Demonstration of RNA polymerase multiplicity in *Trypanosoma brucei*

Characterization and purification of α -amanitin-resistant and -sensitive enzymes

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We have isolated, characterized and substantially purified two distinct RNA polymerase activities from the flagellate protozoan parasite *Trypanosoma brucei*. RNA polymerases from this organism were resolved poorly on DEAE-Sephadex, but could be separated with CM-Sephadex. One form was totally resistant to α -amanitin, whereas the second was 50% inhibited by 10–20 μ g of the drug/ml. The enzymes had different salt optima, but both were of high M_r ($> 480\,000$) and demonstrated the template preference: poly[d(A-T)] $>$ denatured DNA $>$ native DNA, and both were more active with Mn^{2+} than with Mg^{2+} . The amanitin-resistant enzyme, polymerase R, was partially purified by chromatography on CM-Sephadex, DEAE-Sephadex and heparin–Sephadex. This enzyme was very labile, and activity yields were around 9%; after purification, one or two protein bands could be discerned after electrophoresis under non-denaturing conditions, but about 20 polypeptides were resolved on denaturing gels, including a major component (not thought to be part of the enzyme) of M_r 65000. Polymerase S, sensitive to low α -amanitin concentrations, was more extensively purified, with an 18% recovery, and yielded a single major band with two minor ones after native gel electrophoresis. Analysis under denaturing conditions permitted a possible subunit structure for this enzyme to be ascribed.

INTRODUCTION

The molecular biology of the flagellate protozoa has received increasing attention in recent years, as a number of unusual features in relation to the control of gene expression have come to light. Notable among the subjects of study has been *Trypanosoma brucei*, now well known for its variable surface glycoproteins (VSGs) produced sequentially during infection to counter immunological responses from the host. It is now clear that activation of a VSG gene often involves physical translocation to an expression site situated in a chromosome telomere (e.g. Pays *et al.*, 1981; Boothroyd & Cross, 1982). Transcription in *T. brucei* seems bizarre in comparison with other eukaryotes; most, perhaps all, mRNAs have spliced to them at their 5' ends a 35-nucleotide leader sequence transcribed separately from a 'mini-exon' elsewhere in the genome (DeLange *et al.*, 1984; Milhausen *et al.*, 1984; Kooter *et al.*, 1984). Moreover, it has been shown that, although normal structural genes in *T. brucei* (such as that for tubulin) are transcribed by an RNA polymerase highly sensitive to α -amanitin, VSGs are transcribed by a totally resistant enzyme and mini-exons by a polymerase of slightly lower sensitivity than that responsible for most mRNA synthesis (Kooter & Borst, 1984; Laird *et al.*, 1985). In typical eukaryotes all structural genes are transcribed by highly amanitin-sensitive (classically type II) RNA polymerases.

The molecular details of transcription and splicing events in *T. brucei* are therefore the subjects of considerable research efforts. It has been shown

previously that RNA polymerase solubilized from either whole cells or isolated nuclei of this trypanosome chromatographs on DEAE-Sephadex as a single unresolved peak of activity sensitive to high α -amanitin concentrations (Kitchin *et al.*, 1984; Earnshaw *et al.*, 1985). In the present paper we describe a further investigation into the RNA polymerase content of *T. brucei*, including the resolution, characterization and substantial purification of two species of enzymes.

MATERIALS AND METHODS

Materials

[3H]UTP (16Ci/mmol) was from Amersham International. DEAE-cellulose DE-52 and glass-fibre (GF/C) filters were from Whatman. Poly[d(A-T)] was from Boehringer and Polymin P from Miles Laboratories. Fine acid-washed sand was from BDH. α -Amanitin was kindly given by Professor Th. Wieland. All other reagents were of analytical or reagent grade and obtained from Sigma or BDH.

Growth and isolation of *T. brucei*

The long slender blood trypomastigote form of *T. brucei* (strain 427) was maintained by syringe passage in mice every 3–4 days. Batches of trypanosomes for enzyme isolation were obtained from infected rats containing about 10^{10} parasites, after isolation from blood by DEAE-cellulose chromatography (Lanham, 1968). A 10–15 ml portion of heparinized (10 μ g/ml) blood was pretreated by centrifugation (MSE Chilsbin) at 300 g for 10 min. The buffy trypanosome-containing

Abbreviation used: VSG, variable surface glycoprotein.

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layer above the blood-cell pellet was removed by Pasteur pipette and diluted (in 8 ml/rat) in buffer containing 60 mM-sodium phosphate, pH 8.0, 45 mM-NaCl and 55 mM-glucose before purification on a 10 ml DEAE-cellulose column equilibrated in the same buffer. Preparations were checked for purity by light microscopy and trypanosomes counted with a haemocytometer.

RNA polymerase solubilization

All operations were at 0–4 °C unless otherwise stated. The optimised solubilization procedure was modified from that described by Kitchin *et al.* (1984). Cells were resuspended in buffer A [50 mM-Tris/HCl, pH 7.9, 25% (v/v) glycerol, 0.1 mM-EDTA, 0.1 mM-dithiothreitol] at 1.5×10^9 – 2.5×10^9 cells/ml and broken by grinding with sand in a pestle and mortar for 3 min. Sand was removed by centrifugation (300 *g* for 30 s) and the lysed cells were then centrifuged at 30000 *g* for 30 min. The supernatant was removed and kept as the soluble ('SL') RNA polymerase pool. High-salt and sonication treatment (Roeder & Rutter, 1969) was used to release chromatin-associated ('CR') RNA polymerase activity from the pellet, which was resuspended in 2 ml of buffer A/rat and made 300 mM with $(\text{NH}_4)_2\text{SO}_4$. After 10 min the extract was sonicated in three 5 s bursts (16 μm peak-to-peak amplitude), with 30 s cooling periods, with the exponential probe of a MSE sonicator. The sonicated preparation was diluted with 2 vol. of buffer A, centrifuged for 30 min at 30000 *g*, and the supernatant used as the 'CR' pool. Both SL and CR polymerase fractions were stable during storage at –196 °C.

Enzyme assays

Assays were in final volumes of 0.1 ml, and generally contained 50 mM-Tris/HCl, pH 7.9, 1 mM-MnCl₂, 1 mM-dithiothreitol, 0.3 mM each of ATP, GTP and CTP, 10 μg of denatured calf thymus DNA and 1 or 2 μCi of [³H]UTP. Assays for quantification of enzyme recoveries also contained 50 μM unlabelled UTP. Final salt concentrations were adjusted to 40 mM- $(\text{NH}_4)_2\text{SO}_4$ where necessary, and up to 50 μl of enzyme fraction was added last. Incubations (single for column fractions, otherwise duplicates) were for 15 min at 37 °C. Precipitation was with 10% trichloroacetic acid in the presence of 50 μg of carrier DNA, samples were pipetted on to GF/C discs and a series of washes (three with 5% trichloroacetic acid, one with ethanol and one with ether) was done before drying and radioactivity measurement.

Purification procedures

In the optimised protocol, starting material was 100–200 ml of fraction CR isolated from 1×10^{11} – 2×10^{11} cells. This was passed through a 40 ml CM-Sephadex column pre-equilibrated in buffer A containing 25 mM- $(\text{NH}_4)_2\text{SO}_4$. This and all subsequent operations were carried out at 0–4 °C. Bound activity (polymerase R) was eluted with buffer A + 0.1 M- $(\text{NH}_4)_2\text{SO}_4$, diluted with 2 vol. of buffer A and loaded on a 5 ml column of DEAE-Sephadex pre-equilibrated in buffer A + 25 mM- $(\text{NH}_4)_2\text{SO}_4$. After thorough washing, enzyme was eluted with a 10 ml 25–200 mM- $(\text{NH}_4)_2\text{SO}_4$ gradient in buffer A, and active fractions (5–6 ml) were pooled and loaded on a 1 ml column of heparin-Sepharose pre-equilibrated in buffer A + 0.15 M- $(\text{NH}_4)_2\text{SO}_4$. Elution was with 0.6 M- $(\text{NH}_4)_2\text{SO}_4$ in buffer A. Activity not bound to the initial CM-Sephadex

Fig. 1. Ion-exchange chromatography of trypanosome extracts

In (a), 4 ml of SL extract was loaded directly on to DEAE-Sephadex A-25, washed extensively with loading buffer and then eluted with a linear 0.025–0.4 M- $(\text{NH}_4)_2\text{SO}_4$ gradient in a total volume of 70 ml of buffer A. In (b), 4 ml of CR extract was diluted with 4 vol. of buffer A before loading, and then chromatographed as above. In (c), 4 ml of CR extract was diluted and then loaded and eluted from CM-Sephadex C-25, otherwise as described above. $\cdots$, A_{280} ; —, RNA polymerase activity; ----, $(\text{NH}_4)_2\text{SO}_4$ gradient.

column (polymerase S) was precipitated by adding Polymin P to a final concentration of 0.05% (v/v), leaving the mixture for 15 min at 0 °C, and collecting by centrifugation at 10000 *g* for 10 min. Precipitates were resuspended in 10 pellet volumes of buffer A, made 0.3 M with $(\text{NH}_4)_2\text{SO}_4$ and then re-centrifuged as described above, leaving polymerase activity in the supernatant. This was chromatographed on heparin-Sepharose as described for polymerase R, except that a larger (10 ml) column of material was necessary. Active fractions were pooled, adjusted to 50% saturation with $(\text{NH}_4)_2\text{SO}_4$, and centrifuged at 30000 *g* for 30 min. Protein pellets were redissolved in small volumes (< 0.3 ml per gradient) of buffer A containing 2.5% glycerol, layered over 4 ml 10–30% glycerol gradients made up in buffer A + 0.1 M- $(\text{NH}_4)_2\text{SO}_4$ and centrifuged in a Beckman SW50 rotor at 50000 rev./min for 6 h. Each gradient was subsequently fractionated and polymerase activity pooled, diluted to

Table 1. Properties of partially purified trypanosome RNA polymerases

Property	RNA polymerase fraction			
	Soluble extract		Chromatin extract	
	DEAE-Sephadex peak I	DEAE-Sephadex peak II	CM-Sephadex flow-through	CM-Sephadex bound
Optimal salt concentration (NH ₄) ₂ SO ₄ with native DNA	25 mM	40 mM	40 mM	25 mM
Template activity proportions {native:denatured: poly[d(A-T)]}	1:1.3:2.6	1:2.0:4.9	1:1.2:1.1	1:1.4:5.2
Bivalent-cation activity ratio (Mg ²⁺ :Mn ²⁺) at optimum concentrations	1:3.4	1:3.2	Negligible activity with Mg ²⁺	1:3.3
Approx. <i>M_r</i> (from glycerol gradients)	480000	480000	585000	605000
Sensitivity to α -amanitin (100 μ g/ml)	< 10% inhibition	> 70% inhibition	60% inhibition	0% inhibition

< 50 mM-(NH₄)₂SO₄ and loaded on a 3 ml DEAE-Sephadex column pre-equilibrated in buffer A + 25 mM-(NH₄)₂SO₄. Final elution was with a 25–300 mM-(NH₄)₂SO₄ gradient made up in buffer A.

Protein determinations

These were done as described by Bradford (1976), but modified as described by Spector (1978).

Polyacrylamide-gel electrophoresis

Non-denaturing gel electrophoresis was carried out on 4% (w/v) polyacrylamide tube gels at 4 °C; 5–10 μ g of enzyme sample was loaded, and run at 1–2 mA per rod. Electrophoresis under denaturing conditions was carried out on 8% polyacrylamide slab gels, and protein samples (20–25 μ g) were precipitated with 5% trichloroacetic acid before denaturation and loading. Staining was with Coomassie Blue, and pulverization of gel slices for RNA polymerase extraction was by the method of Kobayashi *et al.* (1985).

RESULTS

Separation and characterization of polymerase activities

RNA polymerase fraction SL was resolved partially on DEAE-Sephadex chromatography into two close-running peaks. Conversely, fraction CR (which accounted for 70–80% of the total polymerase activity extractable from *T. brucei*) yielded only a single gaussian peak on this resin (Fig. 1). In all cases the enzymes were eluted at very low salt concentrations [certainly less than 0.15 M-(NH₄)₂SO₄]. Mixing experiments with rat liver RNA polymerases, which revealed the three typical activity peaks when chromatographed under identical conditions (Earnshaw *et al.*, 1985), demonstrated that trypanosomes did not contain general polymerase inhibitors that were co-eluted at higher salt concentrations from DEAE-Sephadex; moreover, binding of the trypanosome polymerase activity to DEAE-Sephadex was good, with little or no

activity detectable in the flow-through, and the elution pattern was not altered by prior removal of nucleic acids [e.g. by precipitating polymerases with (NH₄)₂SO₄] (results not shown). However, application of fraction CR to CM-Sephadex separated activity into a major component which failed to bind under any conditions tested (including lower pH and re-passage through a fresh column) and a minor one which bound weakly and could be eluted by 0.1 M-(NH₄)₂SO₄.

Subsequent study confirmed that at least two types of polymerase activities were present in both SL and CR fractions. Peak 1 of the SL extract on DEAE-Sephadex shared a low [< 25 mM-(NH₄)₂SO₄] salt optimum with the CM-Sephadex-binding component of the CR fraction. Moreover, SL-fraction DEAE-Sephadex peak 2 and CM-Sephadex-non-binding component of fraction CR had similar, higher (40 mM), salt optima. More convincingly, there were corresponding differences in α -amanitin sensitivity. Both SL DEAE-Sephadex peak 1 and CR CM-Sephadex-binding component (henceforth referred to as polymerase R) were essentially resistant to all concentrations of α -amanitin tested. On the other hand, SL DEAE-Sephadex peak 2 and CR CM-Sephadex-non-binding component (henceforth polymerase S) were progressively inhibited by 10–100 μ g of α -amanitin/ml. Both enzymes were apparently of high *M_r* (> 480000), since they sedimented as fast or faster than *Escherichia coli* RNA polymerase on glycerol density gradients. Chromatin-derived enzymes consistently sedimented faster than those from the soluble pool, with apparent *M_r* values in the region of 580000–610000. Recoveries of form-R activity from these gradients was however always low (< 10% of load). Table 1 summarizes a general characterization of the RNA polymerases isolated from *T. brucei*. Features shared by both enzymes included marked preferences for Mn²⁺ (optimally 1–2 mM) rather than Mg²⁺, and for single- rather than double-stranded DNA; poly[d(A-T)] was in most cases the best template *in vitro*, and neither enzyme showed any activity in the absence of DNA.

Table 2. Summary of RNA polymerase purification

Enzyme activity was not measured directly in crude homogenates; data for polymerase R (in parentheses) are the assumed minimum content (i.e. the same as eluted from CM-Sephadex in the first purification step) of enzyme units necessary for the estimation of subsequent purification factors (1 unit of RNA polymerase activity represents incorporation of 1 nmol of UMP into RNA in 15 min at 37 °C).

RNA polymerase	Purification stage	Total enzyme activity (units)	Total protein (mg)	Sp. activity (units/mg of protein)	Yield (%)	Purification factor
R	Crude homogenate	(0.744)	94.000	(0.0008)	(100)	1
	CM-Sephadex eluate	0.744	1.800	0.411	100	53
	DEAE-Sephadex eluate	0.420	0.100	4.160	56	520
	Heparin-Sepharose eluate	0.064	0.025	2.580	9	323
S	CM-Sephadex flow-through	8.90	92.100	0.097	100	1
	Polymyxin P pool	14.31	16.300	0.877	161	9
	Heparin-Sepharose eluate	9.39	2.100	4.470	105	46
	Glycerol gradient pool	2.54	0.350	7.250	28	75
	DEAE-Sephadex eluate	1.58	0.036	43.800	18	453

Purification of RNA polymerases

A number of protocols were tested to try to purify the trypanosome RNA polymerases. CM-Sephadex was used to make an early separation of polymerases R and S. Polymerase R proved too labile for techniques based on differences in M_r (i.e. gel filtration or gradient

centrifugation), probably on account of the dilution inevitably involved, a problem that we were unable to overcome by the use of lower temperatures, addition of carrier protein or the proteinase inhibitor phenylmethane-sulphonyl fluoride. Polymyxin P precipitation also yielded poor recoveries of this enzyme, all of which limited the types of approaches we were able to apply. A more extensive procedure was possible with polymerase S, owing to an apparently lower intrinsic lability.

Table 2 summarizes the results of these efforts. Polymerase R was purified some 500-fold, with an apparent yield of about 9%. This estimate is necessarily approximate, because it was not possible to measure this enzyme reliably before CM-Sephadex chromatography. Polymerase S was purified to a similar extent, but ended up with a substantially higher specific activity, partly because its higher stability resulted in a better yield (18%) even after five rather than three purification steps.

Polymerase R remained totally resistant to α -amanitin after purification. Polymerase S, however, was still sensitive, with 50% inhibition caused by 10–20 μ g of the drug/ml (Fig. 2).

Maximally purified polymerase fractions were electrophoresed under non-denaturing conditions and then stained for protein with Coomassie Blue. Alternatively, batches of enzyme were precipitated and electrophoresed under denaturing conditions, with the results shown in Fig. 3. Polymerase R preparations yielded a major protein band remaining at the gel top, and a minor (scarcely visible) one, labelled '1', within the gel under non-denaturing conditions. This pattern was seen with a range of electrophoretic conditions, polyacrylamide concentrations etc. and contrasted with the behaviour of *E. coli* RNA polymerase (results not shown) and polymerase S. The latter preparation electrophoresed as one major and two minor bands, also with some protein remaining at the gel top. Attempts to extract active enzymes yielded low recoveries, but sufficient to demonstrate that > 90% of polymerase R activity recovered from the gel was associated with the major (top) band, with a small amount commensurate with the minor band and none elsewhere. Conversely, > 90% of recovered polymerase S activity was from the bands

Fig. 2. α -Amanitin-sensitivity of purified RNA polymerases

●, RNA polymerase R; ○, RNA polymerase S. Enzymes were purified through to the heparin-Sepharose and DEAE-Sephadex stages respectively. Incorporations by uninhibited controls were 1620 c.p.m. (polymerase R) and 1280 c.p.m. (polymerase S).

Fig. 3. Electrophoretic analysis of purified RNA polymerases

Samples (5 μg) of α -amanitin-resistant (R) and -sensitive (S) RNA polymerases purified through to the heparin-Sepharose and DEAE-Sephadex stages respectively were electrophoresed through 4% -polyacrylamide gels under non-denaturing conditions and stained with Coomassie Blue (a). Bands visible on the gels are indicated 1 (R-enzyme) and A, B, C (S enzyme). In addition, 20–30 μg samples of purified polymerase were precipitated with 5% trichloroacetic acid, heat denatured in the presence of SDS and mercaptoethanol and electrophoresed on 8% -polyacrylamide slab gels under denaturing conditions followed by Coomassie Blue staining (b). Tracks M show markers, with M_r values ($\times 10^{-3}$) shown alongside.

(apparently present in all three) within the gel, with the remainder at the top and none elsewhere (results not shown). Since essentially all activity was sensitive to low concentrations of α -amanitin, these bands must represent variants of enzyme S. No activity was seen if enzymes were heat-denatured before loading. Under denaturing conditions the polymerase R preparation was resolved into about 20 bands, and about 50% of the protein was constituted by a single polypeptide of M_r about 65000. Apart from the lack of general stoichiometry, the banding pattern was not readily reconciled with any of the well-characterized eukaryotic RNA polymerases. Essentially the same pattern was seen if the main band from the native gel was run subsequently under denaturing conditions in a second dimension; the M_r 65000 component remained associated with polymerase activity at the top of the native gel. Polymerase S was resolved into about 16 bands under denaturing conditions, yielding a pattern in which many of the major components were present in the stoichiometry expected of polymerase subunits as deduced from gel scanning. The most probable combination on this basis was subunits of M_r 176000 or 170000, 136000, 89000, 51000, 44000, 39000, 34000, 32000. Two smaller subunits ($M_r < 20000$), visible by silver staining but not by Coomassie Blue staining, might also be involved, yielding a total M_r of about 600000.

DISCUSSION

We report here the first demonstration of RNA polymerase multiplicity in *T. brucei*, and the first attempt at purification of the trypanosome enzymes. Clearly the polymerases from this organism are unusual in their chromatographic properties on DEAE-Sephadex, as previously reported by Kitchin *et al.* (1984). It now appears that the single DEAE-Sephadex peak of activity, which exhibits intermediate sensitivity to α -amanitin, is in fact a composite of resistant and sensitive forms. Both of the enzymes so far identified are weakly charged at neutral pH and do not bind strongly to anionic or cationic exchangers. Nevertheless, CM-Sephadex is able to separate the two species if used at an early stage in the purification (presumably while polymerase S is still bound to nucleic acid fragments). We have observed that partially purified polymerase S does bind to CM-Sephadex and is eluted at a salt concentration much the same as for polymerase R. Fraction-SL polymerases did not bind to CM-Sephadex at all unless first bound to and eluted from DEAE-Sephadex, again indicating that material in trypanosome extracts interferes with RNA polymerase chromatography on cation- (but not anion-) exchange resins. In our hands polymerase R activity constituted $< 10\%$ of the total solubilized, but this could be a feature of assay or extraction bias. In particular, we

have found that inclusion of phenylmethanesulphonyl fluoride in extraction buffers decreases apparent polymerase-R activity substantially. It may therefore be that release of this enzyme is facilitated by limited proteolysis, as seems to be the case with mammalian cells (Lindell, 1975). The two *T. brucei* enzymes were of similar size and had similar template and bivalent-cation requirements, differing only in their salt optima and, more dramatically, in their sensitivities to α -amanitin. For polymerase S this sensitivity increased with purification, as indicated by comparing Table 1 and Fig. 2. RNA polymerases from *Trypanosoma cruzi* have been resolved into two species by phosphocellulose chromatography (Hodo & Hatcher, 1986). However, both of these enzymes were sensitive to α -amanitin (albeit to different degrees), and thus would not seem directly analogous with those found in this study.

We have developed a procedure for the purification of two major trypanosome RNA polymerases designed to avoid as far as possible chromatography media previously shown to separate functional subunits from polymerase enzymes. In particular we chose not to use phosphocellulose, since this resin removes sigma subunit from prokaryotic RNA polymerase (Burgess *et al.*, 1969) and the unusual properties of the trypanosome enzymes on DEAE-Sephadex precluded safe comparison with polymerases from any previously studied eukaryotic sources. Polymerase R proved very labile, and separation based on molecular size (gradients, gel filtration) had to be omitted. Various attempts to improve stability on columns (such as presaturating heparin-Sepharose with serum albumin) failed to improve recoveries; the decay of activity was time- rather than method-dependent. Polymerase S was easier to work with, and the final product had a specific activity of the same order as numerous other eukaryotic RNA polymerases purified to homogeneity (e.g. Engelke *et al.*, 1983).

Native-gel electrophoresis indicated that our protocol resulted in useful purifications for both types of enzyme. However, complete purification can be difficult to establish with eukaryotic RNA polymerases; there are examples of secondary bands on non-denaturing gels which are inactive but turn out to be polymerase lacking a single subunit (Schwartz & Roeder, 1974), and there is a notorious predisposition of the enzyme to disintegrate during electrophoresis (Sentenac, 1985). Clearly this form of gel analysis can be deceptive, and denaturing conditions revealed a complex mixture of polypeptides still present in the polymerase-R preparation. We suspect that this enzyme remains less than 50% pure, but the co-purification of the M_r -65000 protein was the most striking feature. This is unlikely to be the polymerase itself, since glycerol-gradient analyses indicated a much larger size, but it could be a protein which binds to the functional enzyme either *in vivo* or during purification. The three bands, all with activity, detected on the native polymerase-S gel could be artefacts of the type described above, or functionally different enzymes. Clearly, though, most of the protein present at this stage was RNA polymerase of some sort. Analysis under denaturing conditions in a second dimension indicated differences in the largest subunits, with the major native-gel band (C) being resolved as in Fig. 3(b), but with band A including a larger subunit of M_r around 190000 (also faintly visible in Fig. 3b) and none at M_r 170000–176000. This could be an example of selective proteolysis of a large

polymerase subunit, a feature observed in many previous studies, especially with polymerase II (Sentenac, 1985). However, purification of polymerase S in the presence of phenylmethanesulphonyl fluoride yielded a polypeptide pattern indistinguishable from that seen in Fig. 3(b) (all results not shown). Since at least three components were detected by non-denaturing-gel analysis, we assume that the putative subunit structure represents the major species (i.e. band C), with presently indeterminate contributions of non-stoichiometric polypeptides from bands A and B. Running these bands under denaturing conditions in a second dimension tended to confirm this inference, though quantities of material were at the limits of detection even by silver staining (results not shown). Clearly the data on subunit structure are indicative rather than definitive at this stage.

It remains to be seen as to how these enzymes relate to the transcriptional activities identified by using 'run-on' assays in intact trypanosome nuclei (Kooter & Borst, 1984), or indeed to the polymerases of other eukaryotes. Our polymerase R may be responsible for rRNA and/or VSG-gene transcription; polymerase S is more problematical, especially as it has been shown that mRNA and mini-exon transcripts differ slightly but significantly in their amanitin-sensitivities (Laird *et al.*, 1985). After purification, the major amanitin-sensitive enzyme that we detected was inhibited by concentrations of toxin similar to those affecting mini-exon transcription in isolated nuclei, and on this basis it may be the equivalent of RNA polymerase II in higher eukaryotes. However, the subunit structure indicated by denaturing-gel analysis is more similar to a classical polymerase III than to a class II enzyme (Sentenac, 1985). It seems likely that at least one more enzyme still awaits discovery in trypanosome extracts, and until functional assays are developed it would be imprudent to assign the trypanosome enzymes R and S to the standard eukaryote pattern at this stage. An urgent requirement is to establish more certainly which of the polypeptides are true polymerase subunits, and towards this end it is desirable to raise monoclonal antibodies against the trypanosome enzymes. Only with such tools and the development of accurate assays of transcription *in vitro* (with cloned genes) is a more definitive study likely to be possible.

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