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Transcription of cloned tRNA and 5S RNA genes in a Drosophila cell free extract

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ABSTRACT

We describe the preparation of a cell-free extract from Drosophila Kc cells which allows transcription of a variety of cloned eukaryotic RNA polymerase III genes. The extract has low RNA-processing nuclease activity and thus the major products obtained are primary transcripts.

INTRODUCTION

RNA polymerase III transcription systems have been prepared from a number of higher eukaryotes. Extracts of Xenopus laevis germinal vesicles (1,2), X. laevis oocytes (3), X. laevis kidney cells (4), human KB cells (4,5), mouse plasmacytoma cells (4) and Bombyx mori posterior silk glands or ovaries (6) transcribe various tRNA genes as well as 5S RNA and VA RNA genes. Since we are interested in identifying the DNA signals which regulate Drosophila tRNA gene expression (7-9) we required an homologous transcription system. In this paper we report the preparation of a cell-free extract from Drosophila Kc cells which transcribes several classes of RNA polymerase III genes.

MATERIALS AND METHODS

Cell Culture. Drosophila Kc cells were grown in suspension culture at 24°C in D20 Eschalier medium (without serum) supplemented with 0.005% (w/v) penicillin and streptomycin (10).

Plasmid DNA. Plasmid DNA was prepared by standard procedures (11). The recombinant DNA plasmid pYH48 contains a single Drosophila $tRNA_2^{Arg}$ gene within a 508bp HindIII fragment inserted into pBR322 (12). Additional cloned tRNA genes and 5S RNA genes used were: pYM3.2, containing a Schizosaccharomyces pombe 5S RNA gene (13); $ptRNA^{Ala}$ 18 containing a B. mori $tRNA^{Ala}$ gene (14); pCRI-MetA1 containing a Xenopus laevis

$tRNA_i^{Met}$ gene (15); psup4-o containing the Saccharomyces cerevisae sup4-o $tRNA^{Tyr}$ (16); pJB20m, containing an S. cerevisiae $tRNA^{Tyr}$ gene (17); pYM7.2, containing an S. pombe $tRNA^{His}$ gene (18); pPW516.6, containing $tRNA_2^{Lys}$ gene (7); p50AB, containing Drosophila $tRNA^{Leu}$ and $tRNA^{Ile}$ genes (19); pPW539, containing a Drosophila $tRNA_i^{Met}$ gene (S. Sharp, unpublished results); p12D1, containing Drosophila 5S RNA genes (20).

Preparation of Drosophila Kc cell extract. Extracts were prepared essentially by modifications of the methods of Wu and Zubay (21) and Weil et al. (4). Cells were harvested by centrifugation at 600 x g and washed three times at 4°C by repeated resuspension in 50 volumes of a buffer containing 30 mM Hepes-KOH, pH 8.0, 120 mM KCl, 5 mM $MgCl_2$ and 0.5 mM DTT. The packed cell volume was determined after centrifugation at 800 x g for 5 min and the cells were resuspended in 2 volumes of a hypotonic buffer containing 10 mM Hepes-KOH, pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$ and 0.5 mM DTT. After swelling on ice for 1 h cells were broken using 12 strokes of the tight pestle of a Dounce homogenizer (Wheaton, USA). Cell lysis was generally greater than 90% with minimal breakage of nuclei (determined by phase contrast light-microscopy). To the lysate 0.125 volumes of a solution of 0.23 M Hepes-KOH, pH 8.0, 1.2 M KCl, 0.04 M $MgCl_2$ and 0.05 M DTT was added and the lysate was centrifuged at 100,000 x g for 60 min. The supernatant was collected and glycerol was added to a final concentration of 20% (v/v). Creatine phosphokinase was added directly to the supernatant to a final concentration of 12 U/ml. The extract was frozen in liquid nitrogen and stored at -70°C.

In vitro Synthesis and Electrophoretic Analysis of RNA. Unless otherwise indicated transcription assays were performed at 24°C in a final reaction volume (0.04 ml) of 30 mM Hepes-KOH, pH 8.0, 100 mM KCl, 3 mM $MgCl_2$ and 3 mM DTT, 0.5 mM of each of the three unlabeled ribonucleoside triphosphates, 0.025 mM of either (α-32P)GTP or (α-32P)UTP (2-10 Ci/mmole), 0.24 units creatine phosphokinase (contained in the cell extract), 8 mM creatine phosphate; 0.02 ml cell-free extract and 0.5 ug plasmid DNA. X. laevis germinal vesicle (GV) extract transcription reactions were performed as previously described (2).

Reactions were started by the addition of cell-free extract and incubated at 24°C for the times indicated. Synthesis was terminated and RNA collected as described previously (12). RNAs were dissolved in 2 ul of a solution containing 80% (v/v) formamide, 1 mM EDTA, o.1% (w/v) bromphenol blue and 0.1% (w/v) xylene cyanol FF and analysed by electrophoresis on 10%

polyacrylamide thin-gels containing 8 M urea (22). Radioactivity was measured by counting Cerenkov radiation of the excised gel pieces.

Fingerprint and Nucleotide Analysis of RNAs. RNAs were eluted from gel slices by incubating overnight at 37°C in a solution of 0.3 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, 1% (w/v) phenol. RNA was collected by repeated ethanol precipitation from 0.3 M sodium acetate, pH 5.5 at -70°C, then digested with appropriate ribonucleases. Transcripts were analyzed by standard fingerprinting methods (23).

RESULTS

Comparison of Primary Transcripts Synthesized by X. laevis GV extract and Drosophila Kc Cell Extract. Cell-free extracts derived by high speed centrifugation of cytoplasmic fractions of cultured cells have been shown to contain 65 - 90% of the cellular eukaryotic RNA polymerase III (see e.g. 24, 25). We adapted these procedures to prepare an extract from Drosophila Kc cells. Nuclei breakage was minimal and confirmed the relative ease of release of RNA polymerase III activity from intact nuclei. The transcripts obtained from both the Drosophila and Xenopus extracts using a cloned Drosophila $tRNA^{Arg}$ gene were indistinguishable in size (Fig. 1) (12). However the predominant RNA (RNA-1)synthesized in the Drosophila extract is precursor tRNA, while in the GV extract significant processing of this product to mature tRNA (RNA-2) occurs. RNA-1 and RNA-2 were recovered by elution and analyzed by RNase T1 fingerprints (Fig. 2). RNA-1 formed in the Xenopus and Drosophila extracts were identical except for some differences in the degree of post-transcriptional base modification. The oligonucleotides designated X and Y were observed in the fingerprints of the Drosophila RNA-1 (Fig. 2B) and the Xenopus RNA-2 (Fig. 2C). These fragments correspond to the modified olignucleotides CAADG and m^1 ACUCCUG, respectively, as judged by nearest neighbor nucleotide analysis. It is known that adenine methyl transferase activity is present in the GV extract and leads to the formation of this modified nucleotide in RNA transcripts (2). The increased post-transcriptional base modification in the precursor RNA synthesized in the Drosophila extract probably arises from the presence of cytoplasmic as well as nuclear components in this extract.

The primary transcription product synthesized in the Drosophila extract in the presence of 5'-(γ-S)ATP and the four ^{32}P-labeled nucleoside triphosphates, was hydrolyzed using RNase A. The (γ-S) oligonucleotide was isolated, analyzed as previously described (12), and found to be pppGpUp.

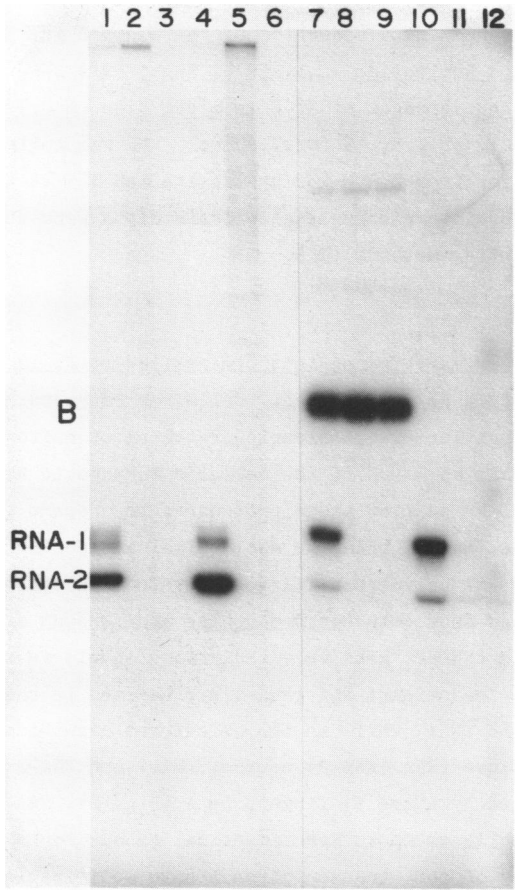

Figure 1. Autoradiogram of in vitro transcription reactions of pYH48 DNA in the Xenopus laevis germinal vesicle system and in a cytoplasmic extract of Drosophila Kc cells. Lanes 1-3 and 7 -9 show reactions using(α-^{32}P) UTP as a radioactive source. In lanes 4 - 6 and 10 -12 (α-^{32}P)GTP was used. Reactions 3,6,9, and 12 contain no DNA, reactions 2,5,8 and 11 contain 0.5 ug of pBR322 and reactions 1,4,7 and 10 contain pYH48 DNA.

Thus the RNA initiation site in this transcript which is the G at position -7 is identical to that formed in the the GV extract. We conclude from 5'-terminal analysis, electrophoretic mobility of the primary transcripts, and the fingerprint comparisons, that transcription of the $tRNA_2^{Arg}$ gene initiates and terminates at the same sequences in the Drosophila Kc cell extract as in X. laevis GV extract.

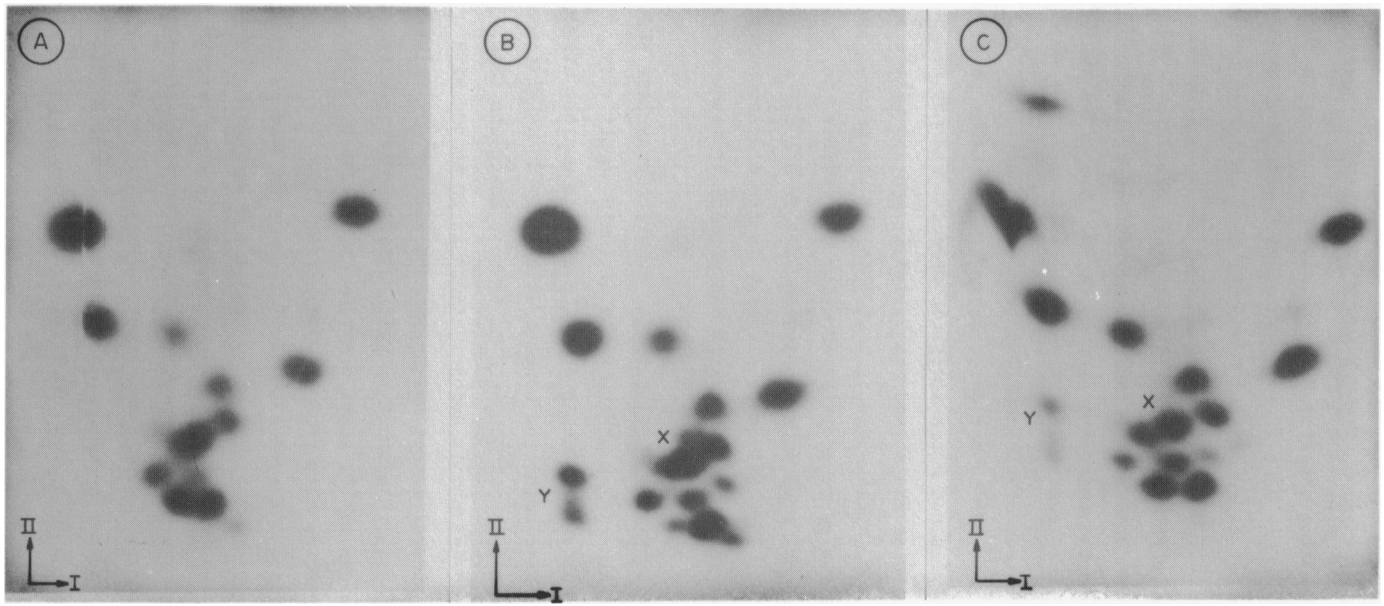

Figure 2. RNase T_1 fingerprints of G-labeled ptRNA^{Arg} (A) and mature tRNA^{Arg} (C) transcribed in the Xenopus laevis GV system and of ptRNA^{Arg} (B) transcribed in the Drosophila system. RNase T_1 fragments were separated in the first dimension by electrophoresis at pH 3.5 on cellulose-acetate and in the second dimension by homochromatography on PEI-TLC plates. The picture given for the mature tRNA^{Arg} from Xenopus GV extract (C) does not show the nucleotide pGp.

Differences between Drosophila and GV Transcriptional Extracts. Compared to the GV extract the Drosophila extract contains a lower level of precursor tRNA processing activity (see above). This suggests that the 5'- and 3'-processing RNases are more strongly associated with nuclear components which are not readily solubilized under the conditions of cell-breakage that we have employed. The nuclear localization of these enzymes in Xenopus was apparent from in vivo (e.g. 26) and in vitro studies (e.g. 2).

Two radioactive RNA species were sometimes observed in the Drosophila transcription system in the absence of any added DNA. Use of (α-^{32}P)UTP in transcription reactions in the absence of DNA resulted in an RNA product which had a slower electrophoretic mobility than precursor tRNA (Fig. 1, lane 3, lanes 7-9). Since this RNA is not detected in transcription reactions with (α-^{32}P)GTP it probably is not a transcription product. Nearest neighbor analysis of the UTP labeled RNA showed 66% U, 16% A, and 16% C labeled whereas G was undetected. This suggests that this RNA was present in the extract and could be labeled by addition of uridylate residues to its 3'-terminus. A different RNA species was observed when (α-^{32}P)GTP was used in transcription reactions. This RNA had a slightly faster electrophoretic mobility than mature-size tRNA and since it was present in trace amounts was not analyzed further.

Properties of Drosophila Kc Cell Extract. Eukaryotic nuclear RNA polymerases have been classified on the basis of their sensitivity to α-amanitin. Transcription activity in the Drosophila extract was not affected by low concentrations of α-amanitin. Concentrations of 200 ug/ml α-amanitin inhibited RNA synthesis only 50% compared to control reactions (Fig. 3). The tolerance of higher α-amanitin concentrations is in accord with other reports on the properties of insect RNA polymerase III (24, 27). The observed specificity of the Drosophila extract to faithfully transcribe a Drosophila $tRNA^{Arg}$ gene leaves little doubt that the major transcriptional activity of this extract results from RNA polymerase III.

The rate of in vitro RNA synthesis was linear up to 2 hours incubation (Fig. 4). After this time a plateau of synthesis was reached which could not be altered by varying the concentrations of added nucleoside triphosphates. However, the addition of an ATP-regenerating system (creatine phosphate/ creatine phosphokinase) to the Drosophila extract stimulated RNA synthesis remarkably (Fig. 4). Whether a component of the RNA polymerase III transcriptional apparatus is energy-requiring or is inhibited by levels of ADP already present in the extract, has not been determined.

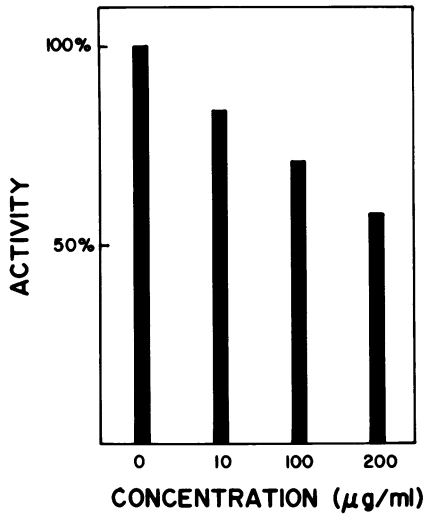

Figure 3. The effect of α-amanitin on pYH48 DNA transcription in the Drosophila extract. The amount of transcription in a reaction without α-amanitin was arbitrarily set as 100%.

Figure 5 demonstrates the effect of changing the concentrations of KCl, $MgCl_2$ and DNA on the transcription efficiency. The optimal concentration of KCl was determined to be 100 mM (Fig. 5A) and the optimal concentration for Mg^{2+} was 3 to 3.5 mM (Fig. 5B). Specific RNA synthesis was linear upon addition of pYH48 DNA up to 25 ug/ml (1 ug/assay). Routinely a template concentration of 12 ug/ml was used (Fig. 5C). Relatively high concentrations (2-5 mM) of polyamines (putrescine, spermine or spermidine) resulted in inhibition of RNA formation, while lower concentrations had no effect (results not shown).

Transcription rates were maintained optimally at 24°C, the temperature of cell growth (Fig. 6). Precursor tRNA processing was accelerated at 30°C, however total RNA synthesis was less than that obtained at 24°C after 2 h of incubation (Fig. 6).

Transcription of Homologous and Heterologous Cloned DNA Templates. In order to check the Drosophila system for its general use we tested a number of RNA polymerase III genes which had previously been transcribed in other systems. The Drosophila extract transcribed all the Drosophila tRNA genes tested (Fig. 7), including a gene for $tRNA^{Leu}$ which contains an intervening sequence (19). Our extract however did not have a high splicing enzyme activity (Fig. 7, lane 9). This is in accord with the finding that

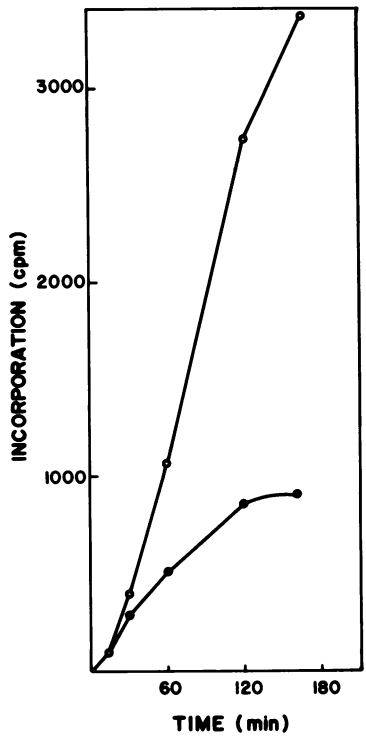

Figure 4. Effect of an ATP regenerating system on transcription in the Drosophila extract. pYH48 was transcribed with (o-o) and without (•-•) the addition of 8 mM creatine phosphate and 12 U/ml creatine phosphokinase. Synthesized RNA (precursor and mature tRNA) was quantitated.

processing nucleases are tightly associated with the nucleus (26). In general the transcription patterns of the various Drosophila templates were identical to those obtained when transcription reactions were performed in Xenopus GV extracts. The size of the primary transcripts were the same in both extracts. 5S RNA genes from Drosophila (Fig. 7, line 11) and Notophtalus vividescens (data not shown), and VA I RNA gene (28) (data not shown) also transcribed efficiently in the Drosophila extract. However, as was found in the GV system (13,29) the S. pombe 5S RNA genes did not transcribe. The ability of the Drosophila extract to transcribe tRNA genes from diverse organisms is shown in Fig. 7, lanes 2-6. The transcription products of genes for B. mori $tRNA^{Ala}$, S. cerevisiae sup4-0 $tRNA^{Tyr}$ and $tRNA^{Trp}$, S. pombe $tRNA^{His}$ and X. laevis $tRNA_i^{Met}$ appear to

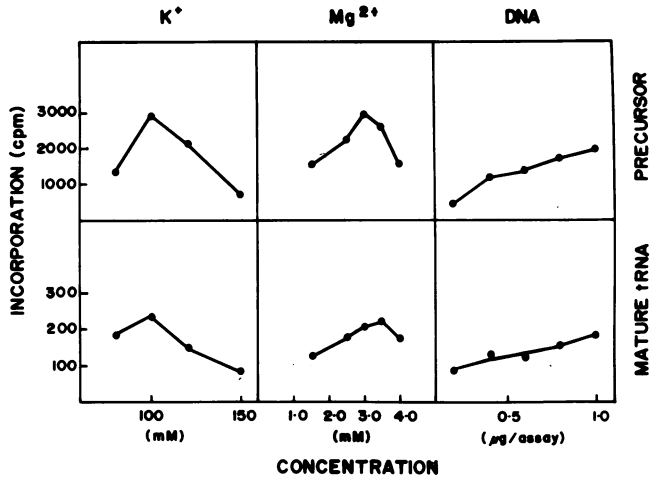

Figure 5. Concentration curves for K^+, Mg^{2+} and DNA in the Drosophila transcription system with pYH48 DNA as template. The precursor RNA and the mature $tRNA^{Arg}$ band were excised and the amount of incorporated radioactivity was determined separately. To determine the K^+ optimum the concentration of Mg^{2+} and DNA was constant at 3 mM and 0.5 ug, respectively. In the reactions with varying amounts of Mg^{2+} the postassium concentration was held constant at 100 mM. To determine the optimal the DNA concentration curve 100 mM K^+ and 3 mM Mg^{2+} were used in transcription reactions.

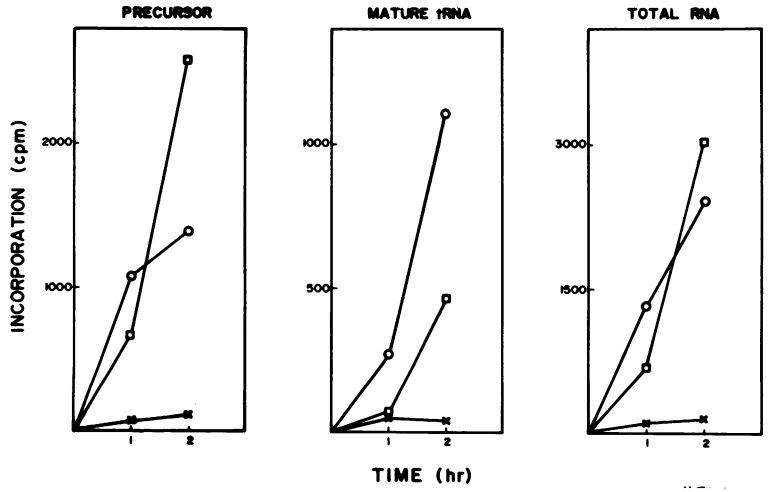

Figure 6. Temperature dependence of pYH48 transcription in the Drosophila extract. Temperature was 12.5°C (x-x), 24°C (o-o) and 30°C (□-□). The amount of radioactivity incorporated into precursor tRNA, mature tRNA and the sum of both were determined.

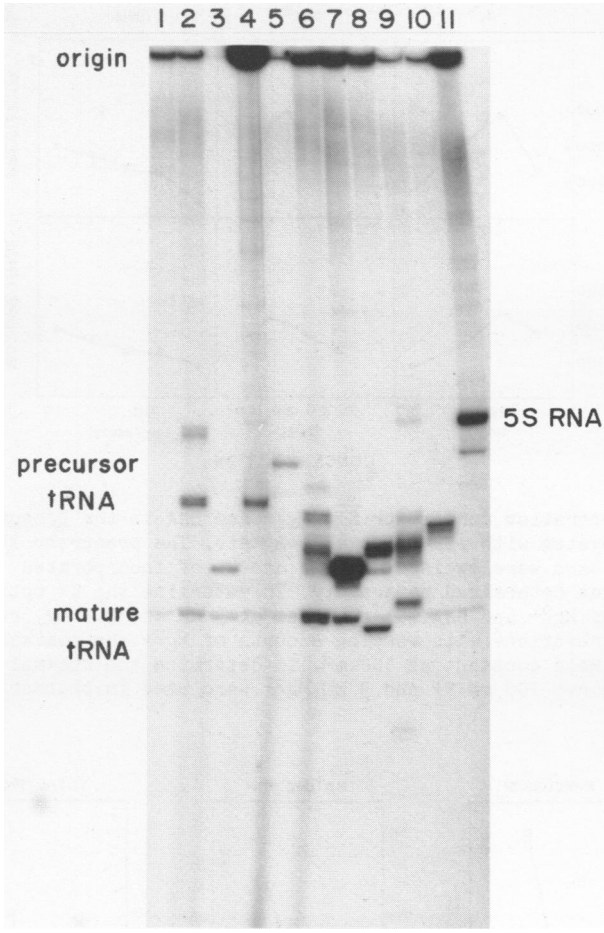

Figure 7. Autoradiogram of the electrophoretic separation of transcription products of heterologous and homologous tRNA and 5S RNA genes in the Drosophila extract. The lanes contain transcripts of the following genes (see Materials and Methods): 1: S. pombe 5S RNA; 2: B. more $tRNA^{Ala}$, 3: X. laevis $tRNA_i^{Met}$; 4: S. cerevisiae sup4-o $tRNA^{Tyr}$; 5: S. cerevisiae $tRNA^{Trp}$; 6: S. pombe $tRNA^{His}$; 7: Drosophila $tRNA_2^{Arg}$; 8: Drosophila $tRNA_2^{Lys}$; 9: Drosophila $tRNA^{Leu}$ and $tRNA^{Ile}$; 10: Drosophila $tRNA_i^{Met}$; 11: Drosophila 5S RNA.

have identical electrophoretic mobilities when transcribed in either the Drosophila extract or Xenopus GV extract. Some S. cerevisiae and S. pombe tRNA genes did not transcribe in the Drosophila extract. However, the same genes also transcribed poorly or not at all in the Xenopus RNA

polymerase III transcription system.

DISCUSSION

We have demonstrated that a cell-free extract derived from *Drosophila* Kc cells faithfully transcribes cloned *Drosophila* tRNA genes. The primary transcripts, as analyzed by RNA fingerprinting, are identical to those obtained in the *Xenopus laevis* germinal vesicle system. This suggests that our earlier data on the transcription of *Drosophila* tRNA genes in the *Xenopus* system can be extended to the homologous system and indicates that our conclusions regarding transcription-initiation and -termination were correct.

The *Drosophila* system was shown to transcribe tRNA genes from *S. cerevisiae*, *S. pombe*, *Xenopus* and *Drosophila*. Transcription of these genes in the *Drosophila* and *Xenopus* systems leads to precursor products of similar size. Since there is much lower processing nuclease activity in the *Drosophila* extract it provides an excellent source of primary transcripts to aid in the study of tRNA processing enzymes.

The *Drosophila* extract transcribes all classes of RNA polymerase III genes tested (adenovirus VA and 5S RNA genes) of higher eukaryotes. However, some yeast tRNA genes and 5S RNA genes are poorly transcribed or not transcribed at all. This indicates that some components of the transcription complex are different in lower eukaryotes from that present in *Drosophila*. Also, we have observed differences in transcription efficiency between other DNA templates. For instance, the *Xenopus* $tRNA_i^{Met}$ gene is transcribed more efficiently in an homologous system than it is in the *Drosophila* system, while *Drosophila* $tRNA_i^{Met}$ genes are transcribed most efficiently in the *Drosophila* system. We are currently studying the components of the *Drosophila* system and *Drosophila* tRNA gene sequences which are responsible for such variation in transcription efficiencies.

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