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The purification and properties of hen oviduct form B DNA-dependent RNA polymerase

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ABSTRACT

Hen oviduct form B DNA-dependent RNA polymerase has been extensively purified and its properties analysed. It seems likely to consist of a mixture of two forms of the type observed in tissues from other species. Furthermore using S1 nuclease to digest single-stranded DNA, we show that although form B can transcribe double-stranded DNA template it has a very strong preference for single-stranded regions. Also the rate of elongation on native DNA *in vitro* was measured and is an order of magnitude slower than that reported to be operative *in vivo.*

INTRODUCTION

Multiple DNA-Dependent RNA polymerases have been observed in a wide variety of eukaryotes on the basis of different affinities towards ion-exchange columns, specific template and ionic requirements, intranuclear localisation, structure, and response to the antibiotic α-amanitin. Using these criteria, the RNA polymerase population from most tissues can be broadly separated into A (or I) and B (or II) forms[1]. This communication reports the solubilisation at high ionic strength of hen oviduct form B and its purification using ion-exchange chromatography[2] and glycerol gradient centrifugation. The purified enzyme obtained appears to be a mixture of BI and BII enzymes[3,4] with subunit structures similar to those found in other species. The properties of this enzyme preparation using hen liver DNA were also studied. With the aid of S1 nuclease[5], a direct comparison between enzyme activity on double and single-stranded DNA templates was obtained. Secondly, the rate of elongation on DNA *in vitro* was measured. Both results indicate that the highly purified enzymes may be lacking certain specific factors present during transcription in vivo.

METHODS

1. Solubilisation of Enzymes

All operations were carried out between 0-4°C and dithiothreitol (DTT) was added just before use. 3 x 100 g batches of oviduct magnum tissue[6] from 'point of lay' White Leghorn hens were chopped, blended in 0.25 l 0.32 M

sucrose, 3 mM $MgCl_2$, 0.5% Triton X-100, pH 6 (SMT) in a Waring blender (2 min ; full speed), homogenised in a Thomas 'C' homogeniser (8 strokes) and filtered through two layers of Nybolt. The pooled filtrate was diluted to 4.5 l with SMT, further diluted with 1.2 l deionised water and centrifuged (1 000 x g ; 15 min). The nuclear pellet was suspended in 2.25 l SMT, diluted with 0.6 l deionised water and centrifuged as before. The resulting pellet was resuspended in 1.5 l 0.25 M sucrose, 3 mM $MgCl_2$, 0.5 mM DTT, pH 6.5, and recentrifuged. The nuclei were then suspended in 0.36 l 1.0 M sucrose, 5 mM $MgCl_2$, 1 mM DTT, 10 mM Tris-HCl, pH 8 and 0.12 l 4 M $(NH_4)_2SO_4$, pH 8 was added followed by 0.96 l 25% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 50 mM Tris-HCl, pH 8 (TGMED) containing 1.0 M $(NH_4)_2SO_4$. The resulting viscous solution was shaken (10 min) and centrifuged (22 000 x g ; 1 h). Batches of supernatant (100 ml) were then sonicated (3 x 20 sec bursts) using a Branson sonicator (full strength, large probe), the sample being maintained below 5°C using an ice/salt bath. Finally the enzyme was precipitated by adding solid $(NH_4)_2SO_4$ (11 g per 100 ml sonicate), stirring for 50 min and centrifuging (22 000 x g ; 30 min).

2. DEAE-Sephadex (A25) chromatography

All subsequent stock solutions were filtered through Millipores (HAWP 025 00) before use. The pellet was suspended in 1.8 l TGMED containing 0.05 M $(NH_4)_2SO_4$ (high salt extract) and stirred for 60 min with 40 g dry wt. of A25 (Pharmacia) previously equilibrated with TGMED/0.05 M $(NH_4)_2SO_4$ for 24 h. After filtering through a sintered funnel (pore size 2) and washing with 2 l TGMED/0.05 M $(NH_4)_2SO_4$, the slurry was poured into a column (25 cm x 4 cm) and a 0.25 l linear gradient of 0.05 M to 0.5 M $(NH_4)_2SO_4$ in TGMED was applied. Fractions (5 ml) were collected and assayed for RNA polymerase activity (see Table 1), and those sensitive to α-amanitin were pooled and precipitated by adding solid $(NH_4)_2SO_4$ (29 g/100 ml), stirring for 50 min and centrifuging (18 000 x g ; 30 min).

3. Phosphocellulose (P11) chromatography

The precipitate was suspended in 45 ml 40% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 50 mM Tris-HCl, pH 8 ($TG^{40}ED$) containing 0.05 M $(NH_4)_2SO_4$ and loaded onto a column (6 cm x 1.5 cm) of P11 (Whatman) previously equilibrated with the same solution. The column was washed with the latter solution until all free protein was eluted, then a 60 ml linear gradient of 0.05 M to 0.5 M $(NH_4)_2SO_4$ in $TG^{40}ED$ containing 1 mM $MgCl_2$ was applied and 2 ml fractions were collected.

4. DEAE-Cellulose (DE23) chromatography

In subsequent steps, all buffers were gassed with N_2 for 60 min before use. Active fractions from the previous step were pooled, and bovine serum albumin (BSA) was added (final concentration 1.6 mg per ml). The pool was then diluted x 4 with $TG^{40}MED$ and loaded onto a column (15 cm x 1 cm) containing DE 23 (Whatman) previously equilibrated with $TG^{40}MED/0.05$ M $(NH_4)_2SO_4$. Unbound protein was washed from the column using the latter solution and B enzymes were eluted using a 30 ml linear gradient from 0.05 M $(NH_4)_2SO_4$ to 0.5 M $(NH_4)_2SO_4$ in $TG^{40}MED$. Fractions (2 ml) were collected in plastic tubes containing 400 ug BSA.

5.Glycerol gradient centrifugation

All glassware and tubes were previously rinsed with a solution containing BSA (50 ug/ml), then dried. Active fractions from the previous step (material from 2-3 preparations) were pooled and BSA added (final concentration 1 mg per ml). 1.5 vol of 4 M $(NH_4)_2SO_4$ were then added, stirred for 45 min and the resulting suspension centrifuged (200 000 x g ; 75 min). The pellet was dissolved in 0.25 ml 0.1 mM EDTA, 0.5 mM DTT, 50 mM Tris-HCl pH 8, containing 100 ug BSA per ml, and layered onto a 5 ml gradient of 15-30% glycerol in sample buffer containing 0.05 M $(NH_4)_2SO_4$ but only 30 ug/ml BSA. Tubes were centrifuged (8 h ; 300 000 x g) in a Spinco SW 65 rotor and 0.3 ml fractions were collected and assayed.

The complete purification takes 3-4 days and yields 57 ug (excluding BSA carrier) of 13 000-fold purified form B RNA polymerase with an optimum specific activity of 17 900 units per mg protein (Table 1). These values compared favourably with those reported previously for rat liver[2]. After A25 chromatography the enzyme activity is completely DNA-dependent and 99% of the glycerol gradient enzyme is inhibited by α-amanitin at a concentration of 0.32 ug per ml on both native and denatured templates.

RESULTS AND DISCUSSION

1. Subunit structure of hen oviduct form B RNA polymerases

Gel electrophoresis of fractions from the glycerol gradient reveals a correlation between enzyme activity and the intensity of staining of six major bands B1 to B6 (Fig. 1) having molecular weights of 223 000, 183 000, 145 000, 33 000, 23 000 and 19 000 respectively. These values are almost identical to those reported for the two calf thymus form B enzymes[3,4] containing subunits B1, 3, 4, 5, 6 (BI enzyme) and B2, 3, 4, 5, 6, (BII enzyme). Four minor bands, labelled B 0, B 2', B 3' and B 3'', with molecular weights of 233 000, 167 000, 132 000 and 123 000, may also correlate with

TABLE 1 Purification of form B RNA polymerases from hen oviduct

Fraction	Volume (ml)	Enzyme Activity (Units x 10^{-3})	Recovery (%)	Protein (mg)	Specific Activity (Units/ mg)	Purification (fold)
Nuclei	360	15.34	100	11180	1.4	1
High salt extract	1800	7.21	47	1619	4.5	3.2
A25 eluate	57	4.4	29	49	90	65
P11 eluate	6.5	1.6	10	2.4	670	480
DE23 eluate	6.0	2.6	17	0.6	4400	3100
Glycerol gradient	0.8	1.02	6.5	ǂ0.057	17900	13000

Assays were performed using method (b) described elsewhere[25] and contained 10 ug native calf thymus DNA and 0.13 M $(NH_4)_2SO_4$. Only activity sensitive to α-amanitin (0.52 ug per ml) is shown. One unit of enzyme incorporates 1 pmole UTP per min into RNA under the assay conditions used. Values given are for 300 g of magnum tissue.
ǂCalculated by scanning stained SDS-gels at 620 nm ; the value is excluding the BSA carrier.

TABLE 2 Effect of heat denaturation and S1 nuclease treatment on the template specificity of purified form B RNA polymerases.

DNA	Nuclease Treatment	RNA Synthesis (pmoles UTP incorporated)	% Transcription after nuclease treatment	% DNA remaining after nuclease treatment
Native	-	7.0		
	+	5.4	76.5	83.0
Denatured	-	58.8		
	+	2.0	3.4	8.0

Hen liver DNA, prepared by the method of Marmur[26], was denatured by heating at 100°C for 10 min. 2.5 ug of either native or denatured DNA were incubated with 0.37 units of S1 nuclease, prepared by the method of Vogt[5], in 25% glycerol, 0.5 mM $ZnSO_4$, 25 mM NaCl, 15 mM sodium acetate, pH 4.6 at 37°C for 60 min in a final volume of 10 ul. Control samples contained no enzyme. Then 240 ul containing standard reaction mixture (see Table 1) and 5 units of purified form B RNA polymerase were added. RNA synthesis was measured after incubation at 37°C for 30 min. S1 nuclease is completely inactive under conditions of RNA synthesis, that is at pH 8.0. The percentage of DNA remaining after S1 nuclease treatment was measured by the method of Burton[27], after incubating S1 nuclease and DNA as described, but scaled up by a factor of ten.

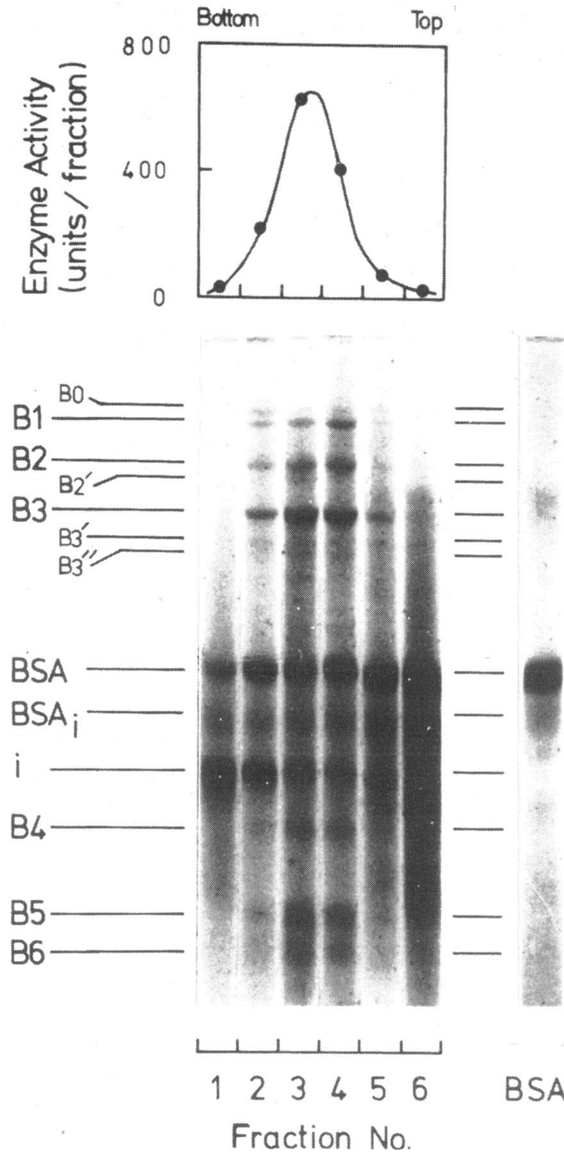

Fig. 1. Aliquots of the active fractions from the glycerol gradient were analysed on 5% acrylamide gels containing 0.1% SDS. Gels were run at 5 ma/gel for 12 h, then stained with 0.25% Coomassie brilliant blue (R250) in methanol : H_2O : acetic acid (5 : 5 : 1) and destained in the same solution without dye. Molecular weights were determined by running marker proteins (E. coli RNA polymerase, β -galactosidase, BSA, ovalbumin, α -chymotrypsinogen A and myoglobin) in parallel gels according to Shapiro et al.[28]. Additional aliquots from glycerol gradient fractions were assayed for RNA polymerase activity as described in Table I and the enzyme activity of the sample applied to each gel is shown in the upper graph.

oviduct form B polymerase activity. BSA, used as a carrier and its associated impurity (BSA_i) were visible on all gels along with band 'i', which is the only main impurity and not a subunit, since its distribution does not correspond with the enzyme activity and its absence has been noted in other preparations.

Quantitation of the protein bands (by scanning the gels at 620 nm) suggests that the molar ratios of the six main subunits are B1 + B2 = 1, B3 = 1, B4 = 1, B5 = 2 and B6 = 2. Furthermore, on sucrose gradients (10 to 30% containing 0.4 M $(NH_4)_2SO_4$) oviduct form B enzyme sediments as a single peak of activity, running slightly faster than E. coli RNA polymerase, corresponding to a molecular weight of approximately 550 000 daltons, and hence all six major subunits are unlikely to exist in the same molecule. It is more probable that in oviduct two form B enzymes exist as appears to be the case in calf thymus[4], rat liver[2,7,8], HeLa and KB cells[9], although in yeast only one form may be present[10,11]. Whether these two forms arise by proteolytic conversion of B1 subunit to B2, or the latter are two unrelated polypeptides, remains unresolved[4,8,9].

When oviduct form B enzyme is subjected to native gel electrophoresis using the high pH discontinuous system[12], two main bands and one diffuse band are seen (Fig.2). These bands contain varying combinations of major and minor bands (see Fig.1) as shown by eluting the protein from each band and analysis on denaturing gels. Since the high pH system may encourage dissociation and aggregation[13,14], the bands observed on native gels do not necessarily represent functionally distinct enzyme species.

2. Template properties of hen oviduct form B RNA polymerases

As can be seen from Table 2, native hen DNA is eight times less efficient as template for oviduct form B enzymes compared to denatured DNA, in agreement with results obtained with mammalian RNA polymerase[9,15,16,17] and also yeast form B polymerase[18] where the poor template activity on yeast native DNA is due to a deficiency in chain initiation.

To determine whether, using 'native' DNA as template, oviduct form B polymerases transcribe truly double-stranded regions of the DNA or whether only single-stranded regions are acting as template, both native and denatured DNA were digested with S1 nuclease prepared by the method of Vogt[5]. This enzyme has both endo- and exo-nuclease activity and under the conditions of incubation employed, efficiently degrades all single-stranded DNA but has no effect on double-stranded DNA (unpublished observations). After treatment

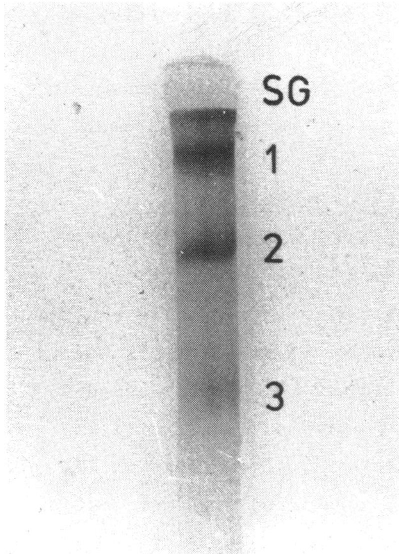

Fig. 2. Polyacrylamide gel electrophoresis under non-denaturing conditions of purified oviduct form B enzymes obtained from a glycerol gradient. 5% resolving gels with 3% spacer gels (SG) were run for 7 h at 80 v and then stained using 0.25% aqueous Coomassie Brilliant Blue-G250[29]. Only the top half of the gel is shown.

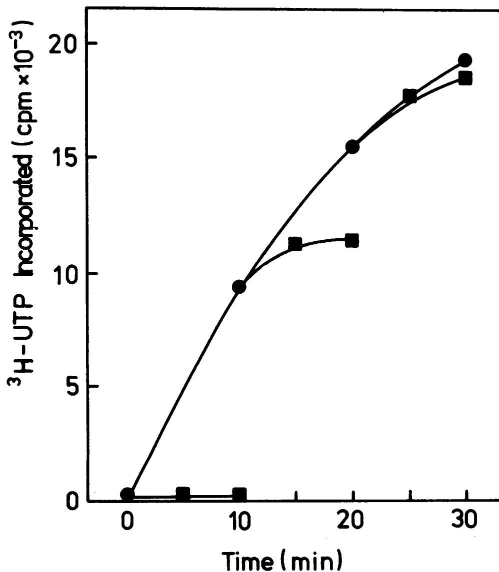

Fig.3. Reaction mixtures containing 10 ug of native DNA and 90 units of purified form B polymerase were incubated for up to 30 min (●——●). RNA synthesis after adding heparin (100 ug/ml) at time 0 (before enzyme) or 10 and 20 min after addition of the enzyme (■——■).

of native DNA with S1 nuclease, 76% of the original activity is retained although the template activity of S1 nuclease-treated denatured DNA is almost completely destroyed. Our results demonstrate that even though single-stranded regions are preferred by oviduct form B polymerase, the homologous double-stranded DNA can be used as template.

3. Rate of elongation of oviduct form B RNA polymerases on native DNA

Previous work[19] has demonstrated that the polyanion heparin inhibits initiation, but not elongation, of oviduct form B RNA polymerases on chromatin templates. Heparin acts in a similar manner using native hen DNA as template. Fig. 3 shows that if heparin is added to the reaction mix before the enzyme, RNA synthesis is completely inhibited. If heparin is added 10 or 20 min after the start of the reaction, then the inhibitory effect is diminished, such that addition after 20 min has negligible effect on RNA synthesis. This and other data (unpublished observations) suggests that once all RNA polymerases have initiated, elongation of RNA chains is unaffected by heparin. By analysing the size of RNA products synthesised by form B polymerases after the addition of heparin, an estimate of the rate of elongation was obtained under conditions in which re-initiation of RNA polymerases was absent. Fig. 4A shows the distribution on polyacrylamide gels of labelled RNA chains synthesised 10 min after heparin addition, after first allowing RNA polymerase to initiate on native DNA in the presence of only three triphosphates. The majority of labelled RNA exceeds the size of 28S ribosomal RNA marker. By estimating the number average molecular weight of RNA chains synthesised at various times, the rate of elongation of oviduct form B polymerases on hen native DNA was obtained, as shown in Fig. 4B ; this value is approximately 1.2 nucleotides per second. It is of interest that similar rates of elongation are observed for endogenous form B RNA polymerases using oviduct chromatin DNA as template[19].

Our present results suggest that purified oviduct form B RNA polymerases lack certain regulatory factors which may be important for the transcriptional process *in vivo*. Although the enzyme will transcribe native double-stranded DNA, the preference for single-stranded DNA suggests the absence of certain components which stabilise the separation of DNA strands *in vivo*. Secondly, the rate of elongation of 1.2 nucleotides per sec observed on native DNA *in vitro* is far below the value of 60-80 nucleotides per sec reported to be operative *in vivo* in eukaryotes[20,21]. Since similar *in vitro* rates are obtained using chromatin templates, it seems that the required elongation factors are not present in chromatin.

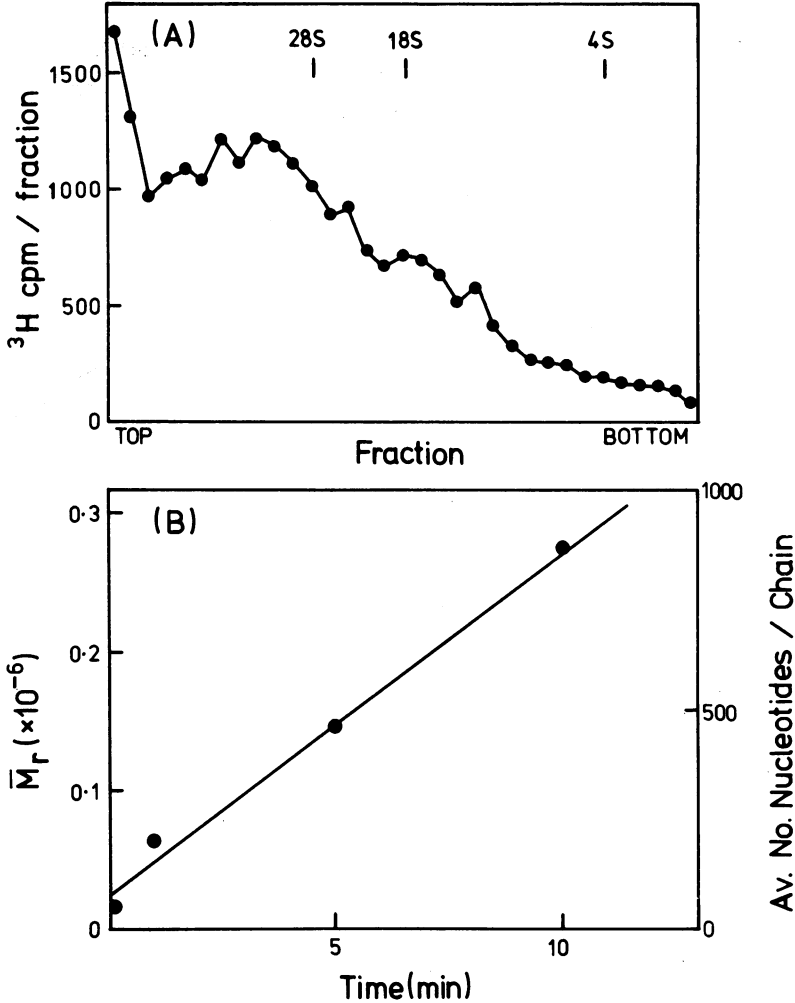

Fig. 4. Reaction mixtures (1 ml) containing 25 ug native DNA and 63 units of purified form B polymerase were incubated for 5 min in the absence of UTP. Heparin (100 ug/ml) and [^{3}H] UTP (1950 dpm per pmole) were then added. After 1, 5 and 10 min of incubation at 37°C, mixtures were frozen at -10°C and labelled RNA was isolated and analysed on 2.7% polyacrylamide gels containing SDS as described previously[19]. The size of RNA chains synthesised after 5 min of incubation in the absence of UTP (time 0) was determined by substituting [^{3}H] ATP (1 000 dpm per pmole) for unlabelled ATP in the reaction mixtures. (A). Distribution of labelled RNA chains synthesised 10 min after the addition of heparin and labelled UTP. The positions to which E.coli tRNA (4S) and chick embryo rRNAs (18S, 28S) migrate under identical conditions are indicated. (B) Estimation of the number average molecular weight ($\bar{M}_r$) calculated as described[19] of labelled RNA chains synthesised after 0,1,5 and 10 min of incubation with heparin and UTP. From this data, a figure of 1.25 nucleotides per sec for the rate of elongation of oviduct form B polymerase on native DNA is obtained.

Certain fractions which stimulate transcription *in vitro* have been reported[9,17,22,23,24]. The search for similar factors affecting both elongation and initiation of oviduct form B polymerases is now in progress.

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