RNA POLYMERASE I FROM *DROSOPHILA HYDEI* PUPAE

Purification and partial characterization

Eckart GUNDELFINGER and Hans STEIN
Max-Planck-Institut für Biologie, Abt. Beermann, Spemannstraße 34, 7400 Tübingen, FRG

Received 14 April 1982

1. Introduction

In vitro systems for selective transcription of DNA by homologous RNA polymerases have been introduced in the study of eukaryotic gene expression [1–4]. In essence these include besides one of the 3 nuclear RNA polymerases (I or A; II or B; III or C) factors of cellular origin serving in promoter recognition. At present the complexity and distribution of these factors are largely unknown. Therefore, we have attempted to establish such systems with cellular components from *Drosophila*, chosen because of the wealth of cytogenetic details known about this organism. In contrast to the relative ease with which RNA polymerases can be isolated from a variety of eukaryotic cells, the isolation of *Drosophila* RNA polymerases is problematic [5–7]. Methods for the isolation of *Drosophila* RNA polymerases II and III are nonetheless available [8,9]; however, RNA polymerase I has been only partially purified [5–7].

Here, we report a method for complete purification of this enzyme from *Drosophila* which is probably applicable to the purification of RNA polymerase I of insects in general. The method is related to the method for isolation of RNA polymerase III from *Drosophila* in [9] and allows the isolation of the class I enzyme on a large scale. Preliminary characterization of the enzyme reveals its close relationship to the homologous eukaryotic enzymes in structural and functional respects. In vitro systems with this enzyme may contribute to our understanding of ribosomal RNA synthesis of *Drosophila* where we are faced by the intriguing problem of how different transcriptional activity on ribosomal cistrons, distinguishable by the presence or absence of intervening sequences, is regulated [11].

Abbreviations: $PhMeSO_2F$, phenylmethylsulfonyl fluoride; buffer A contains 50 mM Tris–HCl (pH 7.8), 10 mM thioglycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, 30% (v/v) glycerol, 0.3 mM $PhMeSO_2F$

Enzyme: DNA-dependent RNA polymerase or nucleoside triphosphate, RNA nucleotidyltransferase (EC 2.7.7.6)

2. Materials and methods

2.1. *Growth of* Drosophila hydei *pupae*

Pupae were grown in mass culture [12] and further processed as in [9].

2.2. *Standard RNA polymerase assay*

The standard enzyme assay was done as in [13] with modifications given in the text. Assays were run at 30°C in 0.05 ml.

2.3. *Enzyme purification*

All steps were done at 4°C. *Drosophila hydei* pupae (1000 g) were homogenized in 2 vol. (w/v) of low salt buffer (10 mM Tris–HCl (pH 7.8), 10 mM thioglycerol, 0.1 mM dithiothreitol, 5 mM 2-mercaptoethanol and 2 mM phenylmethylsulfonylfluoride ($PhMeSO_2F$); 6 min; Waring blendor; high speed). After addition of ammonium sulphate to 70 mM, the homogenate was centrifuged (70 min; Sorvall rotor GSA; 12 000 rev./min). The resulting pellet was redissolved in 50 mM Tris–HCl (pH 7.8), 10 mM thioglycerol, 0.1 mM dithiothreitol, 0.1 mM EDTA, 30% glycerol and 0.3 mM $PhMeSO_2F$ (buffer A) containing 0.5 M ammonium sulphate, rehomogenized (4 min), and centrifuged as above. The resulting high-salt extract was combined with the low-salt extract and submitted to

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

DEAE–Sepharose and heparin–Sepharose chromatography as in [9].

Separation of class I RNA polymerase from RNA polymerase II and III activities was done by DEAE-cellulose chromatography. Enzyme activity was quantitatively adsorbed to DEAE-cellulose at 0.04 M ammonium sulphate in buffer A (2–3 mg/ml column vol.). Using a step of 0.09 M ammonium sulphate, RNA polymerase I activity was specifically eluted from this resin in the same buffer. This was followed by absorption and elution of enzyme activity from DNA–agarose at 0.07 M and 0.25 M ammonium sulphate in buffer A essentially as in [9]. Most active enzyme fractions were pooled, diluted to 0.05 M ammonium sulphate with buffer A and applied to phosphocellulose (3 mg protein/ml packed ion exchanger), extensively pre-equilibrated with the same buffer. Elution was with a gradient from 0.05–0.3 M ammonium sulphate.

For final purification the major peak of phosphocellulose enzyme was concentrated by pressure dialysis (collodium bags, Schleicher and Schüll), subjected to a 5–20% sucrose gradient, sedimented for 28 h at 55 000 rev./min (Beckman rotor SW 55) and concentrated again.

2.4. *Test for contaminating nuclease activities*

DNase activity was monitored by determining the conversion of *E. coli* plasmid pBR 322 from form I DNA to form II DNA, as in [9] and by the conversion of ^{3}H-labeled adenovirus 2 DNA into acid-soluble form. RNase H and RNase A activities were determined by measuring the conversion of RNA (spec. act. 2×10^6 dpm/mg) synthesized on denatured calf thymus DNA by calf thymus RNA polymerase II before or after denaturation of the resulting DNA: RNA hybrid, respectively [14].

3. Results and discussion

3.1. *Purification*

The α-amanitin resistant RNA polymerase fraction of *D. hydei* pupae comprises 2 major subfractions eluting at different ionic strengths from ion-exchange columns [9]. They can be further distinguished, e.g., by their activity ratio on native *vs* denatured DNA. This ratio is found to be near unity in one and <0.2 in the other instance. As shown in [9], the *D. hydei* enzyme with an activity ratio of ~1 is RNA polymerase III. The other activity appeared to be a possible candidate for RNA polymerase I. This enzyme was purified to homogeneity using 6 purification steps (table 1). Purification starts with a combined low and high salt extraction of *D. hydei* pupae. High salt extraction appears to be the most efficient solubilization method for RNA polymerase I [15]. According to [9] considerable RNA polymerase I activity is also present in a low salt extract of *D. hydei* pupae. As a high level of proteolytic activity exists in *Drosophila* extracts [7,10], the overall RNA polymerase activity was first enriched by two chromatography steps, which by batchwise handling can be performed in a minimum of time. From the total RNA polymerase pool an enzyme fraction is then separated (DEAE-cellulose chromatography) which exhibits high α-amanitin resistance and an activity ratio on native *vs* denatured DNA of ~0.2. After an additional purification step (DNA agarose), this activity separates into 2 activities,

Table 1
Purification of RNA polymerase I from *D. hydei* pupae

Fraction	Vol. (ml)	Protein (mg)	Total RNA polymerase activity (units)	Recovery (%)
Homogenate (low salt + high salt)	3900	67 800	60	100
DEAE–Sepharose	665	6680	217	362
Heparin–Sepharose	116	464	104	173
DEAE–Cellulose step	145	34.8	16.5	27.5
DNA–Agarose	4	13.0	11.0	18.3
Phosphocellulose[a]	5	0.38	5.4	9.0
Sucrose gradient	0.5	0.16	2.3	3.8

[a] Only the most active fractions of DNA–agarose enzyme were further purified

The data refer to the purification of the enzyme from 1 kg pupae

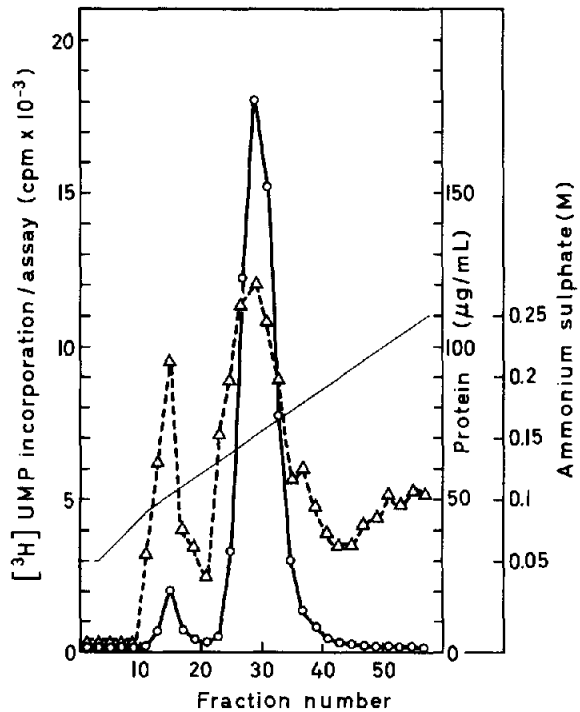

Fig.1. Phosphocellulose column chromatography. DNA–agarose enzyme fraction (15 μl, 9 mg protein) was applied to a 3 ml column of phosphocellulose in buffer A containing 0.05 M ammonium sulphate. Elution was carried out with 30 ml of a gradient from 0.05–0.3 M ammonium sulphate in the same buffer. [^{3}H]UMP incorporation was assayed with 5 μl aliquots of column fractions tested under standard assay conditions (section 2) but with denatured calf thymus DNA as template (o——o). Protein concentration (△– – –△) and conductivity (——) were determined as in [9].

eluting at 0.1 and 0.15 M $(NH_4)_2SO_4$ from a phosphocellulose column (fig.1). Both of these activities have the same activity ratio on native *vs* denatured DNA (not shown) and thus probably belong to the same enzyme class. Upon sucrose gradient centrifugation of the major enzyme fraction eluting from phosphocellulose, a correlation between the enzymatic activity and a protein peak is observed which suggests that this enzyme is essentially pure (not shown). The sucrose gradient enzyme preparation is free of contaminating DNases and RNases, assayed as in section 2. Moreover, tests for RNase H, an enzyme found in RNA polymerase I specimens of yeast [16] performed as in section 2, were clearly negative.

3.2. *Structure and catalytic properties of* Drosophila *RNA polymerase I*

The multimeric composition of the enzyme, revealed

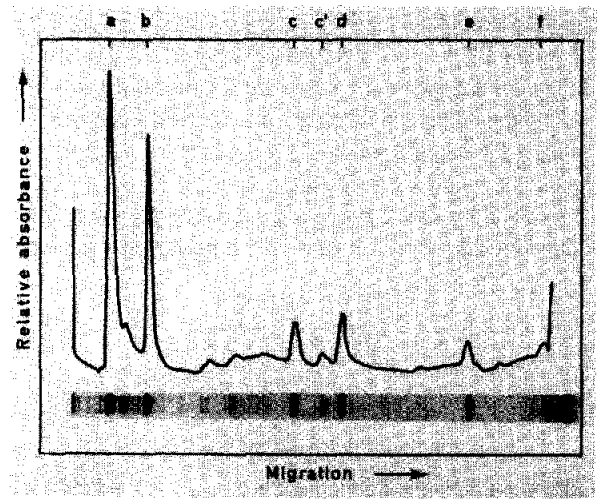

Fig.2. SDS–polyacrylamide gel electrophoresis of sucrose gradient purified RNA polymerase I in 12% gels according to [24] below the corresponding densitometer tracing is shown.

by SDS–polyacrylamide gel electrophoresis, is shown in fig.2. The enzyme is composed of 6 subunits, the largest of which exhibit app. M_r of 195 000 and 125 000. The smaller ones range between ~50 000–20 000 M_r (table 2). Five of the 6 subunits (a–e) are found in ~1:1 molar ratio. The molar proportion of the smallest of the 6 subunits has not been determined, and it is at present unknown, whether subunit c', present in sub-equimolar amounts (0.3–0.7) is a real

Table 2
Subunit composition of RNA polymerase I from *D. hydei* pupae

Subunit	M_r	Molar ratio
a	195 000	1.0
b	125 000	1.1
c	48 000	0.9
c'	41 000	0.3–0.7
d	38 000	1.4
e	25 000	1.1
f	18 500	n.d.

The subunit composition of RNA polymerase I was examined on 12% acrylamide gels as indicated in the legend to fig.2. As standards myoglobin (17 200), trypsin inhibitor from soybean (21 500), glyceraldehyde phosphate dehydrogenase (36 000), bovine serum albumin (68 000), phosphorylase *a* (100 000), β-galactosidase (120 000) and the 2 large subunits of *D. hydei* RNA polymerase II (135 000 and 175 000) were used. Molar ratios were normalized to polypeptide a; that for subunit f was not determined

enzyme subunit in an heterogeneous RNA polymerase I preparation, a loosely bound enzyme component or a contaminant. In any case, the structure of the enzyme is in excellent agreement with that of other class I RNA polymerases [17] and clearly distinct from the α-amanitin resistant *Drosophila* RNA polymerase III isolated [9]. The enzyme eluting first from phosphocellulose, not purified to homogeneity, seems to lack the 48 000 M_r subunit (c) (not shown), a phenomenon also described for other class I enzymes [17].

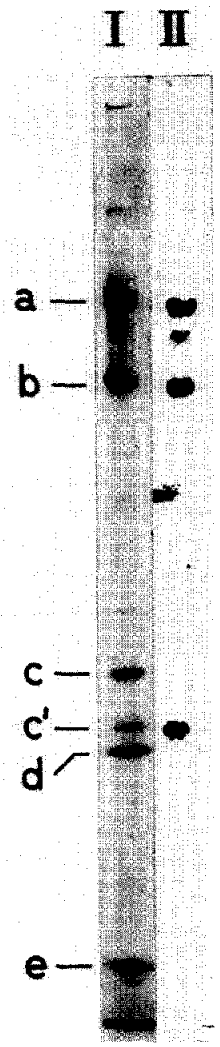

Fig.3. DNA binding capacities of *D. hydei* RNA polymerase I subunits. RNA polymerase I (15 μg) (sucrose gradient fraction) were electrophoresed on a 10% gel under denaturating conditions [24] (lane I) and blotted to nitrocellulose filters [25]. For DNA binding, filters were incubated at room temperature with 0.1 μg tritium-labelled adenovirus 2 DNA (spec. act. 3 × 10^7 dpm/μg) in 0.25 ml DNA binding buffer [18]. Filters were washed and processed for fluorography according to B [18] and exposed for 2 days to a Kodak X-ray film (lane II).

With regard to presumptive functions of the different subunits in the RNA polymerase I reaction, we note a strong binding capacity of the 2 large subunits of *Drosophila* RNA polymerase I for DNA as determined by the protein blotting method [18] (fig.3). For the smaller subunits listed in table 2, no DNA binding capacity was observed, except for the minor peptide component (c', see above), which effectively binds native DNA. Based on indirect evidence, an interaction of the 2 large subunits of yeast RNA polymerase I with DNA was suggested to be essential in the RNA polymerization process [19]. Our results directly support this view and additionally demonstrate the capacity of each of the 2 large subunits to individually bind DNA. (A more detailed characterization of DNA-subunit interactions will be published elsewhere.)

Preliminary characterization of the catalytic properties of the isolated enzyme supports its classification as a class I enzyme. A main characteristic is that the enzymatic activity is resistant to α-amanitin to a degree typical for the enzymes of this class [17] (fig.4a). Due to the exceptionally high α-amanitin

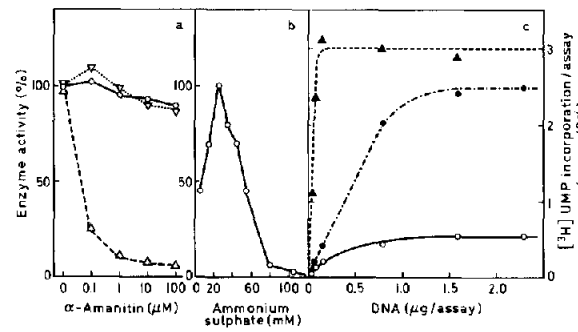

Fig.4. α-Amanitin resistance, salt requirement and template dependence of [^{3}H]UMP incorporation catalyzed by RNA polymerase I. (a) α-Amanitin resistance of the RNA polymerase I reaction was determined with 5 μl enzyme (phosphocellulose fraction) under standard assay conditions and increasing amounts of α-amanitin (o——o). α-Amanitin resistances of *D. hydei* RNA polymerase II (△– – –△) and III (▽. . .▽), determined under the same conditions are shown for comparison. (b) Ammonium sulphate dependence of the RNA polymerase I reaction was assayed under standard assay conditions with various salt concentrations (o——o). (c) Template dependence was determined with 0.5 μg RNA polymerase I (sucrose gradient fraction) in the presence of increasing amounts of recombinant plasmid pDH2-B6, bearing *D. hydei* ribosomal DNA [26]. (o–.–o) plasmid form I, (o——o) plasmid linearized with restriction endonuclease *Bam*HI and (▲– – –▲) heat-denatured linearized form.

resistance of insect class III enzymes [9,20], a distinction between insect class I and III RNA polymerase actually cannot be made using inhibitor resistance as a criterion as illustrated in fig.4a. Like many other enzymes of this class [21], *Drosophila* RNA polymerase I has a sharp salt optimum in the low salt range (fig.4b). In apparent contrast to the behavior of animal class I enzymes (e.g., [22]), the *Drosophila* RNA polymerase I activity is clearly higher on a denatured template than on a native one, both at non-saturating and saturating template concentrations. Since this low activity ratio on native *vs* denatured DNA was found both with standard DNA preparations (calf thymus; not shown), and with a largely intact linearized plasmid DNA (fig.4c), the observed difference between the insect and the mammalian enzymes is probably not due solely to differences in the integrity of the native DNA template used. In agreement with [23], the tertiary structure of the DNA appears to greatly influence the DNA template activity, since supercoiled DNA was found to be transcribed significantly better than a linear DNA duplex by *Drosophila* RNA polymerase I.

In summary, a method for the successful preparation of *Drosophila* RNA polymerase I, in its structural and functional characteristics presumably characteristic for insect class I RNA polymerases in general, has been described. In view of the instability of *Drosophila* RNA polymerases in the initial stages of purification [6,7], a purification scheme is proposed which allows rapid processing particularly during the early steps of enzyme preparation. As a result, a homogeneous enzyme preparation is obtained in a yield comparable to that for RNA polymerases II or III isolated from the same organism, with spec. act. ~15 units/mg*. The functional and structural characterization shows it to be similar to previously isolated homologous animal enzymes and clearly distinct from *Drosophila* RNA polymerase III. The binding capacities of the large subunits of RNA polymerase I for DNA could be demonstrated by the separation of individual subunits and using the protein blotting method.

Acknowledgements

The generous support of this work by Professor W. Beerman is gratefully acknowledged. Thanks are due to R. Renkawitz-Pohl for providing pDh2-B6 plasmid, and to P. Hardy and P. Symmons for critically reading the manuscript.

* One unit of enzyme converts 1 nmol UTP into acid-precipitable material in 20 min in the standard assay [8]

References

[1] Wu, G. J. (1978) Proc. Natl. Acad. Sci. USA 75, 2175–2179.
[2] Luse, D. S. and Roeder, R. G. (1980) Cell 20, 691–699.
[3] Grummt, I. (1981) Proc. Natl. Acad. Sci. USA 78, 727–731.
[4] Kohorn, B. D. and Rae, P. M. M. (1982) Proc. Natl. Acad. Sci. USA 79, 1501–1505.
[5] Philipps, J. P. and Forrest, H. S. (1973) J. Biol. Chem. 248, 265–269.
[6] Adouette, H., Clément, J. M. and Hirshbein, L. (1974) Biochimie 56, 335–340.
[7] Gross, R. H. and Beer, M. (1975) Biochemistry 14, 4024–4028.
[8] Greenleaf, A. L. and Bautz, E. K. F. (1975) Eur. J. Biochem. 60, 169–179.
[9] Gundelfinger, E., Saumweber, H., Dallendörfer, A. and Stein, H. (1980) Eur. J. Biochem. 111, 395–401.
[10] Greenleaf, A. L., Krämer, A. and Bautz, E. K. F. (1976) in: RNA Polymerase (Losick, R. and Chamberlin, M. eds) pp. 793–807, Cold Spring Harbor Lab., New York.
[11] Long, E. O. and Dawid, I. (1979) Cell 18, 1185–1196.
[12] Boyd, J. B. (1975) Methods Cell Physiol. 10, 135–146.
[13] Lukács, N. and Stein, H. (1976) FEBS Lett. 69, 295–299.
[14] Hausen, P. and Stein, H. (1970) Eur. J. Biochem. 14, 278–283.
[15] Chambon, P., Gissinger, F., Kedinger, C., Mandel, J. L. and Meilhac, M. (1974) in: The Cell Nucleus (Busch, H. ed) pp. 270–307, Academic Press, New York.
[16] Huet, J., Wyers, F., Buhler, J. M., Sentenac, A. and Fromageot, P. (1976) Nature 261, 431–433.
[17] Chambon, P. (1975) Annu. Rev. Biochem. 43, 613–637.
[18] Bowen, B., Steinberg, J., Laemmli, U. K. and Weintraub, H. (1980) Nucleic Acids Res. 8, 1–20.
[19] Valenzuela, P., Bull, P. and Zaldivar, J. (1978) Biochem. Biophys. Res. Commun. 81, 662–666.
[20] Sklar, V. E. F., Jaehning, J. A., Gage, P. and Roeder, R. G. (1976) J. Biol. Chem. 251, 3794–3800.
[21] Roeder, R. G. (1976) in: RNA Polymerase (Losick, R. and Chamberlin, M. eds) pp. 285–329, Cold Spring Harbor Lab., New York.
[22] Gissinger, F., Kedinger, C. and Chambon, P. (1974) Biochimie 56, 319–333.
[23] Mandel, J. L. and Chambon, P. (1974) Eur. J. Biochem. 41, 367–378.
[24] Laemmli, U. K. (1970) Nature 227, 680–685.
[25] Risau, W., Saumweber, H. and Symmons, P. (1981) Exp. Cell. Res. 133, 47–54.
[26] Renkawitz-Pohl, R., Glätzer, K. H. and Kunz, W. (1980) Nucleic Acids Res. 8, 4593–4611.