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ARTICLE *in* MOLECULAR AND BIOCHEMICAL PARASITOLOGY · AUGUST 2001

Impact Factor: 1.79 · DOI: 10.1016/S0166-6851(01)00294-8 · Source: PubMed

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Unusual diversity in α-amanitin sensitivity of RNA polymerases in trichomonads

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Received 13 February 2001; accepted in revised form 20 April 2001

Abstract

Previous studies in the parasitic protist *Trichomonas vaginalis* have revealed that protein coding genes are transcribed by an α-amanitin-resistant RNA polymerase (RNAP) II. To investigate whether this unusual property is a general characteristic of trichomonads, we addressed the physiology of RNA synthesis in lysolecithin-permeabilized cells. Unlike in *T. vaginalis*, RNAP II in *Tritrichomonas foetus* was highly sensitive to the inhibitor α-amanitin. On the other hand, RNAP III, identified by its sensitivity to the specific inhibitor tagetitoxin, was found to be resistant to α-amanitin in *Tritrichomonas foetus*, but showed a typical intermediate sensitivity in *T. vaginalis*. Extension of this study to an additional seven trichomonad species confirmed this genera specific pattern of α-amanitin sensitivity and highlighted an unusual diversity in RNAPs among trichomonads, a closely related group of unicellular eukaryotes.

Keywords: *Trichomonas vaginalis*; *Tritrichomonas foetus*; Permeable cells; Gene transcription; α-amanitin; Tagetitoxin

1. Introduction

In eukaryotic cells transcription is carried out by three different RNA polymerases, each mediating the synthesis of a unique set of RNAs. RNAP I is responsible for the synthesis of the large ribosomal RNAs, RNAP II transcribes protein-coding genes and RNAP III catalyses the synthesis of tRNAs, 5S rRNA and a number of other small cytoplasmic and nuclear RNAs. RNAPs are large multimeric proteins consisting of two large subunits (125–240 kDa) and 4–12 small subunits (10–90 kDa). The classification of the RNA polymerases is based on the differential sensitivity to the drug α-amanitin, the cyclic oligopeptide toxin produced by *Amanita* spp. [1]. In animal cells, RNAP I is resistant to concentrations of α-amanitin up to 1 mg ml^{-1}. RNAP II is generally very sensitive, showing 50% inhibition at 2–20 μg ml^{-1} α-amanitin, whereas RNAP III is only moderately sensitive with 50% inhibition at 50–150 μg ml^{-1} α-amanitin. On the other hand, in *Saccharomyces cerevisiae* RNAP I is inhibited at 300–600 μg ml^{-1} α-amanitin, whereas RNAP III is resistant to the toxin. In addition, an α-amanitin insensitive RNAP III has been found in the silkworm *Bombyx mori* [2]. Inhibition of RNAP II is associated with the binding of α-amanitin to the conserved F region of the largest subunit RPB1, which results in a blockage of RNA elongation [3]. Subsequently, RPB1 with bound α-amanitin is degraded by the proteolytic cell machinery [4].

Only limited information is available concerning the function and properties of RNAPs in parasitic protists. The sensitivity of RNAPs to α-amanitin has been investigated in *Trypanosoma brucei* [5–7], *Plasmodium falciparum* [8], *Leishmania* sp. [9], *Crithidia fasciculata* [10] and *Acanthamoeba castellanii* [11]. These organisms showed a pattern of α-amanitin sensitivity similar to that observed in higher eukaryotes. However, unlike other organisms, *Trypanosoma brucei* utilizes an α-amanitin insensitive RNAP for transcription of a set of

Abbreviations: LSU, large subunit; RNAP, RNA polymerase; SSU, small subunit.

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0166-6851/01/$ - see front matter
PII: S0166-6851(01)00294-8

developmentally regulated protein coding genes, namely the variant surface glycoprotein and procyclic acidic repetitive protein genes [6,12]. To date all the available evidence is consistent with RNAP I being responsible for transcription of these genes [5,13]. Another exception among protists has been found in *Trichomonas vaginalis*, *Giardia lamblia* and *Entamoeba histolytica*, where transcription of protein coding genes was relatively insensitive to α-amanitin. RNAP II in *T. vaginalis* showed 50% inhibition at 250 μg ml^{-1} [14], whereas the *G. lamblia* and *E. histolytica* enzymes were insensitive to concentrations up to 1 mg ml^{-1} of the toxin [15,16]. Naturally occurring resistance of RNAP II to α-amanitin has also been reported in the free-living flagellate *Euglena gracilis* [17]. Taken together, these findings might suggest that the properties of RNAPs are much more diverse among unicellular eukaryotes when compared to those in metazoa.

To further address the properties of RNAPs in trichomonads, we established conditions to study transcription in lysolecithin-permeabilized cells. On the basis of α-amanitin and tagetitoxin sensitivity, we uncovered an unexpected diversity of RNAPs in trichomonads.

2. Materials and methods

2.1. Strains and cultivation

The following strains were used in the study: *T. vaginalis* TV 10-02 [19] and *Trichomonas tenax* Hs-4:NIH (ATCC 30207) isolated from human, *Trichomonas galinae* TGK isolated from *Columbia livia* in the Czech Republic, *Tritrichomonas foetus* LUB-1 MIP isolated from *Bos taurus* [20], *Tritrichomonas mobilensis* TM-776 (ATCC 50116) isolated from *Saimiri boliviensis boliviensis* [21], *Tetratrichomonas gallinarum* A6 isolated from *Anas platyrhynchus* in the Czech Republic [22], *Pentatrichomonas hominis* PH-KT isolated from a human in Slovakia, *Trichomitus batrachorum* BUB isolated from *Bufo bufo* in the Czech Republic, and *Monocercomonas colubrorum* R 183 isolated from *Tropidophys melanurus* in Cuba. The last four species were axenized by J. Kulda, Prague, Czech Republic. Axenic cultures were maintained in trypticase-yeast extract-maltose medium (TYM) supplemented with 10% heat inactivated horse serum [23]. The pH of the medium was adjusted to 6.2 for *T. vaginalis* or 7.2 for all other organisms. Trichomonads from mammals and birds were maintained at 37°C and those from amphibians and reptiles were cultured at 27°C. All strains were deposited in the culture collection at the Department of Parasitology, Charles University in Prague, Czech Republic.

2.2. Preparation of DNA probes

Probes were prepared by PCR amplification of *T. vaginalis* or *Tritrichomonas foetus* genomic DNA. The DNA was prepared by a modified guanidinium thiocyanate procedure [24,25]. The following primer pairs were used in the PCR amplifications: β-tubulin, 5′-CATCGTCCCATCTCCAAAGG-3′, 5′-AATGGAACAAGGTTGACAGC-3′; hydrogenosomal malic enzyme, 5′-AGGAAGAACGTGACCGCC-3′, 5′-GTTGCCGATATCGTGGTC-3′; small subunit (SSU) rRNA, 5′-GGTGGTGCATGGCCG-3′, 5′-GTAGGTGAACCTGCAGAAGGATCA-3′. The sequence of all PCR products was verified by sequencing using ABI Prism 310 (Perkin–Elmer). The PCR products were purified on agarose gels followed by phenol-chloroform extraction and inserted into a pGEM vector (TA cloning kit, Stratagene).

2.3. Preparation of permeable cells

Trichomonads in the logarithmic phase of growth (10^8 cells in 100 ml of culture) were harvested by centrifugation ($1500 \times g$ for 5 min at room temperature) and washed three times in Eppendorf tubes with 1 ml of transcription buffer (150 mM sucrose, 20 mM potassium glutamate, 3 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg ml^{-1} leupeptin, 20 mM HEPES–Tris pH 7.9). Final cell pellets were resuspended in two volumes of transcription buffer, chilled for 5 min on ice and permeabilized for 1 min in the presence of lysolecithin (L-α-lysophosphatidylcholine palmitoyl; Sigma) at a final concentration of 1 mg ml^{-1}. The cells were washed twice with two volumes of transcription buffer. Permeabilization of the cell membrane was monitored microscopically using phase and Nomarski contrast (Nikon Microphot-FXA microscope). The integrity of the nuclei was monitored by fluorescence microscopy using DAPI staining.

2.4. RNA transcription

To initiate transcription, permeable cells ($0.5–1 \times 10^7$ in 25 μl) were mixed with an equal volume of transcription mixture (4 mM ATP, 2 mM GTP, 2 mM CTP, 500 μCi ml^{-1} [α-^{32}P]UTP (3000 Ci $mmol^{-1}$, Amersham), 50 mM creatine phosphate, 1.2 mg ml^{-1} creatine phosphokinase, 5 mM potassium glutamate, 1.5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM HEPES–KOH) and incubated for 20 min at 37°C. After the incubation, 1 μl of the reaction mixture was removed to estimate [α-^{32}P]UTP incorporation by TCA precipitation, and the remainder was lysed by TRIZOL–chloroform extraction according to the manufacturers protocol (Gibco-BRL). Total RNA was precipitated with one volume of isopropanol, spun at $10\,000 \times g$ for 15 min, washed

with 70% ethanol and resuspended in 100 μl of water. To remove most of the unincorporated ribonucleotides, labeled RNA was re-precipitated with ethanol and the final RNA pellet was resuspended in 20 μl water.

2.5. Effect of transcription inhibitors

Serial dilutions of transcription inhibitors were prepared in transcription buffer to make the following final concentrations in the reaction: 1.5, 3, 7.5, 15, 30 and 60 μM tagetitoxin; 0, 5, 50, 250 and 1000 μg ml^{-1} of α-amanitin; and 10, 50 and 100 ng ml^{-1} of actinomycin D. Aliquots of permeable cells (10^7 cells in 24 μl) were mixed with 1 μl of inhibitor and preincubated for 1 min at 37°C. As a control, 1 μl of transcription buffer was added instead of inhibitor. Subsequently, 25 μl of the transcription mixture, prewarmed to 37°C, was added and the reactions were processed as described above.

2.6. RNA analysis

Radiolabeled RNA (1 μl out of a total of 20 μl) was fractionated by electrophoresis through 6% polyacrylamide–7 M urea gels as described [18]. After electrophoresis the gels were dried and exposed to X-ray film (Kodak). To quantify labeled tRNAs, gels were analyzed by storage phosphor autoradiography (PhosphorImager SI, Molecular Dynamics or Fluorescent Image Analyzer FLA-2000, FUJI) and signals were quantified using Multi Analyst software (BioRad) or AIDA 2.0 (Advanced Image Data Analyzer).

Dot-blot analysis of ^{32}P-labeled transcripts were performed using DNA specific probes immobilized on nitrocellulose filters. Plasmid DNAs containing PCR amplified fragments of β-tubulin, malic enzyme or SSU rRNA were incubated at 55°C in 0.3 N NaOH for 30 min, diluted with one volume of 2 M ammonium acetate and spotted at 5 μg per dot onto nitrocellulose filters (SERVA) using a BioRad filtration apparatus. The dot-blots were prehybridized at 65°C for 2 h in prehybridization solution (5 × SET, 10 × Denhardt's solution, 1% SDS, 100 μg ml^{-1} carrier tRNA). ^{32}P-labeled transcripts were denatured at 70°C for 5 min, added to the prehybridization mixture and the incubation was continued at 65°C for 72 h. Filters were washed in 2 × SSC with 0.2% SDS at 65°C and analyzed by storage phosphor autoradiography.

3. Results

3.1. RNA synthesis in detergent permeabilized trichomonad cells

As a method to study transcription and to address the properties of RNAPs in trichomonads, we decided to explore lysolecithin permeabilization in two species of trichomonads, *T. vaginalis* and *Tritrichomonas foetus*. To establish optimal conditions for permeabilization, we incubated different cell numbers (0.5, 0.75, 1 and 1.5×10^8 cells ml^{-1}) with a series of lysolecithin concentrations (0.1–4 mg ml^{-1}) and examined the effect microscopically. In the course of these experiments we made the following observations. In both trichomonad species, the detergent treatment resulted in the loss of mobility. At the same time, the nuclei enlarged and changed in shape from ellipsoidal to round forms (Fig. 1). *T. vaginalis* cell membranes appeared to be more resistant to lysolecithin: following permeabilization the volume of the cytosol increased, but the cells maintained the integrity and the shape of the cell body (Fig. 1A). On the other hand, lysolecithin-treated *Tritrichomonas foetus* cells appeared more fragile and they typically lost most of the cytosolic volume (Fig. 1C). To obtain permeabilization efficiencies of ≈95%, while keeping 100% of the nuclei intact, *Tritrichomonas foetus* cells at a density of 10^8 cells ml^{-1} needed to be treated with 0.5 mg ml^{-1} lysolecithin, whereas 5×10^7 *T. vaginalis* cells ml^{-1} needed to be incubated with 1 mg ml^{-1} lysolecithin. In addition to lysolecithin, digitonin was tested for trichomonad cell permeabilization. However, using this detergent we did not observe any effect on *T. vaginalis* or *Tritrichomonas foetus* cells, even at a concentration of 200 μg ml^{-1}, which is in excess of the 20–40 μg ml^{-1} required to permeabilize plasma membranes of other eukaryotic cells.

Next, to establish parameters for efficient RNA synthesis, we measured the incorporation of radiolabelled UTP into TCA-insoluble material under a variety of conditions and analyzed the quality of the RNA synthesized by gel electrophoresis (Fig. 2A and B, and data not shown). In addition, we quantified the accumulation of various transcripts by dot blot hybridization to cloned *T. vaginalis* and *Tritrichomonas foetus* DNAs coding for the small ribosomal RNAs, β-tubulin and malic enzyme (Fig. 2C and D, and data not shown). Guided by our previous experience in setting up a permeable cell system in *Trypanosoma brucei* [18], we examined the effect of different concentrations of potassium and magnesium ions on RNA synthesis (data not shown). In *T. vaginalis*, the synthesis of all transcripts tested remained unaffected by varying the concentration of K-glutamate in the transcription cocktail from 10 to 150 mM. On the other hand, raising the concentration of $MgCl_2$ from 2 to 35 mM gradually increased the accumulation of rRNA and protein coding gene transcripts. Interestingly, an inverse effect was observed in *Tritrichomonas foetus*, where the accumulation of rRNAs and mRNAs was stimulated by increas-

ing potassium concentration up to 150 mM and a reduction of transcription by ≈50% was observed in the presence of 35 mM $MgCl_2$.

Temperature does not appear to affect transcription efficiency in the range tested (22–37°C) and an almost linear rate of RNA synthesis was maintained for about 20 min at 37°C (Fig. 2E and F). At later time points the amount of β-tubulin and malic enzyme transcripts decreased to undetectable levels (Fig. 2C and D). Finally, in *T. vaginalis* rDNA transcripts appeared to be more stable than those in *Tritrichomonas foetus*, since we obtained a strong hybridization signal even after 90 min of incubation (Fig. 2C).

3.2. Effect of transcription inhibitors

To begin to address the physiology of RNA synthesis in trichomonads, we incubated permeable *T. vaginalis* and *Tritrichomonas foetus* cells with increasing concentrations of the transcription elongation inhibitor α-amanitin. As previously reported [14], transcription of protein coding genes in *T. vaginalis* was highly resistant to α-amanitin and the inhibition profile was very similar to that obtained for the ribosomal RNA genes (Fig. 3C). The synthesis of β-tubulin and malic enzyme RNAs was inhibited by only 30–40% in the presence of 1 mg ml^{-1} α-amanitin (Fig. 3C). The α-amanitin inhibition curve obtained for tRNA genes resembled that of a typical RNAP III transcription unit with intermediate sensitivity. To corroborate the latter result, we used tagetitoxin, a bacterial phytotoxin which specifically inhibits RNAP III in other eukaryotic systems. Indeed, in permeabilized *T. vaginalis* cells, tagetitoxin preferentially inhibited tRNA transcription, but had no significant effect on rRNA and mRNA synthesis (Fig. 3A).

In contrast to the results obtained with *T. vaginalis* permeable cells, transcription of protein coding genes in *Tritrichomonas foetus* was highly sensitive to α-amanitin. Synthesis of β-tubulin and malic enzyme transcripts was inhibited ≈90% by the addition of 5 μg ml^{-1} of the toxin (Fig. 3D), similar to what is known to occur in other eukaryotic organisms. A second unexpected result was the effect of α-amanitin on the synthesis of tRNAs. Transcription of these genes was not affected even at concentrations of 1 mg ml^{-1} of α-amanitin (Fig. 3D). Under the same conditions α-amanitin had only a moderate effect on the transcription of rRNA genes.

The effect of transcription inhibitors on RNA synthesis in *Tritrichomonas foetus* was even more unconventional, when we incubated permeable cells with tagetitoxin. Whereas transcription of tRNA genes decreased to 50% in the presence of 2 μM tagetitoxin, indicative of a typical RNA polymerase III-like enzyme, the accumulation of mRNA and rRNA species

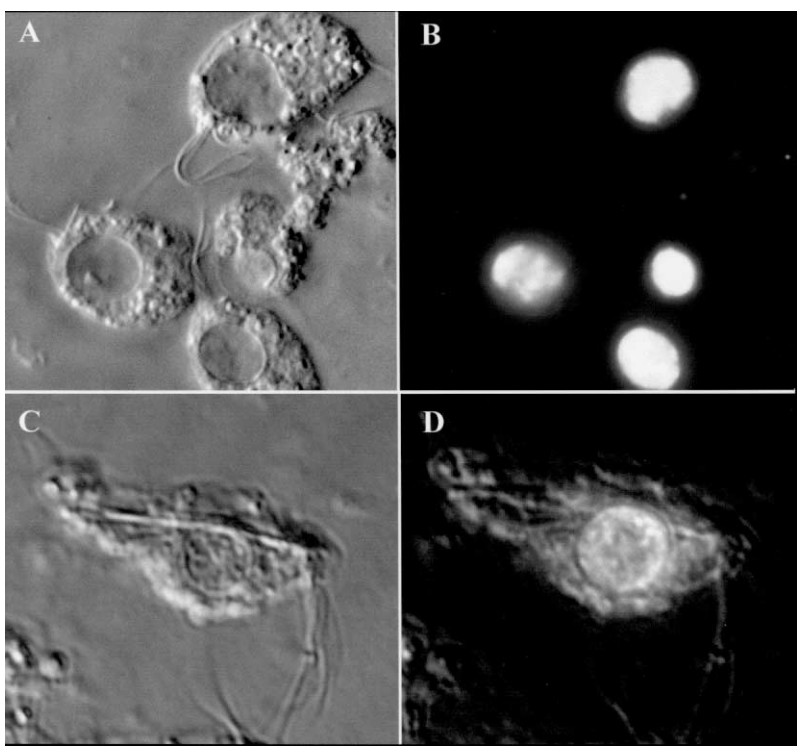

Fig. 1. Microscopic analysis of lysolecithin treated trichomonads. Cells were permeabilized in the presence of 1 mg ml^{-1} (*T. vaginalis*) or 0.5 mg ml^{-1} (*Tritrichomonas foetus*) of lysolecithin and the cell morphology was monitored by Nomarski microscopy (A, C) and the integrity of the nucleus was visualized by DAPI fluorescence (B, D). *T. vaginalis* cells (A, B), *Tritrichomonas foetus* cells (C, D).

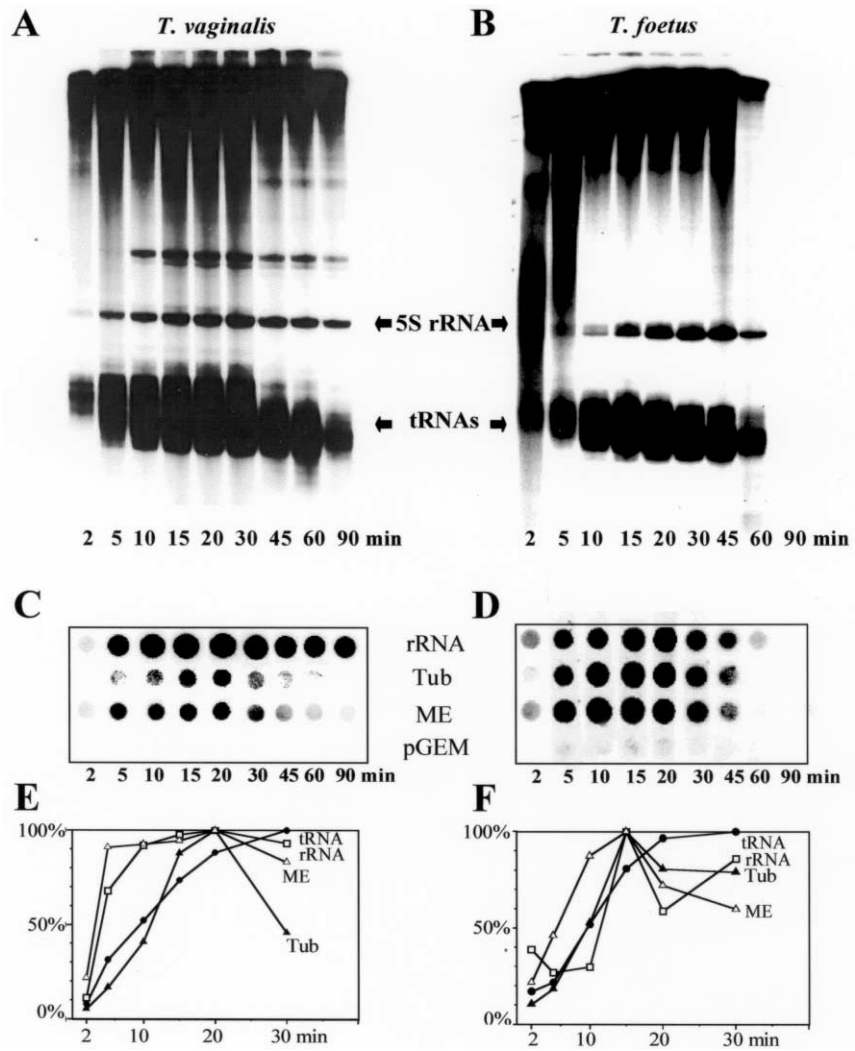

Fig. 2. Time course of RNA synthesis. Aliquots of a transcription reaction were removed at each indicated time point and the reactions were terminated by adding TRIZOL. Aliquots of the radiolabeled RNA of *T. vaginalis* (A) and *Tritrichomonas foetus* (B) were separated on a 6% polyacrylamide gel containing 7 M urea and visualized by autoradiography. The position of transcription products of RNAP III-transcribed genes (5S RNA and tRNAs) are indicated by arrowheads. Radiolabeled RNAs of *T. vaginalis* (C) and *Tritrichomonas foetus* (D) were hybridized to dot blots of plasmid DNAs coding for the small ribosomal RNAs (rRNA), β-tubulin (Tub), hydrogenosomal malic enzyme (ME) and pGEM vector DNA. (E, F) The amount of hybridized RNA was determined by phosphorimaging and for each series the highest value was set at 100%, whereas the other values were expressed as percentages of that number. tRNA synthesis was quantified from the autoradigraph shown in panels (A) and (B).

was reduced by 85–90% at 30 μM tagetitoxin. We can rule out the possibility that this effect is due to the particular batch of tagetitoxin used, since under the same conditions mRNA and rRNA synthesis was not affected in *T. vaginalis*.

Finally, in both species actinomycin D was a highly effective inhibitor and transcription was abolished in the presence of 10 ng ml^{-1} (data not shown).

3.3. Comparison of RNAP sensitivity to α-amanitin in various trichomonad species

The highly unusual spectrum of RNA polymerase sensitivity in *T. vaginalis* and *Tritrichomonas foetus* led us to extend our studies to an additional seven trichomonad species of five genera. For each species, optimal cell permeabilization procedures with lysolecithin were established by microscopic examination and RNA polymerase sensitivity to α-amanitin was followed by dot blot analysis with DNA fragments coding for *T. vaginalis* hydrogenosomal malic enzyme, β-tubulin, and *Tritrichomonas foetus* small subunit rRNA. Preliminary experiments showed that these probes crosshybridized efficiently with RNA of all selected species (data not shown).

RNAP II of the members of the genera *Trichomonas* (*T. vaginalis*, *T. tenax* and *T. galinae*) was α-amanitin resistant, whereas RNAP III was sensitive to this toxin

(Table 1). In contrast, both members of the genera *Tritrichomonas* (*T. foetus* and *T. mobilensis*) possessed an α-amanitin-sensitive RNAP II, whereas RNAP III was resistant. It is interesting to note that RNAP I was inhibited at 1 mg ml^{-1} α-amanitin in *Tritrichomonas foetus*, and a highly sensitive RNAP I was found in *Tritrichomonas mobilensis*. In addition to tritrichomonads, a resistant RNAP III was also detected in *Trichomitus batrachorum*. In *M. colubrorum*, RNAP III was inhibited by 50% at 50 μg ml^{-1}, but its activity was not abolished, even in the presence of 1 mg ml^{-1}. Transcription of protein coding genes in this organism was considerably less sensitive to α-amanitin than in tritrichomonads. On the contrary, unusually low concentrations of the toxin inhibited RNAP I and RNAP III in *P. hominis*. RNAPs sensitivities to α-amanitin in *Tetratrichomonas gallinarum* were comparable with those observed in most eukaryotic cells. Thus, our comparison of RNAP properties within the tested group of trichomonads revealed genera specific patterns of α-amanitin sensitivity.

4. Discussion

To study gene transcription in trichomonads, and in particular to address the properties of RNAPs, we are using lysolecithin permeabilized cells. This procedure has been previously established by some of us for *Trypanosoma brucei* [18] and provides a rapid and efficient method as an alternative to the conventional nuclear run-on assay. In this method the cell integrity and overall cell morphology is retained and 10^7 trichomonads will incorporate as many as 2×10^6 cpm of radiolabeled UTP into newly synthesized RNA.

In contrast to trypanosomes, trichomonad transcription carried out by all three RNAPs required higher concentrations of monovalent cations and RNAP activities did not vary substantially in a broad range of potassium and magnesium concentrations. It is interesting to note that, in the first few minutes of transcription in *Tritrichomonas foetus* permeable cells, some of the synthesized RNA appeared as a continuum of bands in the size range of $\approx 80-300$ nucleotides (Fig. 2B), which

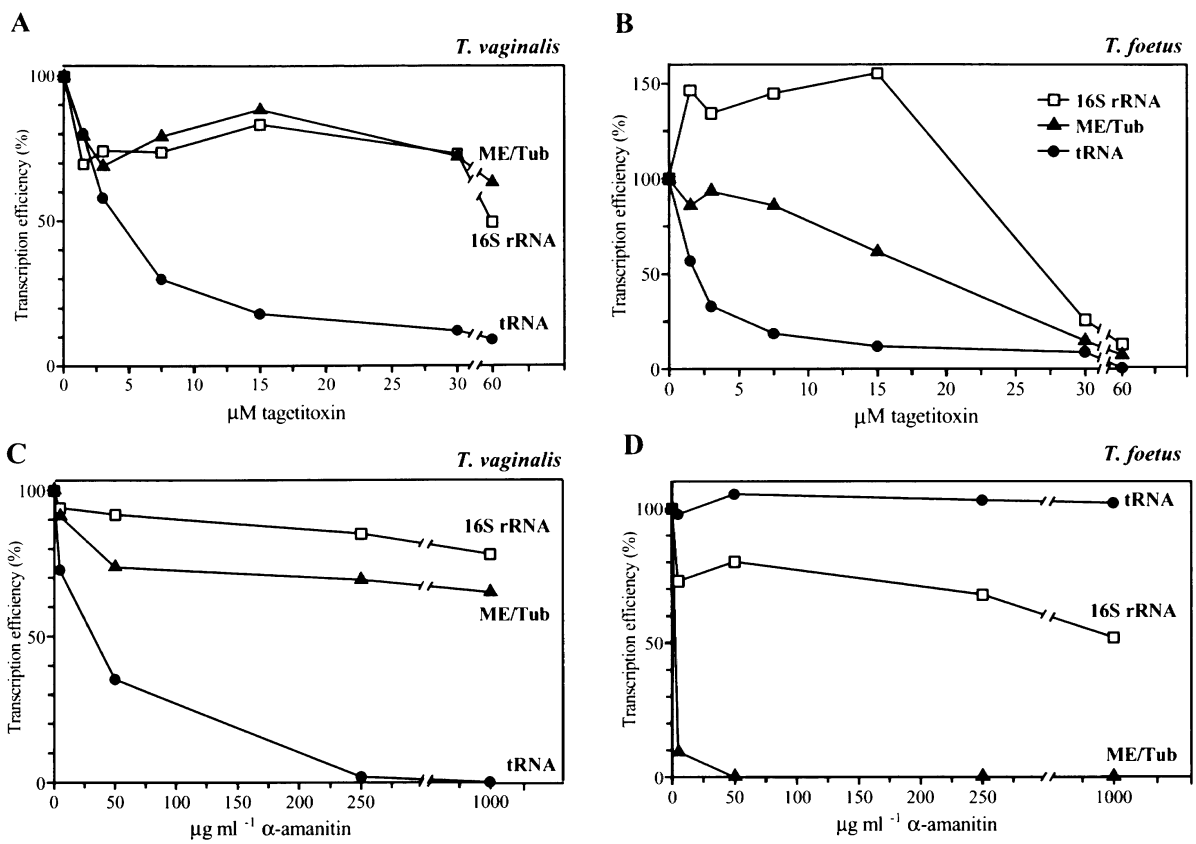

Fig. 3. Effect of tagetitoxin (A and B) and α-amanitin (C and D) on RNA polymerase activities in *T. vaginalis* (A and C) and *Tritrichomonas foetus* (B and D). The synthesis of 16S rRNA (□), hydrogenosomal malic enzyme and β-tubulin (ME/Tub, ▲), and tRNAs (●) was quantified in the presence of increasing concentrations of inhibitors as described in Section 2. The extent of RNA synthesis is expressed as percentage of the value observed in the absence of inhibitor. The data are based on two and three experiments for tagetitoxin and α-amanitin, respectively.

Table 1
RNAP I, II and III sensitivities to α-amanitin in nine trichomonad species

	RNAP I	RNAP II	RNAP III
Trichomonas vaginalis	Resistant	Resistant	20–50 μg ml^{-1}
Trichomonas tenax	Resistant	Resistant	2–5 μg ml^{-1}
Trichomonas galinae	Resistant	Resistant	2–5 μg ml^{-1}
Monocercomonas colubrorum	Resistant	50–250 μg ml^{-1}	50 μg ml^{-1}
Pentatrichomonas hominis	50–250 μg ml^{-1}	5 μg ml^{-1}	<5 μg ml^{-1}
Tetratrichomonas gallinarum	Resistant	5–20 μg ml^{-1}	5 μg ml^{-1}
Tritrichomonas foetus	1 mg ml^{-1}	<5 μg ml^{-1}	Resistant
Tritrichomonas mobilensis	5–50 μg ml^{-1}	<5 μg ml^{-1}	Resistant
Trichomitus batrachorum	Resistant	20–50 μg ml^{-1}	Resistant

The respective RNAP activities were inhibited by 50% at the indicated α-amanitin concentrations. Resistant: no effect on RNAP activity was observed at 1 mg ml^{-1} of α-amanitin. Each experiment was done twice, except for *Monocercomonas* and *Trichomitus*, which were analyzed once, due to very difficult culture conditions.

was not observed in *T. vaginalis* cells. Although this observation could be indicative of precursor RNAs, preliminary pulse-chase experiments in combination with transcription inhibitors (data not shown) do not support this view and further investigation will be necessary to clarify this point.

Our study of transcription in permeable cells revealed the presence of a tripartite RNAP system in trichomonads on the basis of sensitivity to class-specific inhibitors and expression of a number of different genes. RNAP III was specifically inhibited in both *T. vaginalis* and *Tritrichomonas foetus* by tagetitoxin. In *Tritrichomonas foetus*, RNAP I and II were distinguished on the basis of their different sensitivity to α-amanitin. Unlike RNAP I and II was inhibited at low concentrations of the toxin. Unexpectedly, RNAP III showed high resistance to α-amanitin in tritrichomonads, whereas RNAP I was inhibited at high concentrations of the toxin. Therefore, the RNAP I and III present in tritrichomonads showed a pattern of α-amanitin sensitivity that is reverse of that observed in vertebrates. However, it is similar to RNAP sensitivities found in yeasts [26] and possibly in insects [2]. Our experiments confirmed previous data of Quon et al. [14], who observed high resistance of RNAP II to α-amanitin in *T. vaginalis*. We further showed that this unusual property is characteristic for other members of the genera *Trichomonas*, namely *T. tenax* and *T. galinae*. RNAP II sensitive to intermediate concentrations of α-amanitin was found in *M. colubrorum*. The α-amanitin resistance of RNAP II has been reported in various organisms. Not surprisingly, such RNAPs have been found in the amanitin accumulator, the *Amanita* spp. [27], in another fungus *Aspergillus nidulans* [28], and in plants [29]. Mutations that confer resistance to α-amanitin have been reported in vertebrates [30,31], *Drosophila melanogaster* [32] and *Caenorhabditis elegans* [33]. All mutations were clustered to the F region of the largest RNAP II subunit RPB1, which is likely to be involved in binding α-amanitin. Although this region is highly conserved between various species, the *T. vaginalis* RPB1 is unusually divergent [34], including the α-amanitin binding region [14]. It would be of a great interest to delineate the primary structure of the largest RNAP II and III subunits in various trichomonads and compare them with regards to their differences in α-amanitin sensitivity.

Trichomonadidae is a well defined monophyletic group of protists, which has been divided into two main subfamilies, namely Tritrichomonadinae and Trichomonadinae. This separation was proposed on the basis of morphological data [35,36], and later supported by phylogenetic analyses of genes encoding ribosomal RNAs [37,38], GAPDH [39], and SOD [40]. Reverse pattern of RNAP II and III sensitivity to α-amanitin between *Trichomonas* sp. and *Tritrichomonas* sp. is in agreement and supports the known dichotomy of the Trichomonadidae group. Interestingly, pivotal evolutionary position among Trichomonadidae was recently proposed for *Trichomitus batrachorum*-type [41]. However, position of other species within the two subfamilies did not clearly correlate with RNAP sensitivities to α-amanitin. *P. hominis* and *Tetratrichomonas gallinarum* belong to Trichomonadinae subfamily according to morphological [35,36] and large subunit (LSU) rRNA sequence data [37]. Although these species possess an α-amanitin sensitive RNAP III similar to that in *Trichomonas* species, the α-amanitin sensitivity of RNAP II is more like that in *Tritrichomonas* species. *Monocercomonas* sp. was previously considered a primitive genus preceding trichomonads and tritrichomonads based on its simple cytoskeleton [35,36]. More recently the phylogenetic analysis based on rRNAs [37,38] and several protein sequence data [39,40] branched the *M. batrachorum*-type together with tritrichomonads suggesting that these two genera might be related. However, the α-amanitin sensitivity RNAPs in *M. colubrorum* resembles that in trichomonas species. Thus, it is difficult to infer which of the various RNAP properties represents the ancestral pattern.

In conclusion, the results presented here proved the reliability of the permeable cell method, which is now available for the study of gene transcription in trichomonads. We further demonstrated an unusual diversity in RNAPs among trichomonads. High heterogeneity of unicellular eukaryotes has been seen in a number of features, including morphology and physiology. However, the observed variability in rather con-

served RNAP properties within a group of closely related organisms was unexpected.

Acknowledgements

This study received support from National Institutes of Health Grant AI28798 to Elisabetta Ullu and Grant 264/1999 to Jan Tachezy. Elisabetta Ullu is the recipient of a Burroughs Wellcome Fund Scholar Award in Molecular Parasitology and Christian Tschudi is the recipient of a Burroughs Wellcome Fund New Investigator Award in Molecular Parasitology.

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