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Review

The transcription machinery and the molecular toolbox to control gene expression in *Toxoplasma gondii* and other protozoan parasites

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Abstract

The phylum of *Apicomplexa* groups a large variety of obligate intracellular protozoan parasites that exhibit complicated life cycles, involving transmission and differentiation within and between different hosts. Little is known about the level of regulation and the nature of the factors controlling gene expression throughout their life stages. Unravelling the mechanisms that govern gene regulation is critical for the development of adequate tools to manipulate these parasites and modulate gene expression, in order to study their function in molecular terms in vivo. A comparative analysis of the transcriptional machinery of several apicomplexan genomes and other protozoan parasites has revealed the existence of a primitive eukaryotic transcription apparatus consisting only of a subset of the general transcription factors found in higher eukaryotes. These findings have some direct implications on development of tools.

Keywords: Protozoan parasites; *Apicomplexa*; *Toxoplasma gondii*; *Plasmodium falciparum*; Transcription; RNA-interference; Inducible system

1. Introduction

Control and fine-tuning modulation of gene expression is employed by all organisms to adapt to the changes imposed by the environment. One of the key regulatory steps chosen by most organisms to control gene expression is the transcriptional level. This involves the interplay of numerous trans-acting factors and a combinatorial array of *cis*-acting regulatory DNA elements, in order to efficiently recruit the RNA polymerase to the right promoter at the right time. However, regulation of gene expression may also be critically controlled at the level of chromatin structure, during transcriptional elongation, post-transcriptonally at the level of RNA processing, transport or stability and eventually during translation or at the level of protein stability. So far the very detailed analysis on eukaryotic gene regulation has been concentrated mainly on animals, plants and fungi, while knowledge on transcriptional regulation in protists, including protozoan parasites, is still fragmentary. Most studies have focused on kinetoplastida and led to the discovery of novel and exotic mechanisms including eukaryotic polycistronic transcription, polymerase I expression of protein coding genes [1], *trans*-splicing and RNA-editing [2]. Until recently, considerably less was known about the control of gene expression in apicomplexans and in amitochondirates; however, the development of DNA transfection techniques and the associated tools have now boosted functional analyses in these microbial eukaryotes. The sequencing of the genomes for *Toxoplasma gondii*, several *Plasmodium* species, *Thrichomonas vaginalis*, *Giardia lamblia* and *Entamoeba histolytica* and other protists offers a unique opportunity to undertake comparative genomics of the genes involved in regulation of gene expression.

The phylum of *Apicomplexa* includes several thousand obligate intracellular protozoan parasites sharing multiple structural and functional features. The most notorious member is *Plasmodium falciparum*, causative agent of malaria, while *T. gondii* is among the most successful parasites, with nearly half of the human population chronically infected. This opportunistic parasite represents a serious cause of neurological birth defects and frequently causes a fatal cerebral toxoplasmosis in association with a variety of immunosuppressive diseases and treatments. Humans and animals become infected mainly via the ingestion of tissue cysts present in contaminated meat or by oocysts released in the feces of cats [3]. Sporozoites and bradyzoites enter the asexual cycle by

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differentiating into rapidly dividing tachyzoites that disseminate throughout the intermediate host. Upon pressure imposed by the host immune system, the parasites switch into bradyzoites and establish a life-long chronic infection. The stage conversion is of biological and clinical significance because the bradyzoite cysts are poorly susceptible to chemotherapy, and considered the source of reactivation causing fatal toxoplasmosis in immunocompromised patients. A more global view of the stage-specificity of gene expression is progressively accessible through the generation of several stage-specific expressed sequence tag (EST) databases [2] and the recent use of microarrays [4] and proteomics technologies [5]. Despite large gaps, the knowledge of the mechanisms and machinery implicated in gene regulation and life-stage differentiation in *T. gondii* and *P. falciparum* clearly emerges and contributes to the development of novel tools necessary to regulate gene expression and study their function.

2. Genome, chromatin structure and remodelling factors

The haploid genome of *T. gondii* consists of ~ 8 10^7 bp. The size of the 11 chromosomes varies between 2 and 8 Mb [6]. A 10X random shotgun genomic sequencing of the parasite genome is available as well as the clustered ESTs assemblies from multiple strains representing all stages of the parasite life cycle on http://toxodb.org/restricted/toxoDBblast.shtml. Most protein-encoding genes are single copy genes. Exceptions are genes encoding some rhoptry proteins, genes encoding nucleotide triphosphatehydrolase (NTPase) [7,8] or the B1-gene, which can be found in 35 copies [9]. Ribosomal genes are present in 110 copies, organised in tandem and separated by non-transcribed sequences. This organisation and conserved regulatory motifs can be found in most eukaryotic organisms and suggest a similar, conserved mechanism of rRNA-transcription [10]. The largest subunit of RNA polymerase I has been characterised in *P. falciparum* [11]; however, factors involved in regulation of RNA polymerase I transcription have not yet been described in apicomplexans.

Although not much is known about chromosomes and telomere organisation in *T. gondii*, it is likely that nuclear architecture of *T. gondii* is similar to *P. falciparum*, where a nucleosomal organisation typical for eukaryotes has been described (for review see [12]). The genes coding for the histone core proteins H2A, H2B, H3 and H4 are present in *T. gondii* genome and have been characterised in *P. falciparum* and shown to share a high degree of homology with histones of other eukaryotes [13–17]. Intriguingly, no homologue to the linker histone H1 can be identified in the database of *T. gondii* or *Plasmodium* species. The organisation of chromatin is known to have a major impact on many nuclear processes and gene expression is highly influenced by the position and density of nucleosomes at promoter elements (for recent review see [18,19]). As shown in other systems, the repressive state of nucleosomes can be altered by modifications of histones by methylation, acetylation or phosphorylation, which modulates the interaction potential with the DNA and thus the accessibility and activity of promoters. Several components of the chromatin remodelling pathways have recently been described in apicomplexans and include a histone deacetylase (PfHDAC1) [20], a homologue of the Snf-2 family (SNF2L), which belongs to the Swi/Snf complex of ATP-dependent nucleosome remodelling factors in *P. falciparum* [21] and a SRCAP (Snf2-related CBP activator protein) homologue in *T. gondii*, *P. falciparum* and *C. parvum*. In other eukaryotes SRCAP I responds to elevated cAMP levels, by interacting with cAMP response element binding protein (CREB)-binding protein (CBP), thereby enhancing CBP mediated transcription. However, CBP- or CREB-like proteins have not been identified in apicomplexans, suggesting that SRCAP is recruited differently to the promoter [22]. Furthermore a homologue to yeast histone acetylase, GCN5, has been characterised in *T. gondii* [23,24] and recently shown to be transported to the parasite nucleus by interaction with importin-alpha [25]. This essential histone acetylase is capable of partially complementing a yeast GCN5-mutant. Yeast GCN5 is a subunit of two high molecular mass histone acetylase complexes that are recruited to the promoter by interaction with transcription factors (for a review see [26]). Interestingly drugs that inhibit histone deacetylases, such as apicidin, show a strong antiprotozoal activity, suggesting that a fine-tuning between histone acetylation and deacetylation is important for the survival of the parasite [27,28]. In this context, recent studies have revealed several hints that chromatin remodelling plays a role in regulation of antigenic variation in *P. falciparum*. The *var* genes are mainly located close to the telomeres that are organised in a non-nucleosomal structure at the chromosome end. Some *var* genes are also present further upstream at the telomere-associated sequence (TAS) together with other gene families involved in antigenic variation, including the *rifin* genes (for a review see) [29]. Telomeres were shown to cluster at the nuclear periphery [30] and this arrangement appears to enable frequent ectopic recombination, which gives rise to a new *var* gene and therefore increases the repertoire of antigenic variants [31]. Expression of *var* genes is mutually exclusive, due to a cooperative silencing mechanism, which is controlled by two *cis*-acting elements. Whereas one element can be found upstream of the *var* promoter, another element is localised in the intron of each *var* gene. In an elegant study, it was further demonstrated that for effective silencing of the *var* promoter, the DNA has to be associated with chromatin, since effective silencing can only be achieved if the cell progresses through the S-phase [32]. A detailed characterisation of the conserved regions within the introns of the *var* genes revealed that the intron can act as functional promoter, which is only active if the *var* promoter is silenced, resulting in the expression of a sterile transcript [33]. This is in good agreement with the identification of a nuclear factor, exclusively expressed in the S-phase, which binds to a conserved region

within the *var* gene promoter [34]. Recently two reports demonstrated the role of alterations in the structure and subnuclear localisation of chromatin, in connection with the binding of the telomere-associated protein PfSir2 (silent information regulator), in *var* gene silencing. Whereas in yeast Sir2 is associated with a rather short subtelomeric region of up to 3 kb, in *P. falciparum* PfSir2 spreads over more then 55 kb, as far as the first subtelomeric genes, whereas acetylated histones are excluded from the chromosome ends [35]. Disruption of PfSir2 resulted in derepression of a subset of subtelomeric *var* genes. Furthermore it was demonstrated using FISH analysis that silenced and active loci occupy different positions within the nucleus and that repositioning occurs upon activation of a gene [36].

Although the phenomenon of antigenic variation has not been described in *T. gondii* it is likely that the importance of epigenetic control of gene expression established for *Plasmodium* will also hold true for other apicomplexan parasites.

3. Transcription and gene regulation

3.1. Promoter structure

In higher eukaryotes, the promoters of protein coding genes consist of different sets of *cis*-acting elements, where gene-specific enhancer elements are combined with basal or core promoter elements, like TATA-box, Initiator (Inr) element or downstream promoter elements (DPE) (for review see [37]). In *T. gondii*, only a few detailed promoter studies have been undertaken and little is known about *cis*-acting elements. The classical eukaryotic elements such as TATA-box, CAAT-box or SP1-motifs are not conserved. Instead, the nucleoside triphosphate hydrolase appears to contain an Inr-element localised at the transcription start site [38] and the promoter of the *SAG1* gene coding for the major surface antigen possesses a positioning *cis*-acting element composed of six tandemly repeated 27-bp repeats directing the initiation of transcription [39]. The central heptamer motif (TGAGAGC) contained within this 27-bp repeat is also found on other promoters [40–42]. This element functions both as enhancer and selector of transcription initiation, since the activity of heterologous promoters can be significantly stimulated when placed under control of the *SAG1* repeats [39]. In a recent study an extensive promoter characterisation of two stage-specifically expressed genes, Enolase 1 and Enolase 2 was carried out, resulting in the identification of stress response elements (STRE), heat shock elements (HSE) and other *cis*-acting elements that are critical for stage-specific expression of these isoenzymes. Furthermore a DNA-binding activity has been characterised that bound to these elements. This is in good agreement with the fact that stage differentiation of *T. gondii* can be induced in vitro using different stress conditions [43].

In *Plasmodium*, little is known at the level of *cis*- and *trans*-regulatory elements. A global analysis of the genome combined with transient transfection experiments recently led to the identification of the so-called dual, palindromic G-box [44]. This analysis again confirmed the absence of typical eukaryotic promoter elements in apicomplexans. Bioinformatic searches also revealed poor homologies with conserved transcription factors from higher eukaryotes and only one transcriptional factor, a Myb related protein, has been recently described and characterised in *P. falciparum* [45,46].

3.2. The basic machinery of transcription in Apicomplexa

Transcription of DNA in eukaryotes is carried out by three distinct multi-enzyme complexes, as was first demonstrated by their different elution from ion-exchange columns and their different sensitivity to α-amanitin [47]. Since then this different sensitivity emerged as one of the main characteristics distinguishing between RNA polymerase I–III. RNA polymerase I (not sensitive) transcribes ribosomal RNAs, RNA polymerase III (moderately sensitive) small RNAs, like tRNAs and RNA polymerase II (highly sensitive) is responsible for the transcription of most protein-encoding genes. Recently a fourth RNA polymerase has been discovered in plants, that is capable of transcribing methylated (silenced) DNA and therefore generating transcripts that are required to maintain RNAi mediated silencing (for a review see [48]).

However, although many components of the transcription machinery appear to be present in protozoa, many exceptions to general rules have been described. For example the transcription of protein-encoding genes is insensitive to α-amanitin, although transcribed by RNA polymerase II in amitochondriate protozoa (for a review see [49]). In kinetoplastida the expression of protein-encoding genes is insensitive to α-amanitin and mediated by RNA polymerase I, producing a pre-mRNA that is subsequently capped by *trans*-splicing [1].

Recently it was demonstrated in *P. falciparum* that transcription of protein-encoding genes and of antisense RNA is sensitive to α-Amanitin [50]. All three RNA polymerases are present in the genomes of apicomplexans and the large subunit of RNA polymerase I [11], RNA polymerase II [51,52] and RNA polymerase III [53] have been described in *Plasmodium* species. The carboxy-terminal domain (CTD), a characteristic feature of the largest subunit of RNA polymerase II (RPB1) has a number of transcriptional and post-transcriptional functions regulating transcription efficiency, splicing, 3′-end cleavage and polyadenylation [54]. The heptapeptide repeats of the CTD becomes highly phosphorylated during initiation and elongation of transcription [55,56]. Phosphorylation of the CTD is a major control of transcriptional elongation and several kinases are involved in this regulatory step (for a review see [57]). The sequence of the repeats is conserved in eukaryotes (consensus YSPTSPS) with the number of repeats varying from species to species but they are less conserved or even absent in most protists [58]. Despite the lack of the typical heptapeptide repeats it was demonstrated in *Trypanosoma brucei* that the CTD becomes heavily

Table 1
Genome features and genetic "toolkit" to control gene expression in parasites

	T. gondii	*P. falciparum*	*L. major*	*T. brucei*	*G. lamblia*	*E. histolytica*	*E. cuniculi*
Haploid genome size (Mb)	80	30	35	35	12	20	2.9
Number of chromosomes	14	14	36	11 + minichr.	nd, Ploidy	4, Ploidy	11
Genome plasticity	n.d.	chromosome breakage	circular DNA amplification	duplicative transposition telomere exchange	Chromosomal rearrangements of rRNA telomere domains	n.d.	Evidences for chromosomal rearrangements of rRNA telomere domains
Transcription of CDSs	monocistronic	monocistronic	polycistronic	polycistronic	Monocistronic (?)	Monocistronic (?)	Monocistronic clusters of 2 CDSs
RNA splicing	Yes, alternative splicing	Yes, alternative splicing	Yes, *trans*- and alternative splicing	Yes, *trans*- and alternative splicing	Yes, only few introns	Yes, only few introns	Yes, only few introns
Selectable markers	positive/negative	positive/negative	positive/negative	positive/negative	positive	positive	–
Stable transfection	integration	episomal/integration	episomal/integration	episomal/integration	episomal/integration	episomal	–
Homologous recombination	yes	Yes	yes	yes	no	no	no
Mutagenesis	random insertion	No	Transposons	Transposon	no	no	no
RNAi/RNA antisense	+	+/?	–	+++	+	+	–
Inducible system	TetR + tetTA	tetTA	TetR	TetR	TetR	TetR	no

phosphorylated during transcription [59]. Giesecke and colleagues have reported that non-canonical heptamer repeats are present in the *P. berghei* and *P. falciparum* CTD [52] and thus it is plausible that phosphorylation of the CTD contributes to transcriptional elongation in apicomplexans as well. One key factor involved in the phosphorylation of the CTD is the general transcription factor TFIIH, which consists of nine subunits including DNA-helicase and cyclin-dependent kinase (CDK) [57]. Putative homologues to six of the nine subunits (including CDK7 and DNA-helicase XPD) have been identified in *T. gondii* genome database (Table 1).

In eukaryotes, the transcription apparatus is a multilayered ensemble of various regulatory multi-subunit complexes that, in addition to histone modification and chromatin remodelling, includes also co-activators that tightly control transcription initiation. The composition of the core promoter directs the formation of the pre-initiation complex close to the transcription start site. This involves the interplay of general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH) with the RNA polymerase (for a review see [60]). For many transactivators including the viral protein 16 (VP16) of the *Herpes simplex* virus, multiple interactions with components of the transcription machinery have been described, such as TFIIA, TFIIB, TFIID or TFIIH [61]. Additionally, it was demonstrated that an interaction between VP16 and the TATA-binding protein (TBP) associated factor 31 (hTAF31 = ScTAF17, Table 2) is essential for activation in vitro [62]. Employing the activation domain of VP16 it was possible to establish tetracycline inducible systems in a great variety of eukaryotes from yeast to man [63]. In contrast, no detectable transactivation has been monitored using the activation domain of VP16 in the protozoan parasites *T. gondii*, *P. falciparum*, *E. histolytica* and *T. brucei*.

Recently two functional but artificial transactivating domains have been generated in *T. gondii* using a genetic approach by insertion [64]. Further characterisation of these transactivators revealed that they do not function in HeLa cells but can activate transcription in *P. falciparum* [65]. This restricted range of activity suggests that considerable differences exist between the core transcription machinery of these protozoa and other eukaryotes. Confirming this hypothesis, factors like TFIIA, TFIIE-beta, TFIIF and most of the TBP-associated factors (TAFs) are not found in the sequence databases of apicomplexan genomes (Table 1). Especially the absence of most conserved TAFs and other GTFs like TFIIA gives a plausible explanation for the failure of VP16 to activate transcription in protozoan parasites.

Interestingly, the survey of protozoan parasite genomes revealed that the setup of their basal transcription machinery resemble a more rudimentary RNA polymerase system, where many factors conserved in higher eukaryotes are absent (Table 1). Protozoan parasites might have maintained more simplistic transcription machinery than one evolved in higher eukaryotes or lost some components during the course of evolution. For example TFIIA-alpha can be identified in the case of *C. parvum* (accession number EAK87567), whereas no homologue can be identified in case of *Plasmodium*, *T. gondii* or other protozoa. Extensive loss of otherwise conserved

Table 2
Repertoire of the general transcription factors in protozoan parasites

Factor	*S. cerevisiae*	*A. thaliana*	*E. cuniculi*	*E. histolytica*	*T. gondii*	*Plasmodium*	*Kinetoplastida*	*G. lamblia*	*Archaea*	Features (in *S. cerevisiae*)
RNA polymerase II										
RPB1	NP_010141	NP_195305	NP_597540	EAL46395	TgTwinScan_5341	NP_473294	P17545	EAA37804	AF1888	Heptapeptide
RPB2	NP_014794	NP_193902	NP_586140	EAL48102	TgTigrScan_6297	AAC71932	CAB95348	EAA38052	AF1888	Shared by Pol I, II, III
RPB3	NP_012243	NP_179142	NP_585816	EAL46103	TgTwinScan_6000	NP_704769	Tb03.5L5.710	EAA37522	AF2282	Shared by Pol I, II, III
RPB4	NP_012395	NP_196554	NP_597206	–	TgTwinScan_5788	NP_472977	–	–	–	Shared by Pol I, II, III
RPB5	NP_009712	NP_188871	NP_586024	EAL45380	TgGlmHMM_3551	NP_705531	AAL96366	EAA36610	AF1885	Shared by Pol I, II, III
RPB6	NP_015513	NP_200007	NP_597161	EAL49133	TgTwinScan_7337	NP_473164	n.a.	EAA39578	AF1131	Shared by Pol I, II, III
RPB7	P34087	NP_200726	NP_586102	EAL50138	TgTwinScan_7385	EAA17978	n.a.	EAA41681 ?	AF1117	
RPB8	NP_014867	NP_188323	–	EAL51470	TgTwinScan_5960	NP_701498	n.a.	EAA40065	–	
RPB9	NP_011445	NP_567490	NP_586446	EAL43704	TgTwinScan_3720	NP_703304	n.a.	EAA40003 ?	AF1235	
RPB10	NP_014853	NP_176363	NP_585788	EAL48839	TgTigrScan_5510	NP_704025	CAB96746	EAA36688	AF1130	
RPB11	NP_014638	NP_190777	NP_597367	EAL45982	TgTwinScan_5077	NP_704947	n.a.	EAA42291	AF0207	
RPB12	AAB68994	NP_198917	–		TgTwinScan_2987	NP_705320	–	–	AF0056	
TFIIA										
TFIIA-alpha	P32774	NP_849434	NP_584572	–	–	–	–	–	–	
TFIIA-beta	P32773	NP_563790	NP_597616	–	–	–	–	–	–	
TFIID										Histone acetyltransferase
TAF150	NP_009971	NP_177536	NP_596932	EAL44144	–	–	–	–	–	Cell cycle progression
TAF130	S50237	NP_188534	NP_586191	EAL51916	TgTigrScan_2065?	AAN36415?	–	–	–	Similar to histone H2B
TAF90	P38129	NP_197897	NP_586481	EAL49492	–	–	–	–	–	Similar to histone H4
TAF67	NP_013954	–	NP_597394	–	TgTwinScan_4518	NP_704828.1	–	–	–	Similar to histone H3
TAF61	NP_010429	NP_564023	NP_586208	–	–	–	–	–	–	TATA-binding protein
TAF60	S64120	NP_849592	NP_586227	EAL45013	TgTwinScan_0507?	–	–	–	–	
TAF47*	NP_015314	–	–	–	–	–	–	–	–	
TAF40	Q04226	NP_193761	NP_584650	–	–	–	–	–	–	
TAF30*	S38568	–	NP_597668 ?	–	–	–	–	–	–	
TAF25	NP_010451	NP_194900	NP_586389	EAL48552	–	–	–	–	–	
TAF19	NP_013611	NP_171768	NP_584778	–	–	–	–	–	–	
TAF17	NP_013963	NP_175816	NP_586747	–	–	–	–	–	–	
TBP	NP_011075	NP_187953	NP_584829	S52407	AAR84226	NP_703407	AAO17360	EAA38645	AF0373	
TFIIB	S26707	NP_187644	NP_585866	EAL45712	TgTwinScan_0303	CAD49065	n.a.	EAA40027	AF1299	Transcription start site selection
TFIIE										
TFA1	NP_012897	AAM20302	NP_585950	EAL51445	TgTwinScan_5763	NP_704106	n.a.	–	AF0757	
TFA2	NP_012988	NP_193766	NP_585791	EAL51158	–	–	–	–	–	
TFIIF										
TFG1	P41895	NP_192998	–	–	–	–	–	–	–	
TFG2	P41896	NP_177683	NP_584604	–	–	–	–	–	–	
TFG3	S38568	NP_179391?	NP_597668	EAL50025?	TgTwinScan_1311?	NP_704482?	–	–	–	
TFIIH										
TFB1	NP_010597	NP_191701	–	–	–	–	–	–	–	Nucleotide excision repair
TFB2	NP_015203	NP_193435	NP_586678	EH01497	n.a.	NP_701785	n.a.	–	–	Nucleotide excision repair
TFB3	NP_010748	–	NP_586328	–	–	–	–	–	–	Nucleotide excision repair
TFB4	NP_015381	NP_564050	NP_586016	EH02001	–	EAA16554	–	EAA41416	–	DNA-helicase/ATPase
RAD3	AAB64698	NP_171818	NP_585776	EH03964	AA012000 (E)	NP_704873	n.a.	EAA38649	NP_127020	Nucleotide excision repair
SSL1	NP_013105	NP_683275	NP_584711	(contig5720)	CB023388	NP_705057	n.a.	EAA41267	AF0358	DNA-helicase
SSL2/RAD25	Q00578	NP_568591	NP_586718	EH05715	CB239159	EAA15889	n.a.	EAA41218	NP_069194	Cyclin dependent CTD-kinase
KIN28	NP_010175	NP_173244?	NP_584670	+?	+?	+?	+?	+?	–	Cyclin C component
CCL1	NP_015350	NP_198114?	+?	+?	+?	+?	+?	+?	–	
SAGA										Histone acetylase
GCN5	NP_011768	NP_567002	NP_586258	EH04598	AAF29981	NP_704321	n.a. ?	EAA38438	–	
ADA1*	Q12060	–	–	–	–	–	–	–	–	
ADA2	NP_010736	NP_566317	NP_584706	Contig6021	n.a.	NP_700617	–	EAA40074	–	
ADA3*	P32494	–	–	–	–	–	–	–	–	

List of genes coding for putative proteins known to be part of the transcription machinery of eukaryotes as identified by homology search using the NCBI-Blast, ToxoDB and PlasmoDB. Protozoan organisms appear to have less highly conserved general transcription factors. Accession numbers are indicated. n.a. = not annotated, but identified with high p-value in ToxoDB or in the *Trypanosoma* database (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/t_brucei/omni). A question mark indicates hits of proteins that although showing a high *P*-value, might not be part of the transcription machinery.

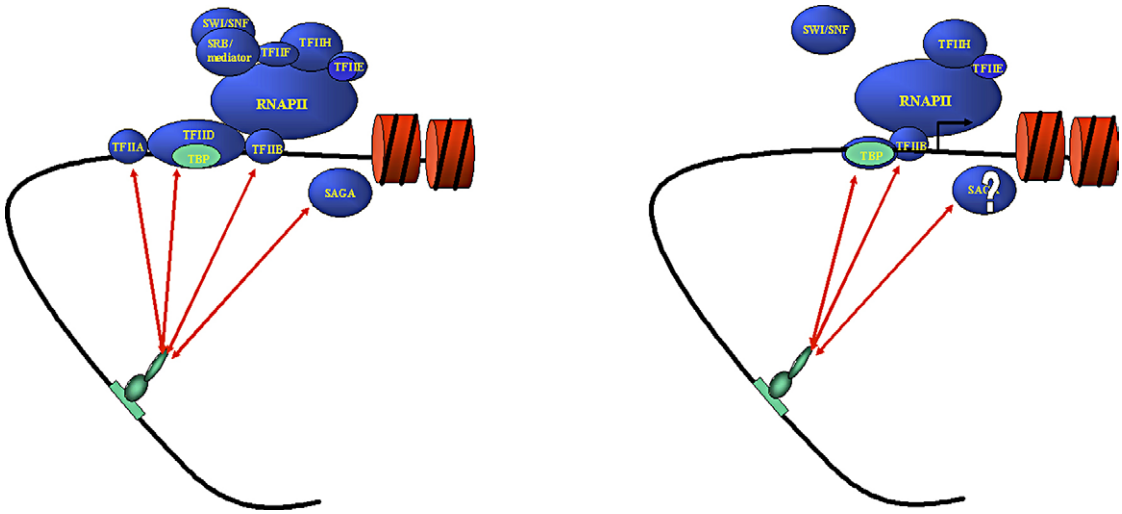

Fig. 1. Schematic promoter in operation in fungi/plants/animals compared to apicomplexans.
The general transcription machinery of higher eukaryotes (left) consists of the core RNA polymerase complex and a set of GTFs. In contrast apicomplexans and other protozoans (right) appear to have only a basic set of GTFs.

genes was indeed recently demonstrated in a comparative analysis of the apicomplexan and eukaryotic genome diversity [66]. Alternatively, some homologous genes might fail to be identified by the search algorithms due to the weak sequence conservation with the higher eukaryotic counterparts. Biochemical and genetic screening approaches will be necessary to identify and complete the panoply of the apicomplexan transcription machinery (Fig. 1).

4. Post-transcriptional control of gene expression

In some organisms regulation of protein expression occurs preferentially at the post-transcriptional level, via alternative splicing, RNA-stability, RNA-interference (RNAi), translation and post-translational events. In kinetoplastida, post-transcriptional control appears to be the major mechanism regulating gene expression probably as consequence of the polycistronic mode of transcription (reviewed in [67]). In *T. gondii*, examples of post-transcriptional regulation has been studied in detail for two bradyzoite specific genes *BSR4* and *LDH1* [68,69]. In *Plasmodium* like in other organisms, the regulation of dihydrofolate reductase (DHFR) involves the binding of DHFR-protein to its own mRNA blocking translation. But in sharp contrast to human DHFR, which binds to the mRNA only in the absence of substrate, the *P. falciparum* enzyme binds to the mRNA also in the presence of natural substrate or the inhibitor pyrimethamine, amplifying the effect of the drug [70].

An increasing number of studies report that many genes in *P. falciparum* are actively transcribed at some stages but not translated into proteins such as the member of the EBA-175 family [71] and the 235 kDa family of rhoptry proteins [72]. Most recently, the application of serial analysis of gene expression (SAGE) to *P. falciparum* has demonstrated that antisense transcription is widespread in the parasite [73]. However, it is not clear if the abundance of antisense transcripts is intentional, leading to regulated overlap between transcripts or simply due to a leakage of the transcription machinery. Recently it has been speculated that two genes that show a sense/antisense relationship are stably linked on the chromosome throughout evolution, since separation would affect the function of both genes [74].

Finally, *P. falciparum* possesses a family of RNA-binding protein (Puf) factors that can recognise specific mRNA targets and repress their translation [75].

5. Experimental approaches and tools developed to regulate gene expression

In order to study the function of essential genes in an organism, tools need to be developed to ectopically and selectively control gene expression while avoiding pleiotropic effects. An overview of the genetic characteristics of protozoan parasites and the tools recently developed to modulate gene expression is presented in Table 2. One of the widely used approaches is the tetracycline-based inducible system controlling gene expression at the transcriptional level. The tetracycline-repressor system of *E. coli* interferes with transcription and has been optimised to tightly regulate gene expression in *T. brucei* [76] and has been also established in other protozoa including *G. lamblia*, *Leishmania donovani*, *E. histolytica* and *T. gondii* [77–81]. Although suitable for the expression of toxic genes and dominant negative mutants, this system proved not to be appropriate to generate conditional knockouts in *T. gondii*. Indeed, the necessity to keep the parasites in the presence of drug (anhydrotetracycline) during a prolonged period in order to maintain the expression of an essential gene led to generation of revertants that lost regulation. In order to improve the system, a genetic screen based on random insertion was designed to identify a func-

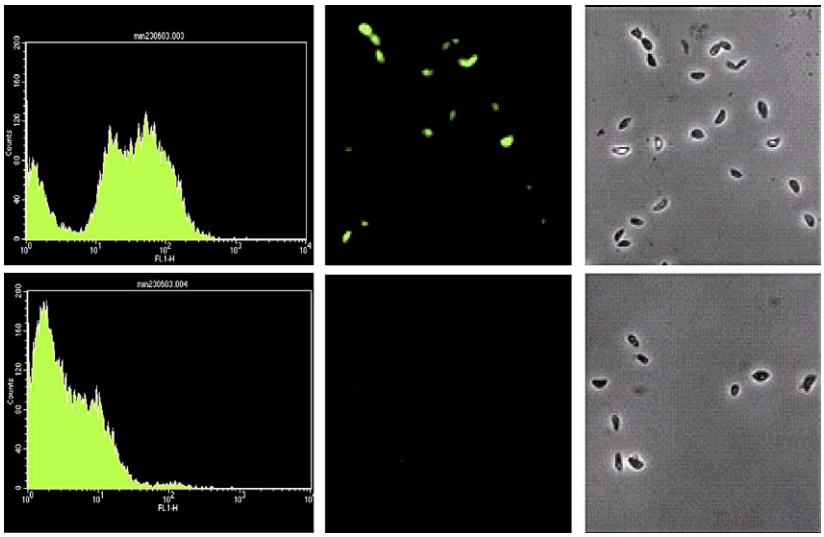

Fig. 2. The Tet-inducible system coupled to FACS sorting.
FACS sorting of parasites expressing a functional Tet-dependent transactivator TATi-1 in *T. gondii*. A recipient strain, where YFP is under control of the inducible promoter TetO7Sag1 [64] was stably transfected with an expression plasmid for expression of the transactivator TATi-1. Only in the absence of ATc parasites, the stable pool expresses YFP and can be easily sorted using FACS.

tional transcriptional activating domain in *T. gondii* and to establish a tetracycline transactivator-based inducible system [64]. Frequently transactivating domains include regions rich in acidic residues, prolines or glutamines without necessarily defined and conserved sequence and tertiary structure [82]. The two artificial transactivators functioning in *T. gondii* and *P. falciparum* correspond to a short stretch of rather hydrophobic amino acids. Neither transactivators function in HeLa cells, underlining the differences between the transcription machinery in apicomplexans and higher eukaryotes [65]. This new system led to the generation of the first conditional knockout for an essential gene with no apparent reversion effect and operates on the parasites in the animal model [64]. In order to learn more about the apicomplexan transcription machinery, it is possible to generate a library of functional transactivators using a *T. gondii* recipient strain, where the gene encoding a YFPYFP-fusion [83] is under control of a tet-inducible promoter. Characterisation of this recipient strain using the previously described transactivator TATi-1 shows that inducible fluorescent parasites can indeed be easily isolated employing FACS (Fig. 2).

Recently in *P. berghei*, a conditional mutagenesis approach has been developed to dissect the function of essential genes. This strategy exploits the flip recombinase Flp/FRT system of yeast to selectively excise a target gene by site-specific recombination. This event is induced after cross-fertilisation in the mosquito vector of two parasite clones, one containing either the target sequence flanked by two FRT sites and the other expressing the Flp recombinase. A tight control of the Flp recombinase activity using a stage-specific promoter to drive its expression should circumvent the step of cross-fertilisation and render this technology more broadly available [84].

Alternative strategies currently used in many organisms rely on the action of RNA/oligonucleotide antisenses, ribozymes, and RNA-interference that specifically lower the level of an mRNA and consequently the level of the corresponding protein. The knocking down of genes should be easier and faster than the generation of conditional knockouts using a tet-regulated system. In *T. brucei*, a combination of efficient RNAi and a tight tet-regulated transcription is routinely applied for large scale analysis of genome function. In contrast, the efficiency of RNAi in apicomplexans is still a matter of debate [85,86], even if previous and more recent studies have reported the successful use of antisense/ribozyme in *T. gondii* [87–89] and some studies have suggested that the mechanism of RNAi can operate in the malaria parasites [90–92].

6. Conclusion

The apicomplexans appear to use the same broad panoply of strategies available in higher eukaryotes to control gene expression even if the current knowledge on the mechanisms and machineries involved in these complex processes is in its infancy. The genome survey revealed that protozoan parasites exhibit a rudimentary basic transcription machinery compared to higher eukaryotes and this major difference can be exploited to develop new tools and to tackle important biological questions in these important human pathogens. However, the use of bioinformatics for the identification of the key players in gene regulation and parasite differentiation appears to be limited, possibly due to sequence divergence. It is now time to go back to the bench and use biochemical and genetic approaches to identify those factors.

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