. Expression of *T. brucei* R2 mRNA in different developmental stages. Total RNA of long slender (ls) and short stumpy (ss) bloodstream and procyclic (pc) insect forms of *T. brucei* was purified, separated and blotted as described under Section 2. The membrane was hybridized with the full length $^{32}$P-labelled *nrd B* probe. A single transcript of 1850 nt is recognized. Subsequent stripping and hybridization with a *T. brucei* actin probe confirmed that equal amounts of RNA were present in the three samples. The complete Northern blot covered RNA molecules between $>10000$ and $<100$ bases. Since there was no additional band and the signal to noise ratio for the R2 transcript was $\gg 100:1$ only the relevant section of the blot is depicted.

tion in the trypanosomal enzyme is similar to that of *E. coli* RR. The conclusion is in accordance with the sensitivity of trypanosomes towards hydroxyurea [17–19] although at low concentrations of hydroxyurea inhibition of ribonucleotide reductase seems not to be the primary effect [19]. The sensitivity of ribonucleotide reductases from different species towards radical scavengers varies remarkably and may be due to the accessibility of the iron/radical center [14]. In the *E. coli* enzyme a channel which connects the surface with the proposed oxygen reaction site is shielded by a tyrosine residue. In contrast, nearly all eukaryotic proteins have a serine at this position (Ser183 in *T. brucei* R2).

Interaction between the R1 and R2 proteins is entirely accounted for by the carboxy terminus of the small subunit [15,20]. Synthetic peptides containing the seven C-terminal residues of R2 are selective inhibitors of mammalian [20], viral [21] and probably plasmodial [22] ribonucleotide reductases.

The C-terminal heptapeptide of *T. brucei* R2 differs by only one Ser/Thr exchange from that of the human enzyme which probably excludes selective inhibition of the parasite enzyme. The heptapeptide can now be used for the preparation of an affinity matrix for purifying the R1 subunit from the parasites [20].

### 3.3. Expression of T. brucei nrd B in E. coli

The *nrd B* gene was cloned into the pQE expression vector. The construct encodes the R2 protein with a 34 residues long N-terminal extention containing a $His_6$ sequence. *E. coli* cells were transformed with the plasmid and expression of the gene was induced with IPTG. The recombinant protein was purified on Ni-NTA Sepharose. SDS-PAGE under reducing conditions revealed a single protein band with a $M_r$ of approximately 42 kDa. From a 100 ml culture of recombinant *E. coli* cells 2–5 mg of protein were purified. Difference absorption spectra of native and hydroxy urea-treated R2 purified under non-denaturing conditions revealed the presence of the characteristic tyrosyl radical in about 5% yield [23].

### 3.4. Northern blot analysis

Total RNA from long slender, short stumpy, and procyclic *T. brucei* was separated and probed with the complete *nrd B*. Long slender parasites are the blood stream forms which are capable of division. Short-stumpy forms do not divide and represent a form with a metabolism intermediate between that of the long slender and the procyclic forms that multiply in the tse-tse fly. A single RNA transcript of 1.85 kb was recognized in all three developmental stages of the parasite (Fig. 2) whereby the hybridization signal was 27% higher in the short stumpy forms when compared to long slender and procyclic forms. The mRNA is about 0.8 kb larger than the gene. This is due to 82 nt at the 5′ end which include the spliced leader sequence and a long 3′ untranslated region [24]. The slightly higher mRNA level in the short stumpy forms does not seem to be significant, but it clearly shows that the mRNA is not down-regulated although these cells do not need deoxyribonucleotides for genome replication. Developmental regulation of *T. brucei* ribonucleotide reductase may occur at the post-transcriptional level as it has been suggested for other proteins of this parasite [25]. Alternatively the levels of the R1 and R2 transcripts are not linked as in *Plasmodium falciparum* [22] and R1 may be regulated.

### 3.5. Outlook

Ribonucleotide reductase is an attractive target molecule for anticancer and antimicrobial drug development [21,22]. The enzyme can be inhibited by nucleotide analogs, by reduction of the essential tyrosyl radical and by preventing subunit interaction [7,20,21]. In addition, interferring with the supply of reducing equivalents should strongly influence the activity of ribonucleotide reductase. Since thioredoxin and glutaredoxin could not yet be detected in trypanosomatids ([1]; Krauth-Siegel et al., unpublished results) we will study if the unique dithiol trypanothione is the donor of reducing equivalents for the parasite ribonucleotide reduction. A link between the trypanothione and the deoxyribonucleotide metabolisms would render inhibitors of trypanothione reductase (TR) even more attractive as potential antiparasitic drugs [26] and nicely fit to the finding that TR is essential for parasite development [27,28].

*Note:* Recently the sequences of the R1 and R2 subunits of *T. brucei* ribonucleotide reductase have been published [29]. There are two discrepancies between the deduced protein sequences of the R2 protein. Arg8 and Lys43 (this paper) correspond to Cys8 and Glu43 [29], respectively. Both differences represent single base exchanges.

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