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Transcription of cloned transfer RNA genes from *Drosophila melanogaster* in a homologous cell-free extract

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ABSTRACT

Cloned Drosophila melanogaster tRNA genes have been transcribed in a homologous cell-free extract isolated from a Schneider II cell line. The major product of the reaction is a tRNA precursor which is processed to a tRNA sized species. The kinetics of transcription has been followed for 5 different valine tRNA gene clones. The results demonstrate formation of a stable transcription complex with at least two kinetic steps. While the rate of formation of the transcription complex is similar for different clones, the ultimate rate of transcription varies dramatically. Comparison of the DNA sequence of the tRNA genes suggests that rate determining nucleotides lie outside the canonical tRNA split-internal promoters.

INTRODUCTION

To identify the components essential for gene expression, cell-free transcription systems in which purified DNA templates can be selectively transcribed have been developed. RNA polymerase III cell-free transcription systems have been prepared from Xenopus oocytes (1), Xenopus kidney cells (2), human KB cells (2,3), mouse plasmacytoma cells (2), HeLa cells (4), Bombyx mori posterior silk glands and ovaries (5), and Drosophila Kc cells (6). These extracts accurately transcribe 5S RNA (1,2,6), tRNA (5, 6,7) and VA RNA (2,3,4,6) genes as well as the Alu family of DNA sequences (8,9).

Fractionation of such cell-free extracts has indicated that there are at least two factors required for tRNA transcription (10) and at least one other factor required for 5S RNA transcription (11,12,13). In the case of 5S RNA transcription, one of the components has been shown to form a stable complex with the DNA and has been implicated in control of 5S RNA gene expression (13).

DNA sequences required for transcription of tRNA genes have been investigated by deleting or altering nucleotides in the genes. Deletions originating at either 5' or 3' end have demonstrated that two internal

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regions of the tRNA coding sequence (the split-promoter) are required (5, 14-17). Nucleotides outside the split-promoter can affect the rates of transcription and the positions of such nucleotides have been set both within the tRNA coding region (17) and in the 5' flanking sequences (4, 5,18). The sequences which alter the rates of transcription are potential targets for regulation of tRNA gene expression.

In this paper we report the characterization of a cell-free transcription system from Drosophila Schneider II cells which accurately transcribes a number of different cloned Drosophila tRNA genes.

MATERIALS AND METHODS

Cell culture. Drosophila Schneider II cells (obtained from C. Laird, U. of Washington) were grown in glass rolling bottles at room temperature (22-25°C) in Drosophila medium (Gibco Lab.) supplemented with 13% fetal bovine serum (Gibco Lab.) and 50 μg/ml gentamycin (Sigma). Cells were harvested at a cell density of approximately 0.5 - 1.0 x 10^7 cells/ml.

Plasmid DNA. The recombinant plasmids used are described in Fig. 5. Plasmids pDt55-0.3 and pDt55-0.6 contain fragments isolated from pDt55 which contained two identical genes coding for $tRNA_4^{Val}$. pDt55-0.3 contains an 0.3 kb fragment bounded by nucleotides 54 and 371 and plasmid pDt55-0.6 carries a 0.6 kb fragment bounded by nucleotide 371 and a HinfI R.endonuclease site at approximately nucleotide 1000. DNA sequences of the $tRNA_4^{Val}$ plasmids are reported in Addison et al. (19) and the sequence of pDt78R has been completed (20). Plasmid DNA was prepared as described by Larsen et al. (21). DNA was purified by two successive cesium chloride equilibrium centrifugation steps.

Preparation of Drosophila cell-free extract (S.100). Cells were harvested by centrifugation and washed three times with 30 mM Tris·HCl (pH 7.9), 120 mM KCl, 5 mM $MgCl_2$ and 0.5 mM DTT. The centrifuged cell pellet was weighed and the cells were resuspended in 2 volumes (1 volume = 1 gm packed cell weight) of a hypotonic buffer [10 mM Tris·HCl (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$ and 0.5 mM DTT]. The cells were allowed to swell for 1 hour on ice and then were broken by 12 strokes of a B-type Dounce homogenizer (cell lysis was generally greater than 95%. To the lysate was added 0.125 cell volume of 0.23 M Tris·HCl (pH 7.9), 1.27 M KCl, 0.04 $MgCl_2$ and 0.5 mM DTT, and the lysate was centrifuged at 100,000 x g for 60 min (2,3). The supernatant was collected, glycerol was added to a final concentration of 20% (v/v) and then stored in aliquots at -70°C.

In vitro transcription and electrophoretic analysis of RNA. Transcription reactions were performed in a final volume of 50 µl containing 20 mM Tris·HCl (pH 7.9), 80 mM KCl, 5 mM $MgCl_2$, 3 mM DTT, 2.5 µg/ml α-amanitin, 6 U/ml creatine phosphokinase, 5 mM creatine phosphate (6), 10% glycerol, 60 µM each of the three unlabelled ribonucleoside triphosphates, 25 µM [α-^{32}P]UTP (3-5 Ci/mmole), plasmid DNA and 25 µl Drosophila S-100 extract.

Transcription reactions were stopped by the addition of 2.5 µl of 2 mg/ml Proteinase K and sodium dodecyl sulfate (SDS) to 0.5%. After 30 min. at room temperature, the mixture was phenol extracted and ethanol precipitated. The RNAs were analyzed by electrophoresis on 10% polyacrylamide gels containing 7 M urea. To determine the radioactivity in specific RNA bands, the appropriate gel bands were excised using the autoradiographs as templates and the Cerenkov radiation was measured.

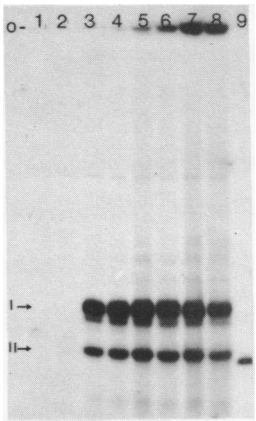
RNA·DNA hybridization. pBR322, pDt41R and pDt78R DNAs were bound to cellulose nitrate filters (Schleicher and Schuell, BA85) and hybridization reactions were carried out as described by Larsen et al. (21).

RNA·DNA hybridizations were performed in 1 ml of a buffer containing 20% formamide, 6XSSC (pH 7.0) and 0.1% SDS at 65°C for 24 hours. The hybridization vial contained 2 blank, 2 pBR322 and either 2 pDt41R or 2 pDt78R filters (6XSSC in 0.9 M NaCl, 0.09 M sodium acetate).

The RNA used for hybridization was obtained from the appropriate excised gel bands by soaking them for 48 hrs. at 4°C in a solution containing 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA and 0.1% SDS and then ethanol precipitation.

RESULTS

Synthesis of distinct RNA species directed by plasmid DNA containing Drosophila tRNA gene. The cell-free extract from Drosophila Schneider II cells was tested for its ability to initiate specific transcription on an exogenous template containing a $tRNA^{Val}_{3b}$ gene. Fig. 1 shows the polyacrylamide gel electrophoresis of the RNAs synthesized by the extract. Incubation with increasing concentrations of DNA containing a cloned tRNA gene (lanes 3 to 8) resulted in the synthesis of two major products (designated RNA-I and RNA-II) which were not present in a minus DNA control (lane 1) or when pBR322 DNA was added (lane 2). The larger RNA, RNA-I, was the predominant product in transcription reactions. The size of RNA-I produced in the reaction varied with the DNA template; for example, the RNA-I product



RNA species	cpm hybridized				% input cpm hybridized	
	Vial 1		Vial 2			
	pBR322	pDt78R	pBR322	pDt78R	pDt78R	pDt41R
RNA-I	220	7517	147	5794	20.0	15.5
RNA-II	159	1408	141	680	3.7	1.8

Figure 1. Transcription of pDt78R and hybridization of *in vitro* products. Left portion shows an autoradiogram of gel analysis of RNA products directed by increasing concentrations of pDt78R DNA (lanes 3-8). Lane 1 shows endogenous transcription and lane 2 shows transcription directed by pBR322 DNA. Lane 9 is a $tRNA_4^{Val}$ marker. Right portion shows the results of hybridization of isolated RNA-I and RNA-II to nitrocellulose bound plasmid DNA. Each hybridization contained approximately 36,000 cpm of transcription product.

directed by pDt55-0.3 DNA was estimated to be 100 nucleotides long, while the RNA-I product in Fig. 1 was 95 nucleotides. However, the size of RNA-II, the smallest of the specific RNA products, was constant for all DNA templates. Different templates showed different patterns of bands between RNA-I and RNA-II and different ratios of RNA-I:RNA-II. Processing experiments in which RNA-I was isolated from acrylamide gels and reincubated with S-100 extract showed that RNA-I could be converted to RNA-II (data not shown).

To demonstrate that RNA-I and RNA-II were copies of the tRNA gene, labeled *in vitro* product directed by pDt78R was eluted from bands on polyacrylamide gels and hybridized to nitrocellulose bound pDt78R and pDt41R plasmid DNA, both of which contain *Drosophila* $tRNA_{3b}^{Val}$ genes (20,22). Analysis of heteroduplexes between pDt78R and pDt41R has shown that the only homology in the *Drosophila* insert lies in the tRNA gene sequence (22). Thus, if RNA-I and RNA-II were authentic tRNA transcripts, they should hybridize to both pDt78R and pDt41R DNAs. The result of one such hybridization analysis is shown in Fig. 1. RNA-I hybridized to pDt78R and pDt41R DNAs 34 and 39 times more than to their respective pBR322 controls. Therefore, most of the RNA contained in the RNA-I band hybridized specifically to *Drosophila* DNA containing $tRNA_{3b}^{Val}$ gene. The efficiency of hybridization of RNA-II to pDt78R and pDt41R

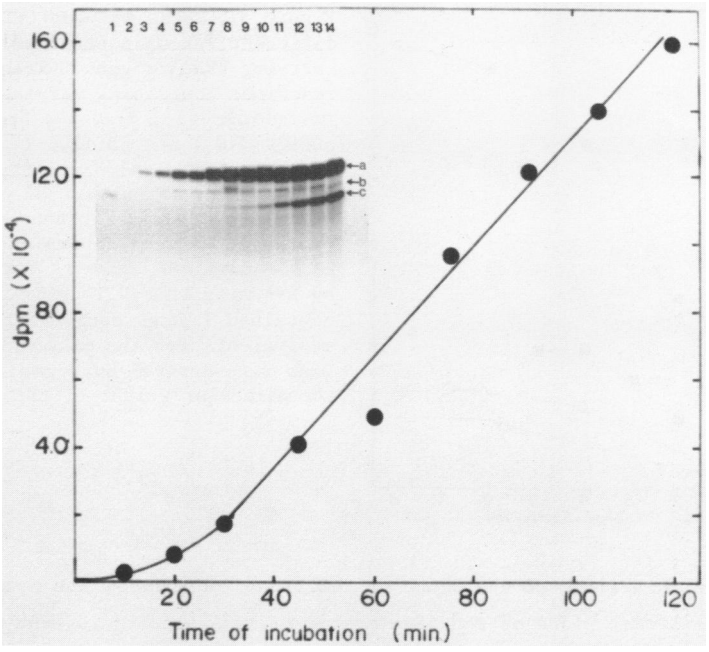


Figure 2. Time course assay with pDt55-0.3 as template. Inset: polyacrylamide gel of transcription products. Lane 1 contains ^{125}I-tRNA$_4^{Val}$ marker. Reactions in lanes 2-14 were stopped after 10, 20, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210 and 240 min. of incubation, respectively. Arrows a, b and c designate the positions of RNA-I, intermediate RNA species and RNA-II, respectively. The RNA bands were excised from the polyacrylamide gel, the sum of the radioactivity present in bands a, b and c was plotted against time of incubation.

DNAs was about 5-8 fold lower than that of RNA-I. The reason for this is not clear; it is possible that RNA-II has a greater secondary structure which makes it less accessible for hybridization. The lower hybridization to pDt41R was not totally unexpected because detailed hybridization studies showed that the efficiency of hybridization of purified tRNA$_{3b}^{Val}$ to pDt41R DNA was 30-50% lower than to pDt78R DNA (23).

Kinetics of transcription from cloned tRNA genes. To investigate the rates of transcription directed by different templates, we first examined the time course of RNA synthesis in detail. A typical time course of synthesis is shown in Fig. 2. A distinct lag was observed in the rate of synthesis lasting approximately 30 min. Following the lag in the reaction, the rate of synthesis was linear for up to two hours. All plasmids tested showed this biphasic time course of synthesis. The length of the lag period was effective-

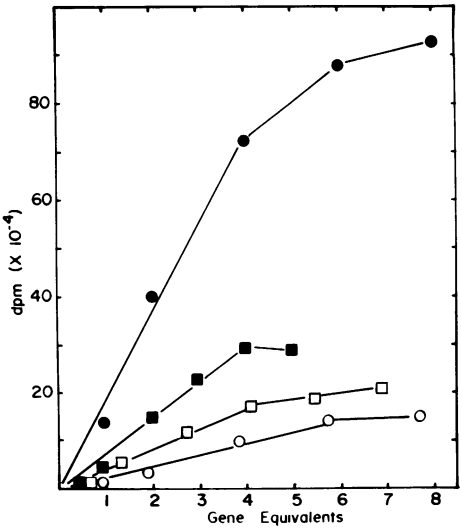


Figure 3. Rates of transcription of different recombinant plasmids carrying $tRNA^{Val}$ gene. Transcription reactions containing varying amounts of pDt55-0.3 (●), pDt78R (■), pDt92R (O) and pDt120R (□). The sum of the radioactivity present in the major transcription products was plotted against DNA concentration expressed as gene equivalents. The gene equivalent numbers were derived by assuming that 0.1 µg of pDt55-0.3 contained 1 gene equivalent. Gene equivalents for the other plasmid DNAs were derived by normalizing to the molecular weight of pDt55-0.3 DNA.

ly the same regardless of the slope of the linear portion of the synthesis curve. The linear slope of the time course extrapolated to 20 min. on the abscissa, indicating that an assembly step requiring approximately 20 min, is necessary for the linear rate of transcription between 40 and 120 min.

The DNA dependence of the transcription reaction was investigated using a 90 min. reaction time since this was well within the linear portion of the time course. The concentration of DNA yielding maximum tRNA transcription varied between plasmids, hence we determined the DNA dependence of the rate of transcription from the initial slope of the DNA curve (Fig. 3). The two $tRNA_4^{Val}$ plasmids derived from pDt55 (22), pDt55-0.3 and pDt55-0.6 (data not shown) transcribed at very high rates (2×10^4 dpm/µg DNA/min.). Plasmids pDt92R and pDt120R carrying variant genes for $tRNA_4^{Val}$ (Fig. 5, 20) directed transcription at rates 8 and 6 fold lower than pDt55-0.3, respectively. pDt78R ($tRNA_{3b}^{Val}$) was transcribed at a rate 3-4 fold lower than that of pDt55-0.3 (or pDt55-0.6).

Stability of the transcription complex. One possibility for the difference in transcription rates for the plasmids was that a complex required for initiation of the linear rate of synthesis was more stable for some plasmids than others. To examine this question, a complex stability experiment was designed using pDt78R and pDt55-0.3 as templates. One of the two templates was added at a concentration just below its optimum DNA concentration. At increasing intervals, the second template was added and each reaction incubated for an additional 90 min. The transcription products (RNA-I) were analyzed on polyacrylamide gels (Figs. 4A and 4B).

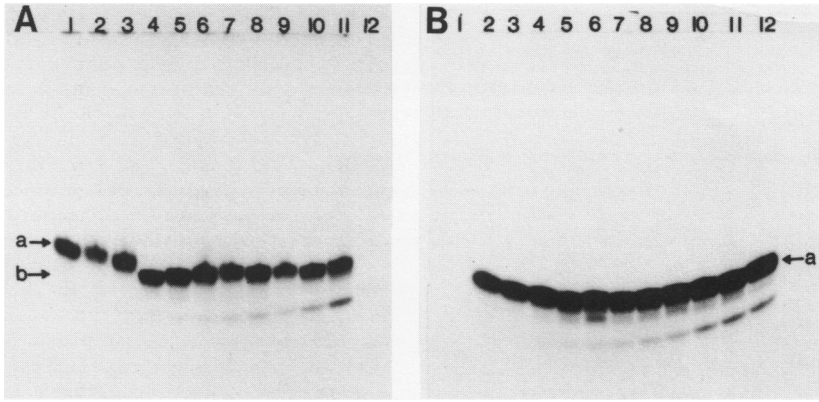


Figure 4. Stability of transcription complex. 0.8 µg of the first DNA was incubated with the S-100 extract for 5, 10, 15, 20, 25, 30, 35, 45, 55, and 65 min. before 0.8 µg of the second DNA was added. All reactions were incubated for 90 min. after addition of the second DNA. RNA-I directed by pDt55-0.3 and pDt78R are indicated by a and b, respectively.

4A. (Left panel): pDt78R as the first DNA; pDt55-0.3 as the second DNA (lanes 1-10, respectively). Lane 11: pDt78R alone; lane 12: ^{125}I-tRNA$_4^{Val}$ marker.

4B. (Right panel): pDt55-0.3 as the first DNA, pDt78R as the second DNA (lanes 2-11). Lane 1: tRNA marker; lane 12: pDt55-0.3 alone.

When pDt78R was added to the reaction first, addition of pDt55-0.3 up to 15 min. later totally inhibited synthesis of the pDt78R primary product (Fig. 4A). After 20 min., addition of pDt55-0.3 DNA had no effect on pDt78R transcription. When pDt55-0.3 was added to the reaction first, no synthesis of of pDt78R was detected, regardless of when it was added to the reaction (Fig. 4B).

DISCUSSION

Sequence dependence of transcription rate. The results presented in this study showed considerable variation in the transcription rate from 5 different cloned Drosophila tRNA^{Val} genes. Two plasmids carrying genes for tRNA$_4^{Val}$ (pDt55-0.3 or pDt55-0.6) were transcribed 3-4 times more efficiently than the DNA containing a tRNA$_{3b}^{Val}$ gene (pDt78R). The tRNA sequence in these plasmids is identical to that of the isoacceptor species to which they have been assigned (19,20). These are, therefore, bona fide tRNA genes, or are homologous to bona fide tRNA genes (21). Two plasmids, pDt92R and pDt120R, which have coding sequences highly related but not identical to tRNA$_4^{Val}$ (Fig. 5;19) were transcribed 8 and 6 fold less efficiently than pDt55-0.3, respectively. The tRNA

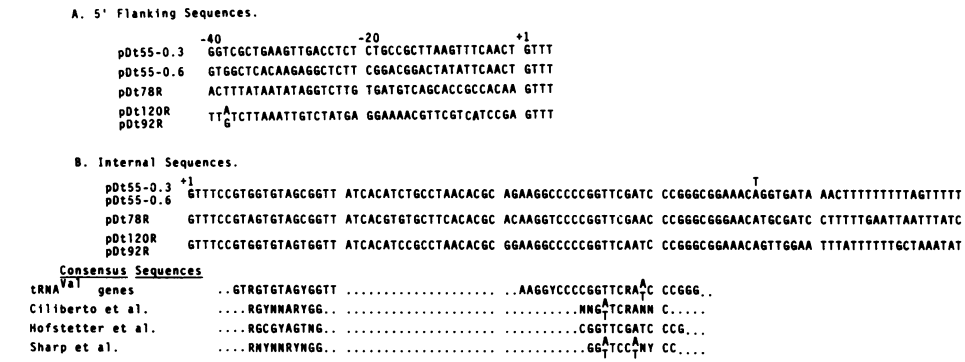


Figure 5. DNA sequences of coding regions of tRNA^{Val} genes. Section A compares the 5' flanking sequences of the tRNA^{Val} genes. +1 denotes the first nucleotide of the tRNA coding region and -40 is 40 nucleotides 5' to the coding region. The difference between pDt92R and pDt120R at position -37 is indicated.

Section B shows the DNA sequences of the tRNA coding regions of the plasmids. The 5' and 3' ends of the coding regions are indicated by +1 and T, respectively. DNA sequences are from references 19 and 20.

The proposed split-promoter regions are shown along with the consensus sequences in the tRNA^{Val} genes for the indicated region. Abbreviations used are R, purine; Y, pyrimidine; N, is any base.

sequence found in pDt92R and pDt120R is not present in any of the three major tRNA^{Val} species isolated from RPC-5 columns (20,24). Whether these variant genes code for any of the minor isoacceptors or whether they are expressed *in vivo* at all is not known.

Previous work on the sequences required for tRNA production has defined two essential regions within the tRNA gene. The nucleotides in these regions have been designated as the internal, split-promoter and have been localized by three groups to lie within nucleotides 8 to 18 and 51 to 62 of the mature tRNA sequence (15,16,17). The split-promoter regions of the tRNA^{Val} genes we have studied share a high degree of homology and correspond to the canonical sequences (Fig. 5). The differences that exist in the split-promoter regions of the tRNA^{Val} genes are found in positions which by consensus show flexibility. For example, base transitions at nucleotides 9, 16 and 57 occur where all three canonical sequences indicate only purine or pyrmidine. At position 59 (base transversion), two of the three canonical sequences indicate any nucleotide (N). We favour the interpretation that the difference in the rates of transcription documented in Fig. 3 is primarily the result of sequences outside the tRNA coding region. Flanking sequences have been shown to affect the rate of tRNA transcription by a number of previous studies (5,14,18). Searches

of the nucleotides upstream from the valine tRNA genes have revealed no common sequences ahead of either efficiently or poorly transcribed genes (Fig. 5;19,20). Plasmids pDt55-0.3 and pDt55-0.6 which are both transcribed at very high rates show very little homology beyond position -7. However, deletion of sequences beyond position -18 in plasmid pDt55-0.3 and subsequent recloning into pBR322 decreased the transcription by two fold (Rajput et al., unpublished observations). This suggests that some modifying sequences are found beyond that point.

Kinetics of tRNA transcription. The experiments reported above on the rate of transcription give kinetic evidence for the multiple components of the transcription complex. Data in Fig. 4 show that the transcription complex is stable during the course of the reaction. Stable association of a specific transcription factor has been shown for 5S RNA genes and has been implicated in a mechanism for regulation of transcription of oocyte and somatic variants of these genes (12,13).

The formation of the transcription complex appeared to be limited by a rather slow event which required 20-30 min. for maximal completion. This time was relatively constant regardless of the final rate of transcription. However, some component acted very quickly with pDt55-0.3 to form an exclusive association. Thus, it appears that before RNA polymerase begins to accumulate transcription products, at least two events must take place: 1) the formation of the exclusionary complex (which was more rapid with pDt55-0.3 than with pDt78R) and 2) the slower step leading to the complex needed to direct the linear rate of transcription.

The cause for the differences in the linear rates of transcription between different $tRNA^{Val}$ plasmids is so far unclear, but we would suggest two possibilities. First, there may be sequence dependent effects on the turnover rate of the polymerase caused by changes in elongation and termination as well as initiation. Second, it is possible that some component of the transcription complex other than the polymerase cycles after each initiation event; kinetics of reassociation of this component could be dependent on the stable portion of the complex and the DNA template sequence.

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