Biochemical and Biophysical Research Communications 451 (2014) 599–602

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Identification of a telomeric DNA-binding protein in *Eimeria tenella*

Na Zhao [a,1], Pengtao Gong [a,1], Zhipeng Li [a], Baiqi Cheng [b], Jianhua Li [a,*], Zhengtao Yang [a], He Li [a], Ju Yang [a], Guocai Zhang [a], Xichen Zhang [a,*]

[a] Key Laboratory of Zoonosis Research, Ministry of Education, College of Veterinary Medicine, Jilin University, 5333 Xi'an Road, Changchun 130062, China
[b] College of Life Science, Jilin University, 2699 Qianjin Street, Changchun 130012, China

ARTICLE INFO

*Article history:*
Received 4 August 2014
Available online 13 August 2014

*Keywords:*
Telomeric DNA-binding protein
EtGbp1p
RRM
*Eimeria tenella*

ABSTRACT

Coccidiosis is considered to be a major problem for the poultry industry, and coccidiosis control is yet urgent. Due to the roles in telomere length regulation and end protection, telomere-binding proteins have been considered as a good target for drug design. In this work, a putative Gbp1p that is similar to telomeric DNA-binding protein Gbp (G-strand binding protein) of *Cryptosporidium parvum*, was searched in the database of *Eimeria tenella*. Sequence analysis indicated *E. tenella* Gbp1p (EtGbp1p) has significant sequence similarity to other eukaryotic Gbps in their RNA recognition motif (RRM) domains. Electrophoretic mobility shift assays (EMSAs) demonstrated recombinant EtGbp1p bound G-rich telomeric DNA, but not C-rich or double-stranded telomeric DNA sequences. Competition and antibody supershift assays confirmed the interaction of DNA–protein complex. Chromatin immunoprecipitation assays confirmed that EtGbp1p interacted with telomeric DNA in vivo. Collectively, these evidences suggest that EtGbp1p represents a G-rich single-stranded telomeric DNA-binding protein in *E. tenella*.



## 1. Introduction

Telomeres consisting of tandemly repeated G-rich sequences are specialized protein–DNA complexes located at the end of linear chromosomes [1]. Proteins that bind to duplex or single-stranded telomere DNA are indispensable components of the telomere complexes and play critical roles in telomere length regulation, end protection and maintenance of specialized telomere chromatin structure [2]. To date, several species of telomeric DNA binding proteins have been identified in parasites. *Leishmania amazonensis* TBP1 (telomere binding protein 1), RPA-1 (replication factor A-1), TRF (TTAGGG repeat-binding factor) and Rbp38 as DNA-binding proteins show interactions with telomeric DNA [3–6]. In *Trypanosoma brucei* (*T. brucei*), TRF and Ku proteins are demonstrated to be an integral component of the telomere complex [7,8]. ST-1, a 39-kDa polypeptide, binds specifically to the trypanosome telomere repeats, and shows a C-strand preference. Another *T. brucei* telomeric protein RAP1 (repressor activator protein 1), which interacts with tbTRF, is essential for silencing variant surface glycoprotein expression sites [9]. Sir2 (silent information regulator 2) and Orc1 (origin-of-recognition-complex 1) were identified as proteins associated with telomere, and colocalized to telomeric and nucleolar regions in *Plasmodium falciparum* [10]. In *Cryptosporidium parvum* (*C. parvum*), Gbp (G-strand binding protein, CpGbp) represents a single-stranded telomeric DNA-binding protein [11].

*Eimeiria tenella* (*E. tenella*) as the most common protozoan pathogen of chickens, can cause coccidiosis, which is considered to be a major problem for the poultry industry [12–14]. Due to the increasing emergence of drug-resistance and the potential reversion to virulence of live or attenuated vaccines in commercial production [15], coccidiosis control is still imminent. The multiple biological functions of telomeres and telomere-associated proteins make these proteins potential drug targets [6]. Previously, the telomere-like sequences (TTTAGGG) in *E. tenella* had been predicted in several reports [16–18], and had been confirmed in our lab (unpublished data). No research on *E. tenella* telomere-associated proteins has been conducted. In the current study, we obtained a CpGbp homologue, *E. tenella* Gbp1p (EtGbp1p). Electrophoretic mobility shift assays and chromatin immunoprecipitation confirmed EtGbp1p is a G-rich single-stranded telomeric DNA-binding protein.

## 2. Materials and methods

### 2.1. Parasites and RNA

*E. tenella* wild strain was isolated from Changchun in China, and was stored in 2.5% potassium dichromate solution at 4 °C. *E. tenella*

* Corresponding authors. Fax: +86 43187981351.
*E-mail addresses:* jianhuali7207@163.com (J. Li), xczhang@jlu.edu.cn (X. Zhang).
[1] These authors contributed equally to this study.

http://dx.doi.org/10.1016/j.bbrc.2014.08.030
0006-291X/

was maintained and propagated in coccidia-free, 2 week-old chickens. Oocysts were purified, and sporulated from feces of chickens 6–10 days after infection as described previously [19]. Sporozoites were prepared from sporulated oocysts according to previously reported method [20]. The experimental procedures were approved by the ethics committee of Jilin University on the use and care of animals. Total RNA was extracted from $2 \times 10^7$ *E. tenella* sporozoites using Trizol™ reagent (Invitrogen, USA), then reverse-transcribed to cDNA using TIANScript RT kit (TIANGEN, China) according to the manufacturer's instructions.

### 2.2. EtGbp1p cloning and sequence analysis

The gene encoding EtGbp1p was amplified from *E. tenella* cDNA using the primers: EtGbp1p-F 5′-GGATCCCTTCCAGCAGATGACGCCAG-3′ (*Bam*H I underlined); EtGbp1p-R 5′-GTCGACTTACATTTCTTTCCGGTCAAAAC-3′ (*Sal* I underlined). The PCR product was inserted into the vector pMD™18-T (Takara Biotechnology, China) and sequenced. Sequence alignments were done with Clustal Omega and DNAMAN 6.0 using default parameters. Bl2seq (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was also used. The molecular mass and isoelectric point were obtained using online ExPASy compute pI/Mw tool (http://web.expasy.org/compute_pi/).

### 2.3. Expression of EtGbp1p in Escherichia coli, protein purification and antibody generation

The gene encoding EtGbp1p was subcloned into the pGEX-4T-1 expression vector and the GST-tagged recombinant protein was expressed in *Escherichia coli* BL21 (DE3) after 5 h of induction with IPTG. The soluble GST-tagged recombinant protein and GST were purified with Glutathione-Triethyleneglycocyl-Sepharose 6B (Beijing CoWin Biotech, China) according to instructions by manufacturers. GST and recombinant GST-EtGbp1p were used to immunize BALB/c mice generating anti-GST and anti-GST-EtGbp1p serums, respectively. Protein G Magnetic beads were used to purify the antiserum according to manufacturer's specification (Thermo scientific, USA).

### 2.4. Electrophoretic mobility shift assay (EMSA)

The digoxigenin (DIG)-labelled probes, Tel-G 5′-$(TTTAGGG)_5$-3′ and Tel-C 5′-$(CCCTAAA)_5$-3′, were synthesized by Sangon Biotech (Shanghai, China). The oligonucleotides, Tel-G and Tel-C, were mixed in equimolar concentrations, degenerated at 95 °C and left to anneal overnight at room temperature to build the double-stranded DNA probe (Tel-ds). The assays were done by mixed 2.5 μg of purified GST-EtGbp1p with 1 pmol of DIG-labeled probes in 20 μl EMSA buffer (10 mM Tris-Hcl [pH 7.5], 50 mM KCl, 1 mM DTT) for 20 min at room temperature. Purified GST protein was subjected to the same procedures to serve as a negative control. For the competition assays, we added 0.2 nmol of unlabeled specific competitor (Tel-G) to the reaction. Supershift assays were done using 50 μg of anti-EtGbp1p serum and 2.5 μg GST-EtGbp1p in the EMSA binding mixture for 5 min on ice prior to the addition of the Tel-G probe. Pre-immune and anti-GST serums were subjected to the same procedures to serve as a negative control. The mixtures were fractionated on an 8% non-denaturing polyarylamide gel (acrylamide:bis-acrylamide, 29:1) and then transferred to a nylon membrane (0.45 μm, GE healthcare, Orsay, France). The nucleotides were cross-linked to membranes by ultraviolet. Subsequently, nucleotides detection was performed using the DIG luminescent detection kit (Roche, Germany) according to manufacturer's protocol.

### 2.5. Chromatin immunoprecipitation (ChIP)

This assay was done as the previously described procedures [21]. DNA was extracted after reversing the cross-linking. DNA samples were slot-blotted and detected with DIG-labelled probes Tel-C 5′-$(CCCTAAA)_5$-3′ and CAG-rich 5′-$(CAGCAG)_5$-3′ using the DIG luminescent detection kit (Roche, Germany) according to manufacturer's protocol. An aliquot corresponding to 1% of the total DNA used in each experiment (input) was tested separately. Control assays were done using mouse pre-immune and anti-GST serums.

## 3. Results and discussion

Telomeres associate with shelterin proteins and form a complex protecting the chromosomes from erosion and end-to-end fusion [22]. Elucidating the relationship between shelterin proteins and telomere maintenance has been the goal of various investigations. CpGbp was identified as a protein that associates with G-rich single-stranded telomeric DNA in *C. parvum* [11]. We therefore initiated our study by searching the *E. tenella* public databases for gene products similar to CpGbp. The search resulted in a putative Gbp1p protein (GenBank accession: CDJ37022), containing two RNA recognition motifs (RRMs). Each of the RRMs contains two RNP submotifs, namely RNP1 and RNP2, which are consistent with CpGbp [11]. In RRM1 (amino acids 31–101), RNPs are located as follows: RNP1 (“KGCGIVVYK”), amino acids 69–77, and RNP2 (“VYVGNL”), amino acids 31–36 (Fig. 1A). In RRM2 (amino acids 163–232), amino acids 201–208 belong to RNP1 (“KGVGTVLF”) and amino acids 163–168 belong to RNP2 (“IFVSNL”) (Fig. 1A). The RRM, known as binding single-stranded DNA, contains two conserved sequence motifs (RNPs) that form the primary nucleic acid-binding surface [23]. The resulting open reading frame (ORF) was predicted to encode a protein of 240 amino acids having a calculated average molecular mass of 25.63 kDa and an average isoelectric point of 7.69. The obtained protein sequence (designated EtGbp1p) shared ~41% identity with CpGbp, according to the bl2seq analysis. Phylogenetic analysis demonstrated EtGbp1p was more closely related to *Babesia bovis* Gbp and CpGbp (data not shown). RRM-containing proteins are involved in telomere maintenance, either through direct binding of the telomeric overhang or an indirect mechanism [23]. Heterogeneous nuclear ribonucleoprotein (hnRNP) has central roles in telomere maintenance as DNA-binding proteins, and transcriptional regulation [24]. Therefore, RRM-containing proteins associated with telomeres could be good targets for regulating telomere length.

Sequence analysis indicated that EtGbp1p shared high sequence similarity with CpGbp, probably because the two species are phylogenetically related. Further studies are required to elucidate the functions of EtGbp1p identified here. EMSAs were done to investigate whether EtGbp1p was able to bind telomeric DNA in vitro. The recombinant protein GST-EtGbp1p was expressed and its purity was verified by SDS-PAGE analysis (Fig. 1B). EMSAs indicated that, purified GST-EtGbp1p protein bound G-rich telomeric DNA (Fig. 2B, lane 3). As a control, binding of GST protein alone to Tel-G could not be detected by EMSA (Fig. 2B, lane 2). In CpGbp, only RRM1 domain has the ability to bind oligonucleotides in vitro [11]. In the case of EtGbp1p, it remains to be determined which part of EtGbp1p contributes to the binding reaction. We also elucidated that EtGbp1p protein didn't bind C-rich telomeric DNA (Fig. 2A) or double-stranded telomeric DNA (Fig. 2C). DIG-labeled Tel-G (1 pmol) was incubated with 0.3125, 0.625, 1.25, and 2.5 μg GST-EtGbp1p, respectively. As illustrated in Fig. 2E, different protein–DNA complexes were formed, suggesting that Tel-G bears several binding sites for EtGbp1p. However, it was difficult to

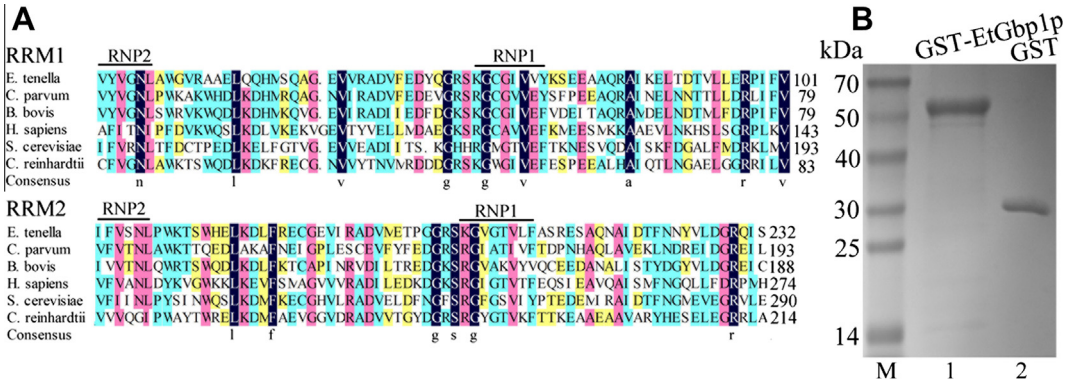


**Fig. 1.** (A) Multiple sequence alignments of RRMs of EtGbp1p with its homologous sequences from *C. parvum* (Gbp, GenBank accession: XP_628165), *Babesia bovis* (*B. bovis*, Gbp, GenBank accession: XP_001610597), *Homo sapiens* (*H. sapiens*, hnRNA-binding protein M4, GenBank accession: AAA36192), *Saccharomyces cerevisiae* (*S. cerevisiae*, Gbp2, GenBank accession: EGA87795), and *Chlamydomonas reinhardtii* (*C. reinhardtii*, Gbp1p, GenBank accession: XP_001690066). The amino acid sequences of RNP1 and RNP2 are highlighted with ledgement. (B) Purified proteins were confirmed by Coomassie Blue staining of SDS-PAGE gel. Molecular weights of each marker protein are shown on the left side of the panel.

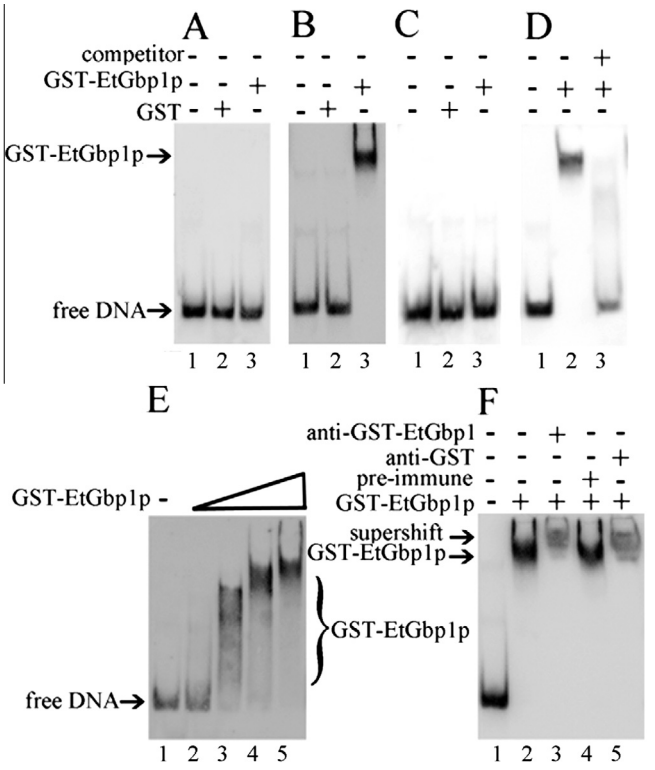


**Fig. 2.** EtGbp1p binds single-stranded telomeric DNA in vitro. (A, B, C) EMSAs were done with purified proteins and DIG-labeled Tel-C, Tel-G, Tel-ds, respectively. Lane 1, no protein. Lane 2, GST used as a negative control. Lane 3, recombinant protein. (D) Competition assay was done in the presence of 0.2 nmol unlabeled Tel-G as specific competitor (lane 3). (E) DIG-labeled Tel-G was incubated with various amounts of GST-EtGbp1p (from 0.3125 to 2.5 μg). Different forms of protein–DNA complexes were indicated (lanes 2–5). (F) Antibody supershift assays done with GST-EtGbp1p and DIG-labeled Tel-G in the presence of anti-GST-EtGbp1p serum (lane 3). Pre-immune serum did not supershift the complex (lane 4), and anti-GST serum also supershifted the complex (lane 5). The free DNA, EtGbp1p, and supershift are indicated.

conclude how many binding sites for EtGbp1p were available at the telomeric DNA in our experiments. The telomere-associated homeobox-containing protein TAH1 shows similar binding pattern with vertebrate telomeric DNA [25]. Competition assays were done to assess the binding specificity of EtGbp1p for G-rich telomeric DNA. Unlabeled Tel-G was used as a specific competitor, and 200-fold excess of competitors relative to DIG-labeled Tel-G was able to totally abolish the EtGbp1p binding (Fig. 2D). Antibody supershift assays confirmed that the DNA–protein complex was formed by the interaction of EtGbp1p with the G-rich telomeric DNA. After addition of anti-GST-EtGbp1p polyclonal serum, the complexes formed with recombinant EtGbp1p showed slow migration profiles in native polyacrylamide gels (Fig. 2F, lane 3). No supershifting was seen in control experiments done with pre-immune serum (Fig. 2F, lane 4), but complexes could also be supershifted in the presence of anti-GST serum (Fig. 2F, lane 5).The results presented above suggest that full length EtGbp1p forms a robust complex with Tel-G.

ChIP assays were used to test the ability of EtGbp1p to bind telomeres in vivo. DNA from *E. tenella* sporozoites was co-immunoprecipitated with anti-GST-EtGbp1p polyclonal serum, anti-GST serum and pre-immune serum, respectively. The immunoprecipitated DNA and 1% input DNA were blotted and hybridized with DIG-labeled Tel-C and CAG-rich (representing a non-telomeric DNA) probes. Anti-GST-EtGbp1p serum immunoprecipitated 0.88% G-rich strand telomeric DNA but did not immunoprecipitate the CTG-rich DNA (Fig. 3). The CAG-rich represents the tandem repeats of the DNA sequence in *E. tenella* [16,18]. Together, immunoprecipitation with anti-GST serum and with pre-immune serum showed no hybridization signals (Fig. 3). ChIP results indicate that EtGbp1p binds telomeric DNA in vivo.

The experiments described here have shown that EtGbp1p is a G-rich strand telomeric DNA-binding protein. G-rich strand-associated proteins are responsible for chromosome end capping and thus, for regulating telomere length and genome stability [26]. The accurate functions of EtGbp1p at *E. tenella* telomeres remain to be determined. Genetic technologies for understanding the function of telomere-associated proteins have been applied. Deletion of Ku demonstrated it is central to telomere length maintenance in trypanosomes [8]. RNAi (RNA interference) knockdown of TbTRF arrested bloodstream cells in G2/M and procyclic cells partly at the S phase caused shortening of G-rich single-stranded telomeric DNA [7]. However, there are no RNAi orthologs or functional studies to date for *E. tenella* [27]. The studies of *E. tenella* are hampered by the difficulty of efficient genetic tools and its complex life cycle. Successful stable transfection of *Eimeria* species constructed by Clark et al. [28], may allow new approaches to functional research of telomere-associated proteins and benefit to develop future vaccination strategies. Undoubtedly, the identification of EtGbp1p as a G-rich single-stranded telomeric DNA-binding protein opens exciting avenues for future research.

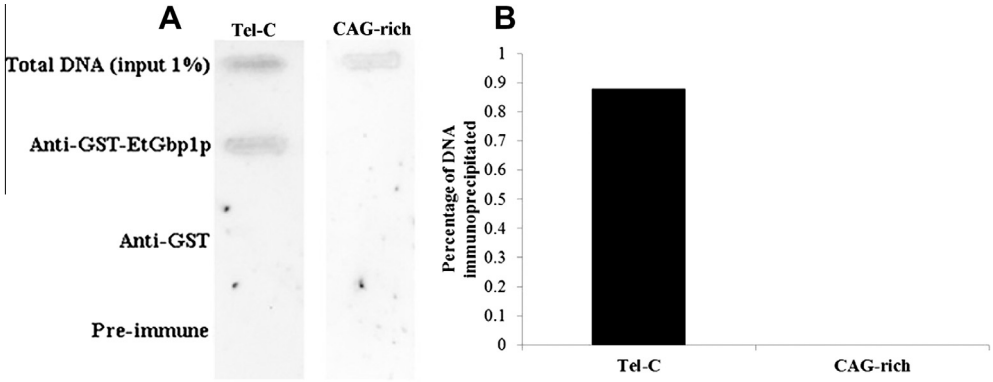


**Fig. 3.** (A) Chromatin immunoprecipitation of *E. tenella* sporozoites using anti-GST-EtGbp1p serum. Control experiments were done in the presence of anti-GST and pre-immune serums. One percent of the total DNA (input) and immunoprecipitated samples were slot-blotted and hybridized with the corresponding probes. (B) Average percentages of the DNA immunoprecipitated relative to the initial input DNA. The results indicate the average ± SEM of three independent experiments.

## Acknowledgment

This work was supported by Grants (nos. 31272550 and 30970322) from the National Natural Science Foundation (NSFC) of China.

## References

[1] E.H. Blackburn, Telomeres and telomerase: their mechanisms of action and the effects of altering their functions, FEBS Lett. 579 (2005) 859–862.
[2] X. Yang, L.M. Figueiredo, A. Espinal, et al., RAP1 is essential for silencing telomeric variant surface glycoprotein genes in *Trypanosoma brucei*, Cell 137 (2009) 99–109.
[3] C.B. Lira, J.L. de Siqueira Neto, L. Khater, et al., LaTBP1: a *Leishmania amazonensis* DNA-binding protein that associates in vivo with telomeres and GT-rich DNA using a Myb-like domain, Arch. Biochem. Biophys. 465 (2007) 399–409.
[4] J.L. Neto, C.B. Lira, M.A. Giardini, et al., *Leishmania* replication protein A-1 binds in vivo single-stranded telomeric DNA, Biochem. Biophys. Res. Commun. 358 (2007) 417–423.
[5] M.S. da Silva, A.M. Perez, C. da Silveira Rde, et al., The *Leishmania amazonensis* TRF (TTAGGG repeat-binding factor) homologue binds and co-localizes with telomeres, BMC Microbiol. 10 (2010) 136.
[6] C.B. Lira, J.L. Siqueira Neto, M.A. Giardini, et al., LaRbp38: a *Leishmania amazonensis* protein that binds nuclear and kinetoplast DNAs, Biochem. Biophys. Res. Commun. 358 (2007) 854–860.
[7] B. Li, A. Espinal, G.A. Cross, Trypanosome telomeres are protected by a homologue of mammalian TRF2, Mol. Cell. Biol. 25 (2005) 5011–5021.
[8] C. Conway, R. McCulloch, M.L. Ginger, et al., Ku is important for telomere maintenance, but not for differential expression of telomeric VSG genes, in African trypanosomes, J. Biol. Chem. 277 (2002) 21269–21277.
[9] J.E. Eid, B. Sollner-Webb, ST-1, a 39-kilodalton protein in *Trypanosoma brucei*, exhibits a dual affinity for the duplex form of the 29-base-pair subtelomeric repeat and its C-rich strand, Mol. Cell. Biol. 15 (1995) 389–397.
[10] L. Mancio-Silva, A.P. Rojas-Meza, M. Vargas, et al., Differential association of Orc1 and Sir2 proteins to telomeric domains in *Plasmodium falciparum*, J. Cell Sci. 121 (2008) 2046–2053.
[11] C. Liu, L. Wang, C.A. Lancto, et al., Characterization of a *Cryptosporidium parvum* protein that binds single-stranded G-strand telomeric DNA, Mol. Biochem. Parasitol. 165 (2009) 132–141.
[12] E. Tabarés, D. Ferguson, J. Clark, et al., *Eimeria tenella* sporozoites and merozoites differentially express glycosylphosphatidylinositol-anchored variant surface proteins, Mol. Biochem. Parasitol. 135 (2004) 123–132.
[13] H. Sun, L. Wang, T. Wang, et al., Display of *Eimeria tenella* EtMic2 protein on the surface of *Saccharomyces cerevisiae* as a potential oral vaccine against chicken coccidiosis, Vaccine 32 (2014) 1869–1876.
[14] Geriletu, L. Xu, Xurihua, X. Li, Vaccination of chickens with DNA vaccine expressing *Eimeria tenella* MZ5-7 against coccidiosis, Vet. Parasitol. 177 (2011) 6–12.
[15] W.C. Li, X.K. Zhang, L. Du, et al., *Eimeria maxima*: efficacy of recombinant *Mycobacterium bovis* BCG expressing apical membrane antigen1 against homologous infection, Parasitol. Res. 112 (2013) 3825–3833.
[16] M.W. Shirley, The genome of *Eimeria tenella*: further studies on its molecular organization, Parasitol. Res. 80 (1994) 366–373.
[17] M.W. Shirley, The genome of *Eimeria* spp., with special reference to *Eimeria tenella*––a coccidium from the chicken, Int. J. Parasitol. 30 (2000) 485–493.
[18] K.H. Ling, M.A. Rajandream, P. Rivailler, et al., Sequencing and analysis of chromosome 1 of *Eimeria tenella* reveals a unique segmental organization, Genome Res. 17 (2007) 311–319.
[19] P.L. Long, B.J. Millard, L.P. Joyner, et al., A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis, Folia Vet. Lat. 6 (1976) 201–217.
[20] F. Tomley, Techniques for isolation and characterization of apical organelles from *Eimeria tenella* sporozoites, Methods 13 (1997) 171–176.
[21] D. Kappei, F. Butter, C. Benda, et al., HOT1 is a mammalian direct telomere repeat-binding protein contributing to telomerase recruitment, EMBO J. 32 (2013) 1681–1701.
[22] H. Oeseburg, R.A. de Boer, W.H. van Gilst, et al., Telomere biology in healthy aging and disease, Pflugers Arch. 459 (2010) 259–268.
[23] T.H. Dickey, S.E. Altschuler, D.S. Wuttke, Single-stranded DNA-binding proteins: multiple domains for multiple functions, Structure 21 (2013) 1074–1084.
[24] E. Tanaka, H. Fukuda, K. Nakashima, et al., HnRNP A3 binds to and protects mammalian telomeric repeats in vitro, Biochem. Biophys. Res. Commun. 358 (2007) 608–614.
[25] X. Feng, Z. Luo, S. Jiang, et al., The telomere-associated homeobox-containing protein TAH1/HMBOX1 participates in telomere maintenance in ALT cells, J. Cell Sci. 126 (2013) 3982–3989.
[26] M.F. Fernández, R.R. Castellari, F.F. Conte, et al., Identification of three proteins that associate in vitro with the *Leishmania* (*Leishmania*) *amazonensis* G-rich telomeric strand, Eur. J. Biochem. 271 (2004) 3050–3063.
[27] K.T. Militello, P. Refour, C.A. Comeaux, et al., Antisense RNA and RNAi in protozoan parasites: working hard or hardly working?, Mol Biochem. Parasitol. 157 (2008) 117–126.
[28] J.D. Clark, K. Billington, J.M. Bumstead, et al., A toolbox facilitating stable transfection of *Eimeria* species, Mol. Biochem. Parasitol. 162 (2008) 77–86.