Vol. 82, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1978 Pages 364-370

IN VITRO TRANSCRIPTION OF DROSOPHILA RIBOSOMAL GENES USING DROSOPHILA RNA POLYMERASES

Donal S. Luse and Michael Beer

Thos. C. Jenkins Department of Biophysics
Johns Hopkins Univ., Baltimore, Md. 21218

Received March 23,1978

ABSTRACT

Drosophila RNA polymerases I & II were used to transcribe a recombinant bacterial plasmid containing one copy of Drosophila ribosomal DNA. Both supercoiled and relaxed, closed circular plasmids were used. With Mg^{+2} as the divalent cation, enzyme I is much more active on both forms of the plasmid; the relaxed form in particular supports almost no RNA synthesis by enzyme II. When Mn^{+2} is present, differences in template efficiencies are minimal. The differences observed in the absence of Mn^{+2} seem to depend only on different preferences for the physical state of the template and not on recognition of specific promotor sequences, since enzyme I shows no strand selection when transcribing these plasmids.

INTRODUCTION

Numerous attempts have been made to devise faithful in vitro RNA transcription systems using eukaryotic RNA polymerases. A central problem in the development of such systems has been determining the appropriate physical state of the DNA to be used. Several recent papers have demonstrated that native (1,2), reconstituted (3), and fixed (4) chromatins all may display some transcriptional specificity with added RNA polymerase. These systems contain the majority of the chromosomal proteins, both the histones, which are involved in maintaining the DNA in a superhelical state in the nucleosome (5), and the acidic proteins, which are presumed to have sequence-specific functions.

It has been shown that the use of supercoiled protein-free DNA templates produces a large increase in transcriptional efficiencies (6). In view of this fact, and in view of the above-mentioned success in faithfully transcribing templates which are supercoiled by chromosomal proteins, it is important to ask whether supercoiled, protein-free DNA templates will support faithful RNA synthesis. In order to address this question, a model system was developed using highly purified Drosophila melanogaster RNA polymerases and Drosophila

0006-291X/78/0821-0364$01.00/0

melanogaster ribosomal genes incorporated into bacterial plasmids. The plasmids were prepared both in the native, supercoiled form and in the relaxed, closed circular form. The use of such templates virtually eliminates DNA nicks and ends, which are known to be aberrant initiation sites (7), and allows one to examine the effects of the state of coiling of the DNA on transcription in a homologous system.

MATERIALS AND METHODS

Crude Drosophila RNA polymerases (Fraction 3) were prepared from third instar larvae as described (8). The enzymes were further purified by polyethylene glycol precipitation and DEAE cellulose step elution (9), and were then separated by elution from DEAE Sephadex (8). The pooled separated enzymes were further purified by elution from phosphocellulose or heparin-Sepharose (10). After concentration, the separate pools were sedimented through glycerol gradients; the final enzyme preparations were essentially free of DNAse activity as assayed by relaxation of supercoiled colE1 DNA. Wheat germ RNA polymerase II was prepared as described by Jendrisak & Burgess (11) and was then sedimented through glycerol gradients.

The recombinant bacterial plasmid, pDmr-Y22, was kindly provided by Dr. Igor Dawid. It contains one repeat unit of Drosophila ribosomal DNA, cleaved at the single EcoR1 restriction site and inserted into the plasmid pMB-9. The repeat unit is diagrammed in Fig. 1; note also from this figure that a restriction fragment, designated "A", containing the promotor region may be prepared with restriction enzyme Hind III. Relaxed, closed plasmids were made from the supercoiled form using an Xenopus laevis nicking-closing enzyme (the method for preparing this enzyme was provided by Dr. R. Reeder); sedimentation of this digest through ethidium Br-CsCl removed contaminating nicked material. Strand separation of the EcoR1 fragment was accomplished by base denaturation followed by gel electrophoresis. DNA was isolated from agarose gels by the freeze-centrifugation method (12). All experiments with recombinant DNA were done in accordance with the published NIH guide lines.

Polymerase assays and RNA syntheses were performed essentially as described (8), except that, for tests involving the plasmid DNAs, DNA concentration was reduced to 0.5μg/assay tube (110μl), $(NH_4)_2SO_4$ concentration was .018M, and Mn^{+2} was not used unless otherwise noted.

RESULTS

With Mg^{+2} as the divalent cation, enzyme I is much more active than enzyme II on both forms of the plasmid. Three separate polymerase preparations were used in these studies; Fig. 2 shows a 40 min. time course of the RNA synthesis for one of the three preparations. In order to compare the behavior of the two enzymes we have computed the ratio of enzyme I to enzyme II activity on each of the templates used, correcting to equal input activities on calf thymus DNA. (This correction is based on the fact that purified eukaryotic RNA polymerases have very similar specific activities on commercial calf thymus DNA,

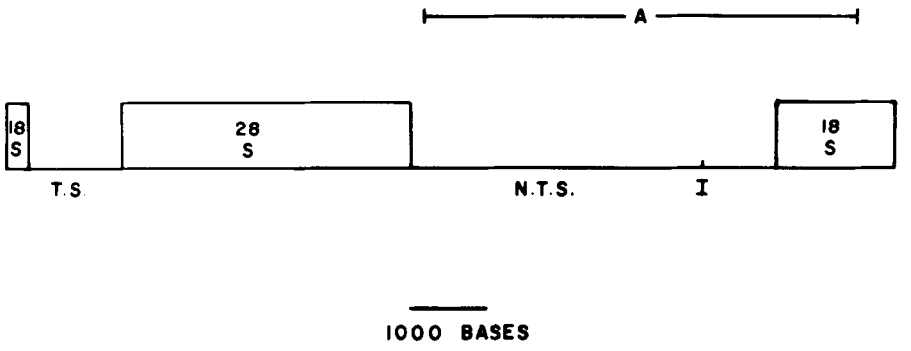

FIGURE 1. The R1 fragment of the plasmid, pDmr-Y22. "A" = subfragment produced by further cleavage with Hind-III. T.S = transcribed spacer; N.T.S. = non-transcribed spacer; I = site of initiation in vivo with transcription proceeding 18S→28S. (see 19)

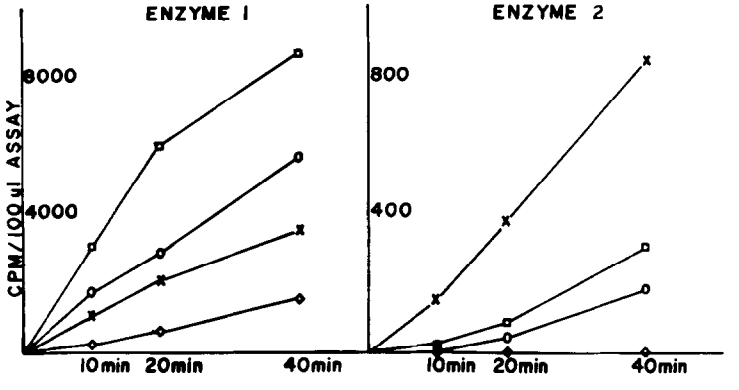

FIGURE 2. Rates of synthesis for enzymes I & II on the following templates: - □ -, Col E1 DNA; -O- Supercoiled plasmid (Y-22) DNA; - ◇ -, Relaxed plasmid (Y-22) DNA; -X- Calf thymus DNA.

probably due to random initiation at nicks (13).) These ratios are presented in Table 1.

The high template efficiencies of enzyme I, relative to enzyme II, on the plasmids could reflect the presence of a physiological initiation site for enzyme I, but not for enzyme II, on these templates. To test this, the activities of the two enzymes were measured on colE1 DNA (a supercoiled bacterial plasmid without Drosophila insertions). As can be seen in Table 1 and Fig. 2, the

TABLE 1. The ratio (activity of enzyme 1/activity of enzyme 2) divided by the ratio (activity of enzyme 1 on calf thymus/activity of enzyme 2 on calf thymus). ∞ = no measurable synthesis by enzyme 2 at the indicated time.

PREP #	TEMPLATE	10 min	20 min	40 min
1	SUPERCOILED	4.9	4.6	3.5
	RELAXED	4.3	8.6	9.7
2	SUPERCOILED	28.8	26.2	20.0
	RELAXED	∞	18.8	12.1
3	SUPERCOILED	∞	12.1	7.8
	RELAXED	∞	∞	∞
	COL E1	20.9	12.9	6.9

activities of the enzymes on this template are very similar to their activities on the supercoiled, ribosomal DNA containing plasmid. This result suggested that the enzymes were responding, not to DNA sequence, but to DNA configurations. To investigate this more precisely, the strand selection of enzyme I under those conditions which most favored transcription by enzyme I relative to enzyme II was examined. RNA complimentary to relaxed plasmids was synthesized and hybridized to separated strands of the ribosomal gene region (the EcoR1 fragment) of the plasmid. No preference for the coding strand was seen, regardless of whether the cRNA used was total cRNA or a fraction of cRNA recovered from a previous hybridization to the HindIII restriction fragment of the plasmid (fragment 'A' in Fig. 1) which bears the in vivo promotor region. The hybridization data are in Table 2. Further, competition hybridization to the gene region of the plasmid with unlabeled Drosophila ribosomal RNA (data not shown) produced a drop in hybridization consistent with uniform reading of both strands.

Mn^{+2} was not used in any of the above experiments because it is thought to promote denaturation of the DNA double helix by intercalation (14); such denatured regions could be aberrant initiation sites. When Mn^{+2} was used in one set of syntheses, the difference in template efficiencies was abolished for the supercoiled template and was greatly reduced for the relaxed template (See Table 3). Since Mn^{+2} is most effective in enhancing enzyme II activity on the supercoiled plasmid, which is already partially denatured by the supercoiling

TABLE 2. Hybridization studies. Hybridization to separated stands of the R1 fragment of Y22. For the total cRNA, 3 sets of filters used (coding, anti-coding and blank), 5000 CPM/set. For the RNA melted off after hybridization to Hind III restriction fragment "A" (see figure 1), only one set of filters used, 500 CPM/set. Hybridization was for 21 hr at 60° C in 3xSSC.

	CPM/filter input : total cRNA	CPM/filter input: melt-off restriction fragment A
CODING STRAND	146	29
ANTI-CODING STRAND	164	29

(14), it seems probable that enzyme II is deficient in its ability to initiate on native (completely base-paired) templates. It could be argued that enzyme II had lost selectively during purification some subunit responsible for initiation on native templates (see 15). To test this possibility and also to see if this effect is peculiar to Drosophila, we purified RNA polymerase II from a different organism (wheat germ) using a different method (polyethylenimine precipitation, (11)). When this enzyme was used to transcribe the plasmids, a very low activity on the relaxed template in the absence of Mn^{+2} was once again observed (Table 3). This argues against the low activities of Drosophila enzyme II on native templates being the result of damage during purification.

DISCUSSION

Our laboratory has recently demonstrated that squash preparations of Drosophila polytene chromosomes will support RNA synthesis by added Drosophila RNA polymerase II, but not by RNA polymerase I (4). The results presented here show that these enzymes (further purified to remove residual DNAse) will under some conditions show differences in transcriptional efficiencies. However, our evidence indicates that specific initiation is not occurring, regardless of the state of coiling of the DNA used as template. (It should be noted that deproteinized, supercoiled DNAs are not exact models for the supercoiled DNA in chromatin; see (16)).

It seems reasonable to conclude that the difference in template efficien-

TABLE 3. Activities of 100 µl assay mixes, incubated for 40 min, using Drosophila enzymes I & II from prep #3, or wheat germ enzyme II.

	Drosophila RNA polymerase I		Drosophila RNA polymerase 2		Wheat germ RNA polymerase 2	
	Mg	Mg+Mn	Mg	Mg+Mn	Mg	Mg+Mn
SUPERCOILED	5781	15624	180	12669	8605	28899
RELAXED	1556	2522	0	606	192	1344
COL E1	8591	24264	304	45588	26974	50916
CALF THYMUS	3451	12691	842	9371	9523	22648

cies between the enzymes is based on the reduced ability of enzyme II, relative to enzyme I, to initiate on templates with no single-stranded character. This is supported by the fact that the relaxed plasmids, which are transcribed at very low levels by II, can be made acceptable templates by either the introduction of supercoils or the presence of Mn^{+2}; both of these perturbations favor the formation of single-stranded regions in the double helix (14). In regard to these points it is important to note a recent finding concerning the state of coiling of the genome. Foe, et al (17) have concluded, on the basis of electron microscopic observations, that in the milkweed bug nucleolar transcription units and adjacent promotor regions are relaxed and show no compaction into nucleosomes whereas non-nucleolar transcription units are compacted into nucleosomes. The different template preferences we observe are consistent with the different configurations of transcriptionally active regions which are described by these authors.

While this work was in progress, several important findings appeared all of which supported the concept that, in addition to the appropriate RNA polymerase and DNA, chromosomal proteins are necessary for transcriptional specificity. Parker and Roeder (1) reported that very specific transcription of 5S RNA could be observed upon adding Xenopus RNA polymerase III to the homologous chromatin; however, no specificity was seen when Xenopus DNA was used as template. Matsui, et al (2) showed highly preferential synthesis of ribosomal RNA in isolated nucleoli with added RNA polymerase I but no specific synthesis on isolated nucleolar DNA. In this laboratory, it was shown that Drosophila RNA polymerases

I and II will show transcriptional selectivity on salivary gland chromosome squash preparations but not on deproteinized squashes (4). With the purified plasmid DNA system described here, we attempted, by the elimination of nicks and ends and by the presentation of the template to the enzyme in various states of coiling, to improve on the results of other purified DNA systems. Our results indicate, unfortunately, that transcription is not detectably better than random. These findings, together with the recent results obtained by Parker, et al (18) using Xenopus 5S DNA plasmids and the homologous enzyme III, suggest that manipulation of the DNA state alone is not likely to yield faithful transcription in vitro.

ACKNOWLEDGEMENTS

We would like to thank Dr. Igor Dawid, who provided us with the recombinant plasmid, pDmr-Y22. We also thank Drs. R. Reeder and E. Weinberg for advice on experiments. This work was supported by NIH grant GM-08968 to Dr. M. Beer.

BIBLIOGRAPHY

1. Parker, C. S. and Roeder, R. G. (1977) Proc. Nat. Acad. Sci. USA 74, 44-48.
2. Matsui, S., Fuke, M. and Busch, H. (1977) Biochemistry 16, 39-45.
3. Daubert, S., Peters, D. and Dahmus, M. E. (1977) Arch. Biochem. Biophysics 178, 381-386
4. Gross, R. H., Luse, D. S. and Beer, M. (1977) Exp. Cell Res. 106, 412-417.
5. Felsenfeld, G. (1978) Nature 271, 115-122.
6. Mandel, J. L. and Chambon, P. (1974) Eur. J. Biochem. 41, 367-378.
7. Chambon, P., Gissinger, F., Mandel, J.L., Kedinger, C., Gniazdowski, M. and Meihlac, M. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 693-708.
8. Gross, R. H. and Beer, M. (1975) Biochemistry 14, 4024-4031.
9. Greenleaf, A.L. and Bautz, E. K. F. (1975) Eur. J. Biochem. 60, 169-179.
10. Greenleaf, A. L., Haars, R. and Bautz, E. K. F. (1976) FEBS Letters 71, 205-208.
11. Jendrisak, J. J., and Burgess, R. R. (1975) Biochemistry 14, 4639-4645.
12. Gross, K., Schaffner, W., Telford, J. and Birnstiel, M. (1976) Cell 8, 479-484.
13. Schwartz, L. B., Sklar, V. E. F., Jaehning, J. A., Weinmann, R. and Roeder, R. G. (1974) J. Biol. Chem. 249, 5889-5897.
14. Hossenlopp, P., Oudet, P. and Chambon, P. (1974) Eur. J. Biochem. 41, 397-411.
15. Huet, J., Buhler, J. M., Sentenac, A. and Fromageot, P. (1975) Proc. Nat. Acad. Sci. USA 72, 3034-3038.
16. Camerini-Otero, R. D., and Felsenfeld, G. (1977) Nuc. Acids Res. 4, 1159-1181.
17. Foe, V. E., Wilkinson, L. E. and Laird, C. D. (1976) Cell 9, 131-146.
18. Parker, C. S., Ng, S., and Roeder, R. G. (1976) Molecular Mechanisms in the Control of Gene Expression (D. Nierlick, W. J. Rutter and C. F. Fox, eds.) pp. 223-242, Academic Press, New York.
19. White, R.L. and Hogness, D.S. (1977) Cell 10, 177-192.