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DEVELOPMENT OF A YEAST TRANSCRIPTION SYSTEM USING A *SACCHAROMYCES CEREVISIAE* ENZYME EXTRACT AND A *DROSOPHILA MELANOGASTER* HISTIDINE tRNA GENE

A Thesis

Presented to the Faculty of the Department of Biological Sciences

of the State University of New York College at Brockport

in Partial Fulfillment for the Degree of

Master of Science

by

Diana M. Parker

December, 1985

THESIS DEFENSE

DEVELOPMENT OF A YEAST TRANSCRIPTION SYSTEM USING A SACCHAROMYCES CEREVISIAE ENZYME EXTRACT AND A DROSOPHILA MELANOGASTER HISTIDINE tRNA GENE

BY

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DEDICATION

This thesis is dedicated to Thomas Parker and Robert Parker who have sacrificed themselves in many ways in order that this final report would become reality. We three have shared the time, effort and commitment that has been necessary to achieve this goal.

ACKNOWLEDGEMENTS

I wish to thank Dr. Larry K. Kline for his supervision during this research project. His candid comments on successes, failures, mistakes and surprises maintained a level of commitment which stemmed from mutual dedication to a goal.

I have enjoyed the opportunity to study with him and have respected the following qualities which describe his commitment to students and education:

- his willingness to answer questions
- his availability at anytime, especially when an experiment was going awry
- his forethinking and
- his patience

My goal has been achieved through his belief in my abilities.

I also wish to thank James Fulginiti and Nancy Sedita for their research support.

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ABSTRACT

This research problem is directed toward the isolation of an enzyme from the yeast Saccharomyces pombe which endonucleolytically processes the 3' terminus of transfer ribonucleic acids.

Plasmids containing tRNA genes were a gift from Dieter Söll, Yale University Department of Molecular Biophysics and Biochemistry. In order to provide increasing amounts of the cloned gene, the bacterium Escherichia coli HB101 was transformed using standard transformation procedures and methods.

The transformed bacteria were cultured, and the plasmids amplified using chloramphenicol. These bacteria were then lysed and cesium chloride-ethidium bromide gradients were run to isolate plasmids containing the desired genes.

The plasmids were then used in a transcription assay following the procedure of Klekamp and Weil (1). The purpose of this transcription was to synthesize precursor tRNA genes which would serve as substrates for the detection of S. pombe processing nucleases. The synthesized [α-^{32}P] labeled precursor tRNA's were purified using polyacrylamide gel electrophoresis. Autoradiographic techniques were employed to identify the location of the precursor on the gel. The precursor was then eluted from the gel.

The initial goal of this thesis project is to obtain sufficient quantities of precursor tRNAs to use in the detection of 3' processing nucleases. Following detection, the processing nucleases will be purified by standard enzyme purification techniques.

INTRODUCTION

The cellular material responsible for carrying genetic information is DNA. In order for DNA to relay information to the cell, messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA) are essential. In the cell, specific tRNAs are responsible for binding with specific amino acids (specificity is essential to reliable read-out of the DNA) after which the aminoacylated tRNAs bind to the mRNA - ribosome complex, making the amino acids available to the growing polypeptide chain.

The tRNAs which carry the amino acids are themselves transcribed from a nucleotide sequence of the DNA. The initial transcription results in a precursor tRNA and as such is not transcribed in the form in which it functions in the cell. In fact, immediately following transcription, processing occurs at both the 5' and 3' ends of the precursor. In addition, in some precursors, processing of introns occurs. The resulting product of these processing reactions is the mature tRNA. The general processing scheme is outlined in figure 1:

Figure 1

Processing Schematic:

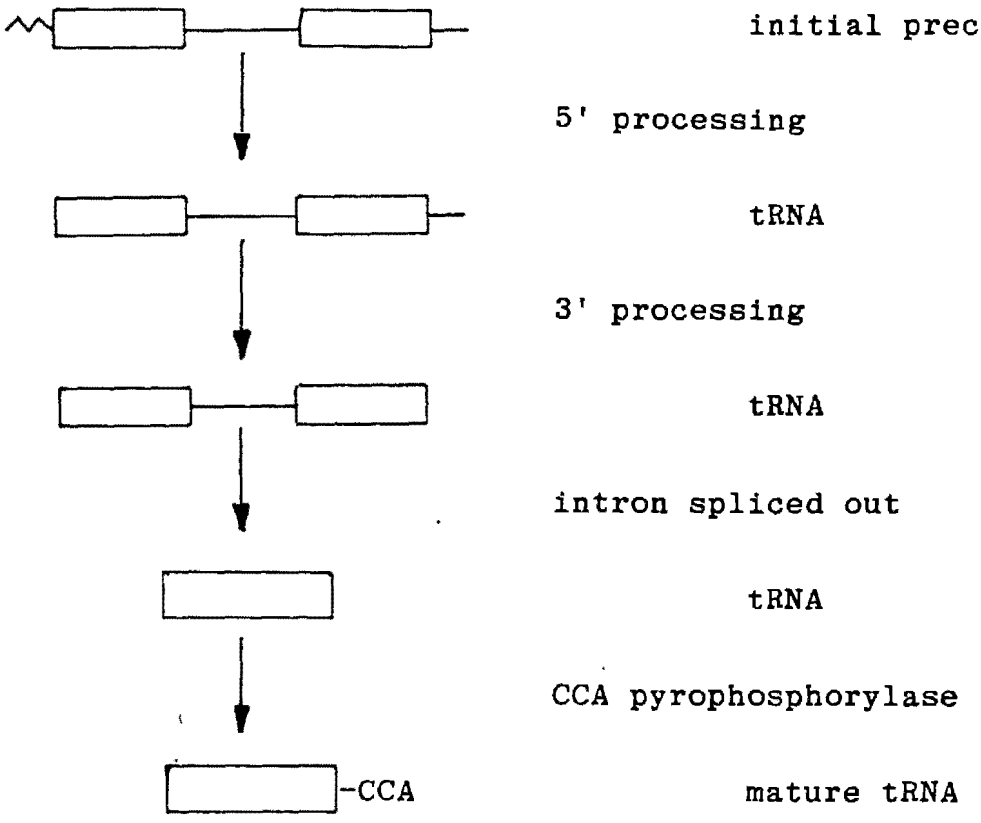


Figure 1. Steps in the processing of a precursor tRNA. 5' processing by RNase P is followed by 3' processing by both endonucleases and exonucleases. Intron splicing usually occursfollowing the addition of the -CCA terminus.

Ribonuclease P (RNase P) is the processing enzyme for the 5' terminus of tRNA. It is an endonuclease which accurately cleaves all tRNA species at the specific 5' nucleotide for the mature tRNA form.

Processing at the 3' terminus has focused on exonucleases which process the precursor tRNA. An exonucleolytic enzyme begins at the 3' end of a precursor tRNA nucleotide sequence and cleaves one base at a time producing mononucleotides. This is similar to a degradation process. A number of exonucleolytic enzymes can provide this degradation. The literature suggests that 3' terminal processing may also result from an endonucleolytic activity. This cleavage creates the 3' terminal end to which the CCA sequence will subsequently be attached.

Maturation of tRNAs differs between prokaryotes and eukaryotes following 3' processing. Since the -CCA bases are sometimes coded for on the gene in prokaryotes, these molecules have a mature 3' terminus when 3' processing is complete. In eukaryotes, the -CCA bases are not coded for in the gene sequence. Following 3' processing, the enzyme CCA pyrophosphorylase adds a -CCA terminus to the tRNA product to make tRNA-CCA, the mature and functional tRNA molecule.

Base modifications occur throughout the tRNA processing sequence perhaps providing internal recognition sites. These modifications are unique to each tRNA. In addition, as transcription proceeds, the tRNA molecules establish secondary and tertiary bonds again serving to expose cleavage sites for processing. Intervening sequences (IS) may be excised before the addition of the -CCA nucleotides in eukaryotes, but the common time of processing for IS follows -CCA addition.

Processing enzymes are essential in the cells's production of mature tRNA for protein synthesis. A better understanding of how the processing enzymes control tRNA production may also provide insights into the regulation of protein synthesis.

STATEMENT OF THE PROBLEM

All RNA products are produced from DNA templates which are transcribed in the cell by necessary polymerases and factors. The RNA product is transcribed in a form that is different from the functioning RNA molecule and is referred to as a precursor RNA (pRNA). Processing enzymes, of which RNase P is the most highly characterized, cleave the molecule at the correct nucleotides to produce a mature, functioning RNA product. These mature RNA products are essential to protein synthesis. This research project is specifically concerned with the 3' endonucleolytic processing of tRNA. Endonucleolytic cleavage signifies that the initial transcript has been cleaved (cut) at some recognition point within the initial transcript. This type of processing is similar to that of RNase P for the 5' terminus of tRNA, except that it occurs at the 3' terminus. In order to study 3' processing, a precursor tRNA substrate is essential. Since processing enzymes work quickly in the cell to process the precursor tRNA molecules, it is difficult to isolate precursors.

Processing enzymes can be studied with the substrates produced by either of two methods or strategies. One method involves the isolation of natural precursors. A plasmid containing a tRNA gene is transcribed <u>in vitro</u> to produce a

precursor tRNA. This precursor tRNA can be isolated and used as a substrate to detect processing endonucleases.

The second method requires the construction of a synthetic precursor tRNA molecule. A radioactive trailing sequence is synthesized and ligased to a tRNA molecule which does not have the -CCA nucleotides at its 3' terminus. This synthetic precursor tRNA can then be used as a substrate to detect processing endonucleases. For this study the use of a natural substrate was selected.

Using a natural substrate to detect a processing endonuclease not only requires that the substrate be synthesized, but also requires that a system to produce the substrate in sufficient amounts is readily available. The choice of substrate is the Drosophila melanogaster tRNA histidine gene. The gene has been inserted into the tetracycline site of plasmid pBR322 to create plasmid pYM10.3T. This tRNA gene contains a 35 base pair trailing sequence at the 3' end. When processing of the gene occurs, the following will be observed:

Figure 1

Processing Schematic:

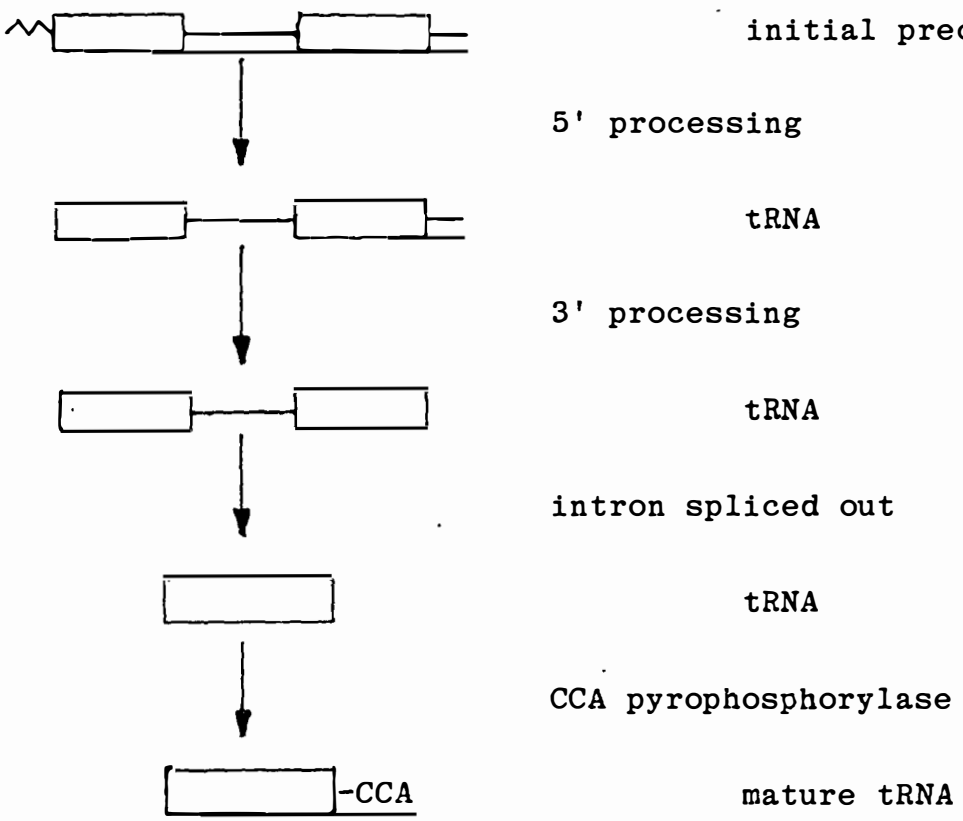


Figure 1. Steps in the processing of a precursor tRNA. 5' processing by RNase P is followed by 3' processing by both endonucleases and exonucleases. Intron splicing usually occursfollowing the addition of the -CCA terminus.

The trailing sequence is easily identifiable because of its size. It can act as an indicator of endonucleolytic processing at the 3' terminus of the RNA transcript because an intact 35 base pair trailing sequence will move rapidly on the gel and will band above the mononucleotide region. In contrast, an exonucleolytic cleavage would remove one base at a time. These individual bases would not be resolved as a separate gel product. In order to conclude that an endonucleolytic cut is being made, the products cannot represent a general degradation process, but must show evidence of cleavage of a large unit which contains the intact trailing sequence.

Once an adequate supply of plasmid DNA is produced, it can be transcribed, purified and used as a template to produce precursor tRNA molecules. The transcription system contains [α-^{32}P]GTP which, when incorporated into ribonucleic acid chains will cause the RNA transcript to be radioactively labeled. Electrophoresis separates the transcription products. Autoradiography identifies the position of labeled products on the gel. These labeled transcription products can be removed from the gel to be used as a substrate in processing assays.

Therefore, the scope of this thesis project involves:

1) isolation of plasmid pYM10.3T which contains the <u>Drosophila</u> histidine tRNA gene;

2) transcription of the plasmid to provide a tRNA precursor for the study of endonucleolytic cleavage and, if possible;

3) the search for an endonuclease activity in S. pombe which produces endonucleolytic cleavage of the precursor tRNA molecule.

LITERATURE SEARCH

The initial transcripts of tRNA genes are processed by a number of nucleases which cleave tRNA at the 3' terminus, at the 5' terminus and on either side of intervening sequences (2). The 5' terminus is processed by the endonuclease Ribonuclease P (3, 4); intervening sequences are processed by an endonuclease and a ligase (5); and the 3' terminus is processed by exonucleases (6). Evidence is also present in the literature for endonucleolytic cleavage of the 3' terminus (7 - 11). Specifically, 3' terminal endonucleolytic processing takes place in a Xenopus laevis extract with human gene precursors (11). The ultimate objective of this project is to identify similar 3' processing enzymes in Saccharomyces pombe.

During the course of this thesis project, Castaño, et al. (11), publishing in the Journal of Biological Chemistry, described an endonucleolytic activity from Xenopus laevis ovaries which accurately processes the 3' terminus of human pre-tRNA$^{met}_{i}$. [α-^{32}P]-labeled transcripts of the human pre-tRNA$^{met}_{i}$ gene were synthesized in a cell-free extract from human KB cells. The 3' processing nuclease was extracted from X. laevis ovaries using the following techniques. Initially an S100 extract was made of the crude extract. The S100 was purified further using phosphocellulose, DEAE-cellulose and Affi-Gel Blue columns.

These purifications initially separated processing enzymes from unwanted enzyme activities. Next the 5' processing activity was separated from the 3' activity. The last column purified the 3' processing activity. An SDS-polyacrylamide gel electrophoresis demonstrated that a single polypeptide co-purified with the 3'-processing activity. The activity was identified to have an endonucleolytic function by using the following techniques. Electrophoresis demonstrated that oligonucleotides of interest in 3' processing increased in amount at identical rates over a period of time. Using an [α-^{32}P] UTP-labeled precursor, 3' processing revealed little accumulation of free labeled UMP during processing. Oligonucleotide identification of gel products using fingerprint analysis indicated the base sequence of the cleaved segment and thereby demonstrated that the final mature product was mature RNA minus the -CCA terminus (11).

The purified enzyme was identified as a single polypeptide having a molecular weight of approximately 97,400 daltons. It processes endonucleolytically and has no requirement for an RNA component.

Its mechanism of action indicates:

1) that the substrate molecule has a mature 5' terminus;
2) that the enzyme is universal, cleaving several tRNA gene products; and

3) that cleavage seems to be based on general properties of the substrate molecule, and is not influenced by peculiar structural or sequence properties (11).

The goal of this present research is to determine if a similar enzyme exists in S. pombe.

There are at least two general methods by which processing of tRNA precursors can be studied. In one instance a natural substrate is generated. By radioactively labeling the substrate, the processing mechanism can be followed. Utilization of a synthetic substrate is a second possible method by which the tRNA processing mechanisms can be studied. The nucleotide sequence of the synthetic substrate is similar to the natural precursor molecule and the processing enzyme recognizes the similarity and processes the synthetic substrate. The synthetic substrate is radioactively labeled allowing detection of endonucleolytic processing (3).

This thesis project uses a natural substrate to isolate precursor tRNAs with which endonucleolytic cleavage of the 3' terminus of tRNAs can be detected. The experimental protocol involves three steps:

1) isolation of plasmid pYM10.3T containing the Drosophila melanogaster histidine tRNA gene,
2) transcription of the tRNA gene from the plasmid using an enzyme preparation which contains

the appropriate enzymes and factors to provide a tRNA precursor, and, if possible,

3) detection of an endonuclease activity in S.pombe which produces 3' endonucleolytic cleavage of the precursor tRNA molecule.

Essential components needed in the study are described below.

PLASMID

The mutant bacterium Escherichia coli, HB101, can serve as the host for a plasmid (12). Standard transformation procedures (13) provide the pathway by which the plasmid is inserted into the bacterium. When using the natural substrate method for isolating initial gene transcripts, it is essential that a convenient, purified, stable gene be available to produce abundant quantities of tRNA precursors. A plasmid system provides such a source (13).

The technology exists by which a gene from a eukaryotic organism can be inserted into a plasmid which has the appropriate restriction sequence, thereby producing a source of genetic material which can be manipulated (14 - 17). Of particular importance in this research were plasmids pYM10.3T (also designated p48FHis) and pYM 205, (18,19,20,21) both gifts from Professor Dieter Söll, Department of Molecular Biophysics and Biochemistry, Yale University. Plasmids pYM7.2, pYM104, and pYM118 were also included as gifts (21 - 23). The properties of these plasmids are noted in Table 1. All tRNA genes were cloned into pBR322.

Table 1

Plasmid Characteristics

Plasmid	Gene(s)	Spacer	Trailing Sequence
pYM7.2	S. pombe tRNA histidine	---	22 nucleotides
pYM10.3T	D. Melanogaster tRNA histidine	---	35 nucleotides
pYM104	S. pombe (separate) tRNA arginine tRNA glutamic acid tRNA lysine	---	5 nucleotides 8 nucleotides 10 nucleotides
pYM118	S. pombe (dimer) tRNA serine tRNA methionine	7 nucleotides	7 nucleotides 10 nucleotides
pYM205	S. cerevisiae (dimer) tRNA arginine tRNA aspartic acid	10 nucleotides	10 nucleotides 8 nucleotides

The Drosophila melanogaster tRNA histidine gene was first cloned into a plasmid vector (9). In this form the D. melanogaster tRNA histidine gene could be grown to high concentrations in a transformed bacterium, thereby providing an easy, readily available source of genetic material for transcription (12).

Plasmid vector pBR322 is the plasmid into which both the D. melanogaster histidine gene (pYM10.3T) and the S. pombe arginine-aspartic acid genes (pYM205) have been cloned. Both genes were cloned into the tetracycline resistant gene of the plasmid (18,19,20,21). pBR322 is widely used as a vector because of its convenient cloning sites. The plasmid is under relaxed control thereby enhancing the effect of chloramphenicol amplification of plasmids. This cloning vector contains both an ampicillin resistant and a tetracycline resistant gene (15, 16). In order to develop a pBR322 plasmid containing the gene of interest, the gene was isolated from plasmid p38B10 (18). The fragment was removed using restriction enzymes BamHI and HindIII and the resulting segment hybridized to Drosophila melanogaster histidine tRNA. pBR322 was cleaved with BamHI and HindIII restriction enzymes forming free ends which would ligate to the extracted gene segment. These two products were annealed to each other making available the D. melanogaster tRNA histidine gene (21). pYM205 was inserted into pBR322 using a HindIII restriction site. The inserted

gene includes both the $tRNA^{arg}$ and the $tRNA^{asp}$ genes separated by a spacer of 10 nucleotides. The 3' flanking sequence contains an oligothymidylate sequence which is probably a transcription termination signal for RNA polymerase III (20).

For this project, it should be noted that plasmid pYM10.3T and pYM205 are inserted into the prokaryotic plasmid pBR322. pYM10.3T and pYM205 are eukaryotic gene inserts. In later discussions, it will become evident that when using a eukaryotic yeast transcription system, only the eukaryotic portion of the engineered plasmid will be transcribed. Therefore the product of the transcription of the plasmids pYM10.3T or pYM205 using a eukaryotic yeast transcription system will be only the eukaryotic tRNA species.

Certain properties of pBR322 also make it useful in isolating plasmid-containing bacterial colonies. pBR322 contains two antibiotic sites, an ampicillin resistant site and a tetracycline resistant site. Bacteria containing pBR322 will grow in the presence of both of these antibiotics.

If a DNA fragment is inserted into the tetracycline gene, the bacterium loses its resistance and becomes sensitive to tetracycline. Therefore it will not grow on media containing the antibiotic. The same is true with ampicillin resistance.

A bacterium containing pBR322 will grow on antibiotic plates. If it can no longer be cultured in the presence of antibiotics, two possibilities exist. It has either lost the plasmid or a gene insert has been made in the antibiotic resistant site on the plasmid.

Growth patterns for plasmid containing bacteria on selective media are represented in table 2:

TABLE 2

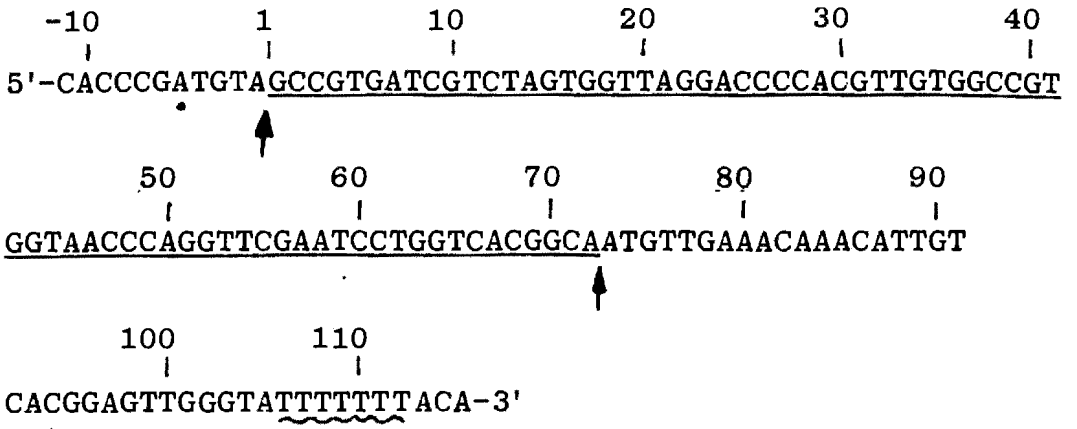
Plasmid Growth Patterns On Selective Media

	Ampicillin media	Tetracycline media	Ampicillin media + Tetracycline media	Media
bacterium	-	-	-	+
bacterium + plasmid	+	+	+	+
bacterium + plasmid insert in tetracycline gene	+	-	-	+
bacterium + plasmid +insert in ampicillin gene	-	+	-	+

From the pattern of growth above, it is clear that one can select for colonies with gene inserts in the proper position. The plasmid containing the gene of interest for the study can then be isolated and amplified.

Several properties of pYM10.3T and pYM205 contribute to their being the plasmids of choice for this research work.

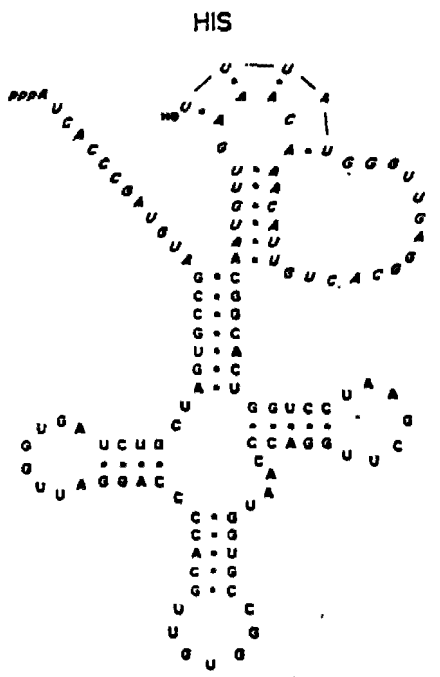
Sequence pYM10.3T



(18,19,21)

Figure 4. Nucleotide sequence of plasmid pYM10.3T, Drosophila melanogaster histidine gene insert. Note the 35-base pair trailing sequence (nucleotides 73f). Arrows mark the gene sequence which has also been underlined. The waved line marks the termination sequence. The dot marks the initiation site.

Cloverleaf Structure pYM10.3T

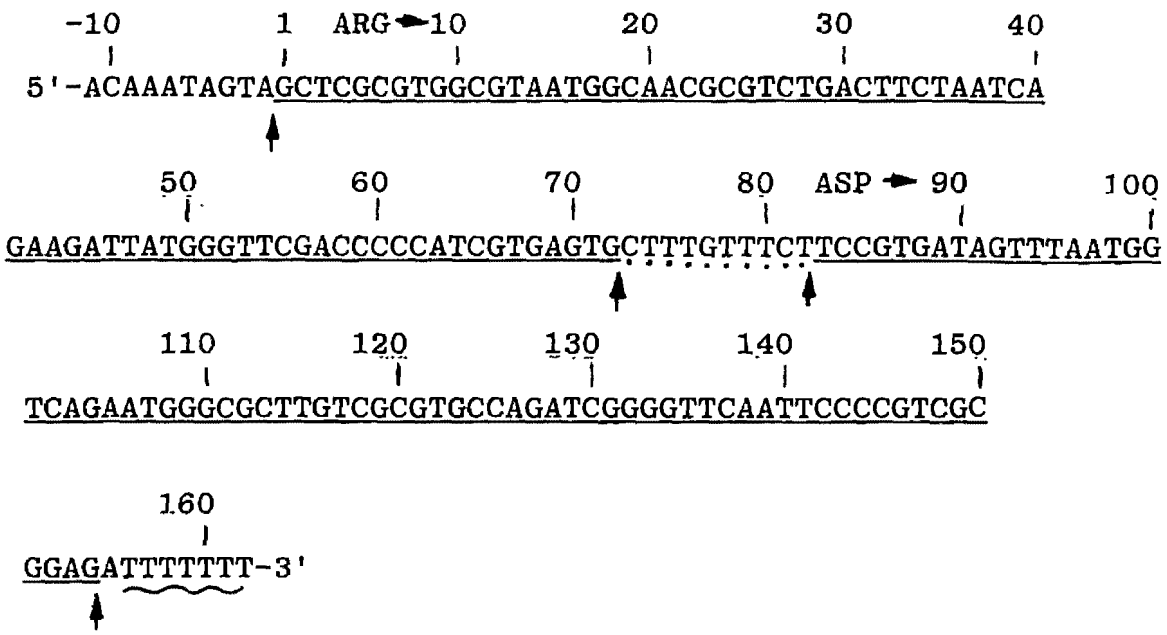


(7)

Figure 5. Possible secondary structure and sequence of a primary transcript of D. melanogaster tRNA histidine gene derived from in vitro transcription. The 3' trailing sequence structure can possibly protect the gene from degradation thereby making it more stable than some other tRNAs.

pYM10.3T (see figures 4 and 5) (18,19,21) has a trailing sequence which extends 35 base pairs beyond the end of the gene. When endonucleolytic processing occurs the 35 base pairs will be cleaved as a unit. If a 35 base pair intact unit is not detected, then cleavage is not endonucleolytic. The 35 base pair unit can be easily identified on a 10% polyacrylamide gel. Because smaller molecules move more quickly, the unit will be visible near the bromophenol blue marker. The dye used in this electrophoresis to identify the position of tRNAs is xylene cyanol FF (XCFF). Bromophenol Blue (BPB) identifies the running front of the gel. Any labeled product near the gel front will be small and can represent either the trailing sequences or mononucleotides. Sequencing would confirm the identity of the 35 base pair trailing sequence. This 35 base pair band is an indication of endonucleolytic processing at the 3' terminus of the tRNA histidine gene. Other bands of interest on the gel would include 5' processed tRNA and 3' processed tRNA. Such easy identification of these bands makes pYM10.3T the plasmid of choice in studying endonucleolytic 3' terminal processing. pYM205 was also selected because it transcribes particularly well in a yeast transcription system (J. Schaack, private communication). The efficiency of transcription using plasmid pYM205 serves as a control for the entire transcription assay (20). Figures 6 and 6a show the nucleotide sequence of pYM205.

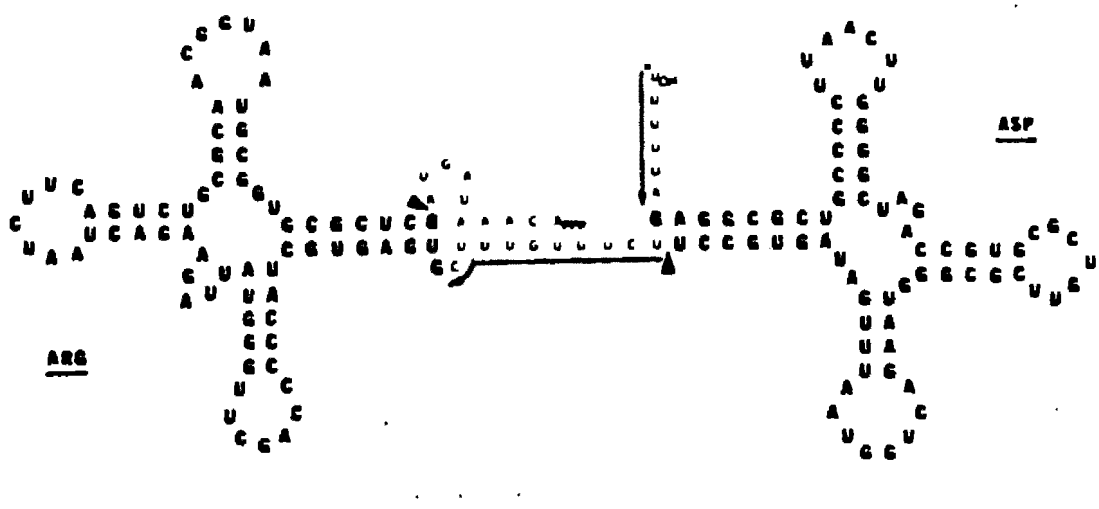
Sequence pYM205



(20, 50)

FIGURE 6. Nucleotide sequence of plasmid pYM205, Saccharomyces cerevisiae arginine - aspartic acid dimer gene insert. Arrows mark the gene sequence which has also been underlined. The waved line marks the termination sequence. The spacer sequence between the genes is marked by dots.

Cloverleaf Structure pYM 205



(50)

Figure 6a. Possible secondary structure and sequence of a primary transcript of S. cerevisiae tRNA arginine/asparetic acid dimeric gene derived from in vitro transcription. Solid triangles indicate endonucleolytic cleavages. Arrows represent 3' - 5' exonucleolytic trimming. A 3' endonucleolytic activity may also contribute to processing at the 3' terminus.

TRANSCRIPTION SYSTEM

In addition to the requirement for plasmids containing tRNA genes it is necessary to have a cell extract which can provide the RNA polymerase and all the factors essential for the formation of an active transcription complex (1). A great deal of effort has been expended in determining the factors necessary in gene transcription as well as the factors necessary for formation of an active transcription complex (24 - 32,1).

Transcription enzymes can be extracted (from: HeLa cells (9), Xenopus laevis oocytes and germinal vesicles (11,33), yeast cells (1), Drosophila KC cells (7,9), and Bombyx mori eggs (34).

If factors are removed during the purification of RNA Polymerase III, they must be added before the transcription system will transcribe well. Without the proper factors, transcription is either random or non-existent (1,9,35,36).

The yeast S. cerevisiae has been used as the source of Polymerase III for the transcription system (1,37). Since a eukaryotic polymerase will only copy the eukaryotic portion of the plasmid, the system will select for the pYM10.3T precursor. The enzyme in this research was prepared according to Klekamp and Weil (1) with modifications by Schaack and Söll (37).

The concentration of the enzyme can influence the ability of the system to transcribe a gene insert accurately (M. Nichols, private communication). It is, therefore,

necessary to determine the most favorable enzyme concentration which enhances transcription, does not inhibit the system, and reduces nuclease activity. Conditions for an assay containing proper component concentrations favoring transcription are described in Klekamp and Weil (1). Their suggested assay conditions were incorporated into these studies with additional modifications as described by Schaack and Söll (37).

It is essential that the results be specific transcription of the desired gene. Initiation and termination sites must be accurately copied so that the correct gene transcript is the resulting product. The conditions of the transcription assay must also favor transcription over degradation of final products. This assures the presence of primary transcripts in the products of the assay.

These crude systems contain additional endonucleases and processing enzymes responsible for general, not specific cleavage, thereby causing a degradation of the precursor tRNA transcripts.

Transcription complex formation has received intensive investigation over the last several years (7,9,25,28-30,32,35,38). Without proper complex formation, the transcript will not be accurate, transcription may not occur, or transcription may be slow.

Stable transcription complex formation involves two steps:

1. a rapid step which binds and sequesters the stable complex-forming factor; and
2. a slower, temperature-dependent step which involves either binding of an additional factor or the rearrangementof the complex into a transcriptionally active form (9).

Formation of a stable complex is not sufficient to promote RNA synthesis (29). Additional sequence specificities are needed (7,9,11,24,31,32,35,39-43).

Factors essential for stable transcription complex formation include RNA polymerase III, a complex enzyme of more than 10 subunits (38), and two factors, factor B and factor C, one which is cycled and one which is stably bound (9,29,35).

Sequence specific factors which influence transcription complex formation may vary slightly from system to system, but include the following:

1. a 3' flanking region (7,9,11,40-42),
2. a 5' flanking region (7,9,11,40-42),
3. an internal control sequence (D-control region and T-control region) to improve efficient site use (7,9,24,31,32,43),
4. a promotor (24,31,32) and
5. an upstream initiation site for which the complex scans the DNA (9).

Once the conditions are set for transcription, specific transcription of the desired eukaryotic gene produces the

desired eukaryotic substrate molecule (44-47). Some of the lag time in initiation of transcription may be due to the requirements of forming a stable complex (37).

In some instances the rate of transcription can be influenced by the system selected. For example, a yeast system has a shorter lag time during the transcription than HeLa cells (37). The rate of transcription can also be influenced by the presence or absence of 5' flanking regions (7). The 5' flanking region enhances the formation of the transcription complex (7,9,40,41).

Literature studies of transcription systems have concentrated on formation of stable complexes (29,30), promotor regions (24,31,32,48), intragenic control regions (24,43), factors required for the functioning of polymerase III (9,25,35,49), and requirements for 3' and 5' flanking regions (9,28,37,40-42). A brief description of each of these areas follows.

Formation of a stable complex directs polymerase III to copy the intended gene segment. A stable complex is composed of RNA polymerase III (38), a cloned tRNA gene (29), two factors (designated B and C) (29), the D-control region and a part of the 5' flanking region encoding the D-stem of tRNA (26,29,30,35). Complexes are formed as a prelude to active transcription.

Promotor regions are a part of the transcribed gene. Two intragenic control regions, designated Box A and Box B (between nucleotides 7 and 58), have enough information to

initiate specific transcription by RNA polymerase III in most systems. These control regions are located in regions of highly conserved sequences. Initiation must occur in a region upstream of the promotor sequences at a fixed distance. Sequences upstream of the initiation site can also modulate promotor activity. The conserved regions influence both promotor activity and processing reactions. Both Box A and Box B sequences are essential for promotion of transcription (24,31,32,48).

There are two intragenic control elements within a eukaryotic tRNA gene. These elements serve as promotor consensus sequences in establishing active transcription at a specified site and function as recognition sequences for tRNA transcription factors. These sequences have been designated the D-control region and the T-control region (24,29,31,32,43,48).

The D-control region codes for the D-stem and D-loop in the tRNA. It encompasses nucleotides 8-25 of tRNA. The T-control region codes for the GTΨC-stem and GTΨC-loop in tRNA. It is composed of nucleotides 50-58 in the tRNA. DNA sequences from nucleotides 7 to 58 possess sufficient information to initiate specific transcription by RNA polymerase. Removal of either the D-region or the T-region results in reduced transcription (29). The distance between the two control regions influences transcription. Distance also influences stable complex formation (24,29,31,32,43,48).

The D-control region has greater significance in stable complex formation (9).

Two factors are required in addition to RNA polymerase III for active and selective transcription. These factors have not been completely characterized, but their requirements for transcription have been established (9,25,35).

Factor B interacts with the D-control region in transcription complex formation while factor C interacts with the T-control region. The presence of these factors inhibit random transcription. If either factor is absent, transcription efficiency diminishes (9,25).

Both the 5' flanking region and the 3' flanking region are necessary for accurate transcription of a tRNA gene to occur. Proper orientation of the 5'- and 3'- flanking regions affect factor binding to create stable complexes. The nature of the 5'-flanking region influences efficient transcription. In some genes the 5'-flanking region can inhibit transcription (9,28,37,40-42). As noted, 5'- and 3'-flanking regions significantly influence processing of a precursor tRNA (7,50).

PROCESSING

The ultimate goal of this research is to isolate an enzyme from a Saacharomyces pombe (51) extract which will endonucleolytically process the 3' terminus of tRNA (11). Since tRNA is the adaptor molecule in protein synthesis, its proper processing is critical to the formation of proteins.

For this reason, tRNA processing has been the focus of the recent research on tRNA (2,5-8,10,11,39,53-63).

In order to study any type of tRNA processing, one must develop a system which will provide a precursor molecule which can serve as a substrate for the processing studies (20,23,34,46,47,64).

Five types of processing, occurring in the proper sequence, produce the mature tRNA: 5' processing, 3' processing, intervening sequence processing, modifications of individual bases and addition of the -CCA end of the molecule in eukaryotes (see Figure 1). The full processing sequence requires numerous enzyme functions. They must be made available to an *in vitro* system for the complete process to occur.

Ribonuclease P is an endonuclease which accurately processes all tRNAs in both prokaryotes and eukaryotes (3,65) to produce the mature 5' terminus of the molecule. Not only has RNase P been characterized by a number of investigators (2-4,66-72), but it also is the only 5' processing endonuclease to have been identified. An exonuclease, which cleaves individual bases from the tRNA initial transcript to produce the mature 5' terminus of the molecule, may also exist (8). Endonucleolytic cleavage by RNase P is the predominant 5' processing mode, however.

RNase P is unique in its structure as an active enzyme, requiring the presence of a large sized RNA molecule for endonucleolytic cleavage to occur. Exposing the enzyme to

micrococcal nuclease and/or pancreatic ribonuclease A inactivates the enzyme, thereby demonstrating a need for the RNA component (2,66). In the absence of the RNA component, no ribonuclease P activity is demonstrated (2).

RNase P is, therefore, a ribonucleoprotein. It contains four major protein components and substantial amounts of RNA (67). The active enzyme can range from 1.35×10^5 daltons to $\geq 2.0 \times 10^5$ daltons (5,67). The RNA component represents approximately 80% by weight of the active enzyme (66,70) and is composed of > 300 nucleotides (2,66,70). The protein component of the enzyme represents approximately 20% by weight of the active enzyme (5) and is composed of four units (67). There is a high affinity between the protein and the RNA component (67). The bouyant density of the active enzyme in $CsCl_2$ is 1.71 g/ml indicating again a need for the RNA component (66).

In considering the role of the active enzyme both the RNA component and the enzymatic component must be evaluated. The RNA component is essential for enzymatic function (72). The RNA molecule could stabilize the protein by fixing the position of the enzyme in space and positioning the nucleolytic subunit so that the substrate is always precisely at the right position to cleave the precursor tRNA at the beginning of the 5' terminus to produce the mature 5' terminus (72). It has been demonstrated that this process occurs before 3' processing (7,11). Changes in the structure of the precursor molecule reduce the rate of RNase

P cleavage (72). The RNA component may activate the nucleolytic function of the enzyme (2,66,71,72). The RNA component could serve as a recognition molecule between the enzyme and the substrate (2,66,72).

The protein component of the enzyme contributes the nucleolytic activity. Without the protein component, cleavage would not occur (2,72). In tandem, the protein component and the RNA component process the 5' terminus of tRNAs. The RNase P is so complex, requiring both five subunits and a mechanism for proper alignment, that it could regulate the rate at which tRNAs mature in the cell (2).

RNase P has been studied in numerous eukaryotic and prokaryotic systems. The enzyme seems to provide universal cleavage at the 5' terminus of all organisms examined, as well as all species of tRNA. The only exception is that in some prokaryotic systems, the RNase P needs the -CCA molecule to function, whereas in eukaryotes the -CCA terminus is added later (2,3). The role of RNase P in the processing of precursor tRNA is a crucial step in tRNA maturation (3).

Literature reviews of RNase P suggest that when studying the isolation of an enzyme with 3' endonucleolytic activity, the following questions should be asked:

1) Will the enzyme responsible for 3' processing demonstrate the same features as RNase P?
2) Will the 3' processing enzyme serve a universal function?

3) Will processing at the 3' terminus require an RNA component for structural recognition of the cleavage site?

4) Will there be a requirement for a mature 5' terminus before cleavage will occur at the 3' terminus?

Processing at the 3' terminus of tRNA has focused on exonucleases and formation of the mature product (2,5,8,50,61,73-75). A number of exonucleases have been characterized. The exonucleases form the 3' terminus by removing one base at a time until the mature 3' end is exposed. 3' processing may also result from an endonucleolytic action (7-11).

Intervening sequences must be spliced out of some tRNA molecules in order to produce the mature tRNA product. This reaction requires the action of two enzymes, an endonuclease which cleaves both ends of the intervening sequence and a ligase which ligates the free ends forming the mature tRNA molecule (5,33,53-56,76-82).

In addition to processing, base modifications occur during the course of tRNA maturation to produce functional tRNA molecules. The types of base modifications are numerous, including alkylations and methylations. Modifications can provide recognition sites that will direct processing of the RNA precursor (6,8,57).

The attachment of the -CCA end to the tRNA molecule is the function of the CCA pyrophosphorylase enzyme. Addition of these bases at the 3' terminus forms the mature attachment arm. This enzyme also maintains the functioning 3' terminus by adding adenine to the CCA terminus so that the CCA end is maintained (8). -CCA addition occurs enzymatically in eukaryotes because the -CCA is not encoded in the gene sequence. In most prokaryotes, the -CCA terminus is encoded in the gene sequence. 3' processing exposes the mature attachment arm with a -CCA terminus.

Dimers and other multiple gene precursors represent a unique entity for the processing nuclease. The original cleavage product for dimers could be the first gene processed at the 5' terminus with the second gene remaining intact; or processing could occur at the 5' terminus of both genes (20,23,50,78,81). Processing is a complex course of events influenced by enzymes, molecular conformation, internal and external factors and the availability of substrates in a given time frame.

The results of the five general processing functions on tRNA is to produce a mature tRNA product which can serve as the adaptor molecule in amino acid transport in protein synthesis (52).

MATERIALS AND METHODS

Reagents. Ribonucleoside triphosphates and ribonucleoside [α-^{32}P] triphosphates were obtained commercially from Sigma Chemical Company, St. Louis, Missouri and New England Nuclear, Boston, Massachusetts, respectively. All chemicals used in this work were reagent grade and obtained from well known suppliers.

Plasmid DNAs. tRNA gene-containing plasmids were a gift from Dieter Söll, Department of Molecular Biophysics and Biochemistry, Yale University. tRNA genes used in this study have been inserted into plasmid pBR322 and include a Drosophila melanogaster histidine tRNA gene (pYM10.3T, also designated p48FHis) (18, 19, 21) which contains a 35 base pair 3' trailing sequence. An S. cerevisiae arginine - aspartic acid dimeric tRNA gene (pYM205, subcloned from pJB19F)(20) was also used. Three additional plasmids were also received from Dr. Söll, inserted into HB101, and used in the research. They are pYM7.2, an S. pombe tRNA histidine gene (21); pYM104, a multimeric plasmid containing S. pombe tRNA arginine, glutamic acid, and lysine genes (22); and pYM118 an S. pombe dimeric tRNA containing serine and methionine genes (23).

Cell Lines. Escherichia coli strain HB101 was used as the host for the transformation and was obtained from Dr. Robert Rothman of the Rochester Institute of Technology.

The yeast strain S.cerevisiae 20B-12 (α-trp 1 pep 4-3)(83) was kindly provided by Dr. D. Söll.

Bacterial transformation. HB101 were transformed according to standard methods (13, 17, 84, 85). HB101 were grown in 50 ml of L Broth [Luria Broth: Bacto tryptone 10 g/l, Bacto yeast extract 5 g/l and sodium chloride 10 g/l, pH 7.5 (13)] to a density of approximately $5x10^7$ cells/ml. 3 ml of cells were used for each transformation assay. Cells were chilled on ice for 10 minutes and then 37 ml of the cells were centrifuged in the Sorvall centrifuge at 4000 x g for 5 minutes at 4°C using the SS-34 rotor. The supernatant was discarded and a sterile solution of 50 mM $CaCl_2$ and 10 mM Tris Cl, pH 8.0, at half the original volume (19 ml) was added to the cells. The cells were placed in an ice bath for 15 minutes and then centrifuged at 4000 x g for 5 minutes at 4°C in the Sorvall, using rotor SS-34. The supernatant was discarded and the cells were resuspended in a 1/15 volume (2.5 ml) of sterile solution of 50 mM $CaCl_2$ and 10 mM Tris Cl, pH 8.0. Cells were stored at 4° for 12-24 hours, in 0.2 ml aliquots. Efficiency of transformation increases during this time (14, 85). Plasmids were added in Tris-EDTA buffer (TE) [10 mM Tris Cl, pH 8.0, 1 mM EDTA] mixed and stored on ice for 30 minutes. Up to 40 ng of DNA in up to 100 ul of T.E. buffer insures best transformation efficiency. Plasmids received as gifts were lyophilized and resuspended in 50 ul T.E. buffer. 10 ul of each plasmid was

used in a transformation assay. The transformation assay was mixed and stored on ice for 30 minutes. The cells and plasmid were placed in a 42°C water bath for 2 minutes. Following the priming bath, 1.0 ml of L broth containing 50 ul/ml ampicillin was added to each tube and incubated at 37°C for 1 hour (ampicillin selection) without shaking. Expression of antibiotic resistance begins with this procedure. Cells were then spread on selective media, (LB plate containing 50 ug/ml ampicillin) at 100 ul or 200 ul of cells per plate, and incubated for 12-16 hours at 37°C. Transformed colonies were visible. All the plasmids used in our studies have the gene inserted into the tetracycline site, therefore colony growth will only occur on ampicillin.

Growth and amplification of the bacterium containing plasmid. 10 ml of LB medium containing the 20 ul ampicillin (50 ug/ml) was inoculated with a single colony from a selective media plate. The culture was incubated with shaking at 37°C 12-18 hours. One-tenth milliliter of the overnight culture was inoculated into 40 ml of L broth containing 80 ul ampicillin (50 ug/ml). The culture was incubated with shaking at 37°C to an $O.D._{600}$ of approximately 0.6. Twenty-five milliliters of this culture was inoculated into 500 ml of L broth containing 1 ml ampicillin (50 ug/ml). Incubation with shaking was for 2.5 hours at 37°C. The $O.D._{600}$ was approximately 0.4. To the cell culture, 2.5 ml of a solution of chloramphenical (CAP), 34 mg/ml in ethanol, was added making the final

concentration of CAP in the culture 170 ug/ml. This solution was incubated with shaking at 37°C for 12-16 hours (13).

Harvesting and lysing of the bacteria. Cells were harvested by centrifugtion at 5000 x g for 5 minutes at 4°C in the Sorvall, using rotor SS-34. The supernatant was discarded. The cells were washed in 40 ml of ice-cold STE buffer [0.1 M NaCl, 10 mM Tris Cl, pH 7.8, and 1.0 mM EDTA] (13).

Lysis of cells was accomplished using lysozyme and sodium dodecyl sulfate (SDS) (13). The bacterial pellet was resuspended in 10 ml of an ice cold solution of 10% sucrose in 50 mM Tris Cl, pH 8.0, and placed in a 30 ml Sorvall centrifuge tube. Two milliliters of a freshly made solution of lysozyme (10 mg/ml in 0.25 M Tris Cl, pH 8.0) was added (13). Immediately 8.0 ml of 0.25 M EDTA was added; the solution was mixed and placed on ice for 10 minutes at 0°C. After 10 minutes, 4 ml of 10% SDS was added and mixed quickly with a pasteur pipette to disperse the SDS evenly throughout the suspension. Gentle mixing prevents liberated DNA from being sheared. Six milliliters of 5 M NaCl (f.c. = 1.0 M) was added, gently mixed, and the extract was placed on ice for at least 1 hour. The suspension was placed in Oakridge tubes, centrifuged in the Beckman centrifuge using the Ti- 50 rotor for 30 minutes at 30,000 rpm at 4°C. The supernatant was retained and the pellet was discarded. The supernatant was extracted twice with

phenol/chloroform (1:1, v:v) saturated with 50 mM Tris Cl, pH 8.0 and then once with chloroform saturated with 50 mM Tris Cl, pH 8.0. After each extraction the suspension was centrifuged in the Sorvall centrifuge using the SS-34 rotor at 8000 x g for 5 minutes at 4°C. After the final extraction the entire aqueous phase was transferred to a 250 ml flask and mixed with two volumes of 95% ethanol (60 ml). The mixture was stored at -70°C for 15 minutes. Alternatively, the mixture could be allowed to stand at -20°C for 1-2 hours, or overnight. Nucleic acids were recovered by centrifugation in the Sorvall centrifuge using the rotor at 6000 x g for 15 minutes at 4°C. The supernatant was discarded and the pellet was washed with 95% ethanol. The DNA pellet was dried by inverting the centrifuge tube on absorbant material. The DNA was dissolved in a total volume of 8.0 ml of TE buffer, pH 8.0.

The volume of the DNA obtained from cell lysis was measured and for every 1.0 ml of DNA, exactly 1.0 g of solid cesium chloride (optical grade) was added. The solution was mixed until all of the salt was dissolved. Eight-tenths milliliter of a solution of ethidium bromide (10 mg/ml in H_2O) for every 10 ml of cesium chloride solution was added and mixed well. Final density as tested on a refractometer read 1.55 g/ml (η = 1.3860) and the concentration of ethidium bromide was approximately 600 ug/ml. The solution was transferred to cellulose nitrate tubes suitable for use in the Beckman type Ti-50 rotor and topped with paraffin

oil. The tubes were centrifuged in a Beckman centrifuge using a Ti-50 rotor at 40,000 rpm for 36 hours at 20°C. When removed, two bands of DNA were visible using UV light. The upper band represents the E. coli chromosomal DNA containing broken fragments, and linear DNA. The bottom band represents the closed circular plasmid DNA in purified form. The tubes were punctured by a syringe with a #21 hypodermic needle entering from the side at the exact level of the lower band of DNA. As much as possible of the band was removed from the tube and subsequently placed in a 15 ml tube. An equal volume of 1- n-butanol saturated with water was added to the plasmid DNA. The two phases were mixed by pipetting vigorously. The tube was centrifuged at 1500 x g for 3 minutes at room temperature in a counter top centrifuge. The upper organic phase was removed and the solution extracted 4-6 times with 1-n-butanol saturated with water until all the pink color of the ethidium bromide disappeared from the aqueous phase. The aqueous phase was then dialyzed against several changes of TE buffer, pH 8.0. Finally, the plasmid DNA was lyophilized and resuspended in distilled water to the appropriate concentration for use in a standard transcription assay. A portion of the dialyzed plasmid was stored in the freezer at -20°C and some was kept at 0°-4°C for immediate use in a transcription assay (13).

Enzyme extraction from Saccharomyces cerevisiae. A culture of S. cerevisiae strain 20B-12 (α -trp 1 pep 4-3) (83) was a gift from Dieter Söll. Transcriptionally active

cell-free extracts from the yeast were prepared according to the procedure of Klekamp and Weil (1) with modifications by Schaack and Söll (37). Cells were grown in YEPD media [1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose (86)] to a density of about 3.0 (37) as measured by absorbance at 650 nm. Cells were harvested and washed twice with distilled water. Eight grams of fresh cells were placed in the 30 ml chamber of the Bead Beater Cell Disrupter (Biospec Products, Bartlesville, OK) using the smooth rotor. Eight grams of glass beads (50 um) were added to the cells. The Bead Beater chamber was filled completely to the top using Solubilization buffer [200 mM Tris Cl, pH 8.1, 10% w/v glycerol, 10 mM $MgCl_2$, 10 mM dithiotreitol, 1.0 mM phenylmethylsulfonyl fluoride]. The unit was assembled with a $CaCl_2$- ice slurry in the jacket. Cells were broken for a total time of 3 min. using 30-second bursts followed by 2 minutes of cooling time. The lysate was recovered by centrifuging in the Sorvall centrifuge using rotor SS-34 at 7000 rpm for 10 minutes. Phenylmethylsulfonyl fluoride and dithiotreitol were added to the lysate to re-establish their final concentrations at 1.0 mM and 10 mM, respectively. Ammonium sulfate (3.7 M, pH 7.9) was added to the lysate to give a final concentration of 0.4 M ammonium sulfate. The lysate was stored on ice for 10 minutes, and then centrifuged in the Beckman ultracentrifuge at 100,000 x g for 60 minutes at 4°C using the Ti-50 rotor. The supernatant volume was measured and solid ammonium sulfate

crystals were added slowly with stirring to a final concentration of 0.35 g/ml. The mixture was stirred for an additional 30 minutes until all the salt was dissolved. The precipitate was recovered following a 12,000 rpm centrifugation for 15 min. at 4°C in the Sorvall centrifuge using rotor SS-34. The precipitate was resuspended in 1.0 ml of Buffer C [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.9, (HEPES), 20% v/v glycerol, 0.2 mM EDTA, 10 mM dithiotreitol, 0.1 mM phenylmethylsulfonyl flouride] and dialyzed overnight against one liter of Buffer C containing 100 mM NaCl. The extract was used fresh or frozen at -80°C in 50 ul aliquots (1, 87).

Protein determination. Amounts of protein in the enzyme preparation were determined by the Bradford method (87) using bovine serum albumin as a standard.

Transcription Assays. Transcription assays were in 50 ul final volumes. The assay contained 12 mM HEPES, pH 7.9, 150 mM NaCl, 10 mM $MgCl_2$, 12% (v/v) glycerol, 6 mM dithiotreitol, 0.6 mM each of CTP and GTP, 1.2 mM ATP (37), 7.5 - 12.5 uCi [α-^{32}P] UTP (760 Ci/mmole), and 25 ug/ml of supercoiled plasmid DNA. An enzyme extract prepared from S. cerevisiae (1 - 5 ul of protein per reaction) was then added. Assays were incubated for 30-60 minutes at 20°C (1).

Reactions were stopped by addition of 40 ul of a solution of proteinase K (1 mg/ml in 0.5% SDS pre-incubated at 37° for 30 minutes) and the reactions were incubated at 37°C for

a further 30 minutes. Following incubation, 100 ul of EBS Buffer [300 mM NaCl, 1.0 mM EDTA, 10 mM Tris Cl pH 8.0, 0.5% SDS] were added. The reactions were extracted twice by the addition of 100 ul of phenol: chloroform: iso-amyl alcohol (v/v/v, 50:48:2) saturated with TE Buffer (86). In some assays, 5 ul of carrier tRNA (2 mg/ml) were also added prior to the first extraction. The RNA contained in the aqueous phase was precipitated by the addition of 2.5 volumes of ethanol and either put in the freezer overnight or stored in the Revco (-80^{o}C) for 30 minutes. The RNA precipitate was recovered by centrifugation in the Sorvall centrifuge for 10 minutes at 4^{o}C and 7000 rpm using rotor SS-24. The supernatant was discarded and the RNA precipitate was resuspended in 200 ul of distilled water. The RNA was reprecipitated by adding 20 ul of a 1.0 M solution of sodium acetate pH 5.4 and 500 ul of ethanol and placed in the freezer overnight. The RNA precipitate was recovered by centrifugation in the Sorvall at 7000 rpm for 10 minutes at 4^{o}C. The RNA was lyophilized to dryness and then redissolved in tracking buffer [7 M urea, 1/2 M Tris-borate electrophoresis buffer (TBE), 10 mM EDTA, 0.1% Xylene cyanol FF (XCFF) and 0.1% Bromophenol blue (BPB)] for electrophoresis. The RNA was then electrophoresed. The voltage applied to 0.3 mm thick gels was 400 V and 6-8 ma for 1.5 hours (86). The voltage for 2 mm thick gels was 150-200 V and 8-10 ma overnight. Overnight autoradiography was used to visualize the transcripts.

Polyacrylamide Gel. The gel used in purifying the substrate RNAs was a 10% polyacrylamide gel containing 8.3 M urea. Gel thickness was either 0.3 mm or 2 mm.

Stock components include:

40% gel - 190 g acrylamide and 10 g bisacrylamide in 500 ml of distilled water, followed by the addition of charcoal and gravity filtration of the entire solution.

10X TEB - 100 mM Tris-borate, 2 mM EDTA; 121.2 g (100 mM) Tris Cl, pH 8.0, 61.8 g (100 mM) boric acid, 7.4 g (2 mM) Na_2EDTA in one liter of distilled water

Ammonium Persulfate solution - (freshly prepared) 0.3 g ammonium persulfate in 10 ml distilled water.

Components were combined as follows: 37.5 ml of 40% gel, 75g urea and 15 ml of 10X TEB were combined and mixed until the urea dissolved. Volume was brought to 148.5 ml with distilled water. Air was removed with a vacuum pump. 45 ul TEMED (N,N,N',N' - tetramethylethylene diamine) and 1.5 ml of the ammonium persulfate stock was added to the gel solution and mixed well. The gel solution was poured into a preassembed mold and allowed to polymerize.

Following electrophoresis, one glass plate was removed from the gel. The gel was carefully wrapped in plastic wrap and the location of the two tracking dyes was marked. The

gel was then exposed to Eastman Kodak X-ray film (X-Omat AR Film, XAR-5, 20.3 cm x 25.4 cm). The film was developed after 24 hours, and evaluated (88,89).

RESULTS

Preliminary. Prior to transforming HB101 cells, it was necessary to determine the concentration of the plasmids obtained from the laboratory of Dr. Dieter Söll. The approximate concentration was determined by constructing a series of samples with known plasmid concentrations. By adding a drop of each standard and the unknown DNA plasmid onto plastic wrap followed by a drop of ethidium bromide, a color change could be noted and concentration of the stock plasmids could be extrapolated (13).

Transformations. The plasmids were then used in separate transformation experiments described previously. The results of these experiments are given in Table 3. Some of the resulting transformed bacterial colonies were picked for subsequent growth experiments and DNA isolation. These transformed bacteria then served as a source of the tRNA gene containing plasmid DNAs used in the various transcription assays.

Some transformed cells were frozen in aliquots and stored in the freezer in a 1:1 solution of dimethyl sulfoxide (DMSO).

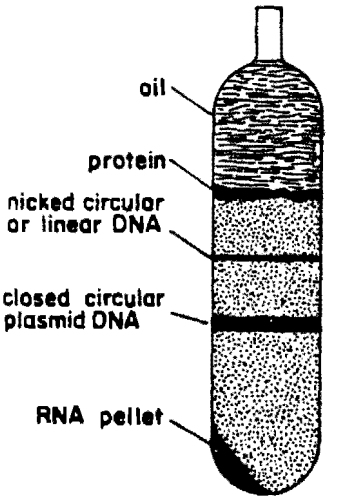
Plasmids. Plasmids were amplified in the E. coli host, HB101, and purified according to standard methods (13). Figure 7 represents a typical profile of a cesium chloride-ethidium bromide gradient. The position of the circular plasmid DNA makes it easy to extract from the gradient in

Table 3

Yield of Transformed Colonies

Plasmid	Number of transformed colonies
pYM7.2	7
pYM10.3T	9
pYM104	2
pYM118	1
pYM205	42

Cesium Chloride Gradient Banding of Plasmid DNAs



(13)

Figure 7. Expected gradient banding pattern following centrifugation. Less ethidium bromide is intercalated in the circular plasmid DNA, so it bands at a lower point in the gradient.

pure form. Table 4 indicates the yields of purified DNA from a number of experiments. The expected yield of plasmid DNA is about 1.0 mg plasmid/500 ml cell culture. Table 4 indicates that the yields of about 31-442 ug DNA/500 ml cell culture were lower than expected (13). There is no apparent reason for this discrepancy. The plasmids were lyophilized to dryness and then resuspended in distilled water to a concentration of 250 ug/ml.

Transcription Extract. An enzyme extract containing RNA polymerase III and all the essential factors for transcription was obtained from S. cerevisiae using the protocol of Klekamp and Weil (1). In some experiments, the experimental design was altered according to Schaack and Söll (37, and personal communications) in amounts of cells homogenized and the ratio of cells to beads. The expected yield of protein from the enzyme extraction was 20-30 mg of protein/ml (1). Experimental data indicated actual yields at 21 ug/ul to 28.5 ug/ul using the Bradford method with bovine serum albumin as standard (89). The concentration of protein was therefore within expected literature values.

Aliquots of the enzyme extract were either used immediately in a transcription assay, or were stored in 50 ul units at -80^{o}C.

TABLE 4

Yields of Purified Plasmid DNA

Plasmids	Prep. No.	ug DNA isolated	tRNA gene
pYM7.2	1	59	S. pombe
pYM7.2	2	442	tRNA (his)
pYM10.3T	1	93	
pYM10.3T	2	35	D.melanogaster
pYM10.3T	3	37	tRNA (his)
pYM10.3T	4	60	
pYM104	1	49	S. pombe tRNA (arg, glu, lys)
pYM118	1	56	S. pombe tRNA (ser, met)
pYM205	1	31	S. cerevisiae
pYM205	2	233	tRNA (arg, asp)

All preparations were from a 500 ml culture of cells. 1 A_{260} unit is equal to 50 ug of plasmid DNA.

Transcription Assay. Initially the procedure of Klekamp and Weil (1) was used in establishing transcription conditions. Results were inconclusive and often resulted in little or no transcription. During the course of this research, an article by J. Schaack and D. Söll was published describing changes for the yeast transcription system. Transcription assays were altered using the published results from Schaack and Söll (37). Conditions altered from those in Klekamp and Weil (1) included enzyme concentration and ATP concentration. Essentially, lower concentrations of enzyme extract were utilized, 1-3 ul vs. 10-30 ul in the Weil assay. In addition, the ATP concentration was changed from 0.6 mM to 1.2 mM. Total assay volume remained at 50 ul. Once these changes were incorporated, the transcription assays gave rise to specific products.

In preliminary experiments it was necessary to ascertain the location of the transcription products on the gel. A brief discussion of the process follows.

Gel Analysis. When a sample is electrophoresed, the molecules move through a gel because of the current applied. Because of the pore size of the gel, molecules will move through the gel according to their size, with the smallest molecule migrating most quickly. Once the electrophoresis is complete, bands in different positions can be identified by making comparisons to standards and marking dyes. The dominant bands of interest are the initial transcript, the 5' processed molecule and the 3' processed molecule.

Standard markers described in the following discussion help to identify these gel bands.

When a sample is loaded on a gel, it has been suspended in tracking buffer. This tracking buffer contains two dyes. One dye, xylene cyanol FF (XCFF) migrates in the region where mature tRNA would be expected; the other dye, bromophenol blue (BPB) runs the most rapidly and tracks the running front of the electrophoresis. At the completion of the electrophoresis, the dyes give a visual indication of the approximate location of the mature tRNA and the gel front.

This tracking pattern was confirmed by a controlled experiment with a known tRNA, E. coli $tRNA^{met}_f$. After electrophoresis, the gel was stained and the location of the $tRNA^{met}_f$ identified. This marks the approximate region in which the purified tRNA molecules will be located, since all tRNA molcules have a similar size. The XCFF marker dye migrated 7.0 cm on the gel whereas the $tRNA^{met}_f$ stained marker migrated 5.7 cm. The rate of migration of tRNA is about .8 that of the XCFF marker. From these controlled data, we would conclude that gel bands located near the R_f region of 0.8 relative to the XCFF position on the gel are mature tRNA products.

The use of [α-^{32}P] ATP in a transcription assay demonstrates some unique characteristics of banding (see figure 8). While [α-^{32}P] ATP shows no specific transcription in this experiment the contaminating tRNA

Transcription of pYM10.3T Using [α-^{32}P] ATP

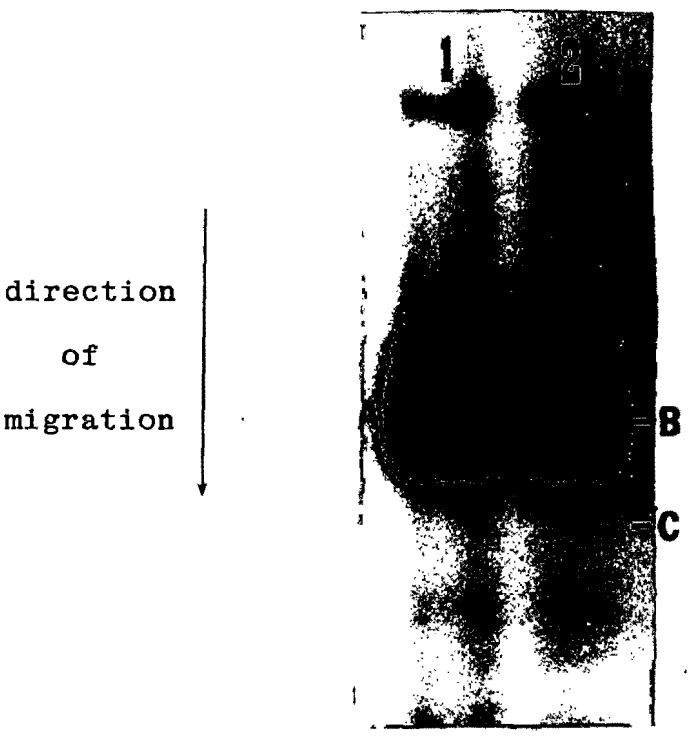


Figure 8. Lane 1, no DNA; Lane 2, 250 ug/ml pYM10.3T DNA A marks the position of the end-labeled mature tRNA. B marks the position of the stained E. coli tRNA $^{met}_{f}$ standard gene marker. C marks the position of the XCFF dye. The BPB dye lies below the region shown on the print.

present in the enzyme preparation is end-labeled due to the turnover of the 3' adenylic acid present on all tRNA molecules. These end-labeled tRNA molecules identify positions of tRNA on a gel. The use of [α-^{32}P] ATP indicates that mature tRNAs migrate somewhat slower than the XCFF marking dye. These indicators demonstrate that mature tRNA on a gel is located slightly behind the XCFF dye marker, near the stained E. coli $tRNA^{met}_f$ standard gene marker, and at the position of the [α-^{32}P] ATP-labeled product. Sequencing is, of course, the ultimate proof by which to identify the mature tRNA product and its location on a gel.

Specific Transcription. Figures 4 and 5 give the nucleotide sequence of pYM10.3T. The sequence demonstrates the importance of selection when choosing a plasmid for use in transcription processing assays.

pYM10.3T is the Drosophila melanogaster histidine tRNA gene. This eukaryotic gene has been inserted into a prokaryotic plasmid, pBR322, at the tetracycline site. Notice the 5' flanking nucleotides which come before the initiation site (indicated by an arrow). These help in transcription complex formation so that the correct initiation site is used. Notice also the 3' flanking sequence which extends beyond the termination site (termination site is identified by an arrow). This is the nucleotide sequence which, when cleaved as a unit, can be easily identified on a gel. The presence of this trailing

sequence as a unit demonstrates that endonucleolytic processing has occurred. The internal portion of the gene contains control regions for complex formation and transcription (nucleotides 8-58). pYM10.3T contains no intervening sequences.

An autoradiograph of a typical transcription of pYM10.3T is shown in Figure 9. As the autoradiograph is analyzed, recall the gel analysis discussed earlier. Band 1 on the autoradiograph represents the initial or precursor tRNA molecule. 5' processing occurs, producing band 2, the 5' processed tRNA molecule. Following 5' processing, the tRNA molecule undergoes 3' processing, producing band 3, the 3' processed or mature tRNA molecule.

Note that in lane 1, which contains no plasmid DNA, there are no bands, indicating dependence on the added pYM10.3T DNA, containing the $tRNA^{his}$ gene. The specific transcription of pYM10.3T is indicated in lanes 2 and 3 which in the assays contained different amounts of pYM10.3T DNA. Lane 4 contained the plasmid pYM118, an S. pombe, dimeric gene containing both a serine and an initiator methionine gene. Note that the banding pattern is different than lanes 2 and 3, again indicating that transcription is specific to the particular tRNA containing plasmid added to the assay.

Conclusions. If an autoradiograph of a transcription reaction, such as the one in Figure 9, is examined carefully, it is evident that the band of interest may be

Transcription of pYM 10.3T and pYM 118

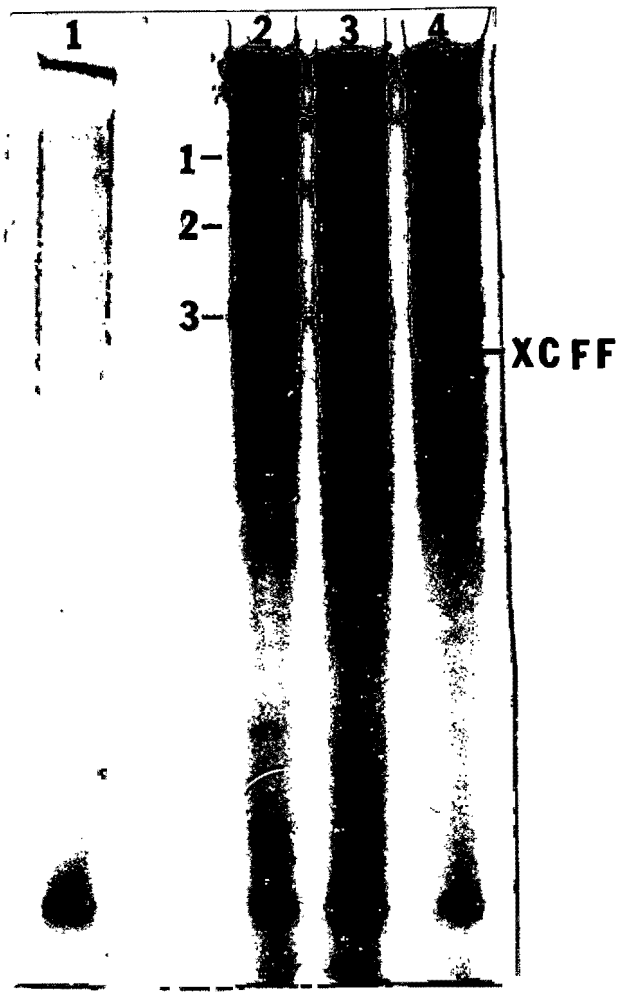


Figure 9. Transcription assays were performed as described in Materials and Methods. Lane 1, no DNA; Lane 2, 250 ug/ml pYM10.3T DNA; Lane 3, 500 ug/ml pYM10.3T DNA; Lane 4, 250 ug/ml pYM118 DNA. Band 1, precursor tRNA; Band 2, 5' processed tRNA; Band 3, 3' processed tRNA or mature tRNA. The BPB dye lies below the region shown on the print.

several bands in close proximity. Since the enzyme preparation used is a crude extract, nuclease activity is present along with the polymerase, endonuclease and ligase activities. Since all enzymes are present, small changes can be represented by a general nuclease activity which has removed one nucleotide from the processed band. Of course, sequencing of gel products will identify the gel bands of interest. This sequencing would confirm the location of the desired substrate. In addition, purifying the crude extract will remove some of the unwanted enzyme activities and precise banding will become more common. The bands on the gel can be extracted and eluted from the gel for the study of endonucleolytic processing of tRNA. This experimentation may identify an enzyme which endonucleolytically processes the 3' terminus of tRNA as RNase P endonucleolytically cleaves the 5' terminus of tRNA.

The goal of this thesis research has been to isolate, from HB101, a plasmid containing Drosophila melanogaster histidine tRNA gene (pYM10.3T), and to transcribe the tRNA gene portion of the plasmid to provide a tRNA precursor molecule for the study of endonucleolytic cleavage in tRNA. Additional studies, which are in progress now, will take the substrate as produced by this transcription system, to identify the enzyme which cleaves the tRNA endonucleolytically at the 3' terminus. This endonucleolytic enzyme acitivty can then be characterized and its properties compared to those of RNase P.

DISCUSSION

The goal of this project has been to establish a transcription system for tRNA genes using a Saccharomyces cerevisiae crude enzyme extract. tRNA genes are transcribed well in the yeast sytem providing a substrate molecule which can be used to study tRNA processing. Transcription systems are very sensitive, so initially, each component of the assay was tested to be sure assay conditions were accurate. Plasmid concentrations were stabilized at 250 ug/ml. The enzyme concentration for the assay was tested using 1 to 30 ul quantities of enzyme. Best transcription results came from lower concentrations ranging from 1 to 5 ul. More enzyme caused greater smearing during gel separation and difficulty in identifying final products. The lower enzyme concentration helped to reduce background nuclease activity as well as reducing smearing on the gel.

Transcription systems are influenced by physical factors in such a way that transcription may be stopped, complex formation may be disturbed, transcription may be reduced, or transcription may be random. In random transcription, a substrate product is being formed but is lost in the general banded smear on a polyacrylamide gel. It has been shown that transcription products form distinct bands and are successfully separated on polyacrylamide gels. This project established conditions which enhance transcription over nuclease activity, which provide the purified molecule

needed and which do this consistently. The next research thrust would be to take the transcribed substrate, subject it to a processing assay, and look for endonucleolytic cleavage at the 3' terminus of the gene. That such an endonucleolytic enzyme exists has been demonstrated by Castaño and colleagues (11).

Recent literature articles have reported on the 5' processing enzyme, RNase P (3,4). It is known that RNase P accurately cleaves the 5' terminus in all tRNAs to produce the mature terminus. The efficiency by which this enzyme carries on its activity suggests that a similar system may function at the 3' terminus in all tRNAs. Costaño's article (11) which not only describes the existence of a 3' processing enzyme but also characterizes that enzyme, raises more questions. Is this 3' activity universal and will its activity correspond to the RNase P activity at the 5' terminus? The thrust of future research for this project will be to isolate a 3' terminal endonucleolytic enzyme and compare its function and characteristics to "3' pre-tRNase" as described by Costaño (11).

The "3' pre-tRNase" isolated by Costaño's group was purified from a Xenopus laevis ovarian extract. This enzyme endonucleolytically processed the 3' terminus of human pre-$tRNA^{met}$ which was generated in a cell-free transcription system from Human KB Cells. The "3' pre-tRNase" isolated by Costaño, et al. was also shown to accurately process the Bombyx mori trailer of the pre-$tRNA^{ala}$.

The processing studied to date has been in animal systems. The 3' endonucleolytic enzyme in this research originates from Saccharomyces pombe (a yeast). Similar activities from the animal system and a plant system would suggest that the 3' endonuclease, as it has been described (11), may provide a universal function.

As can be seen by previous descriptions of the future research thrust, tRNA processing extends control even over its own production. One type of control stems from regulating the amount of RNA available for processing. This is a function of the transcription system. Stable complex formation, 5' and 3' sequences, and an internal control system can cause transcription to produce less tRNA. Enhancing the system produces more tRNA. These are natural controls. In the processing system, a large investment is made in both processing and modifying enzymes. The use of numerous enzymes provides control over the amount of tRNA produced. If any enzyme in the process is limiting, the amount of tRNA produced by the processing system is lower. This would then limit protein sythesis because of the lack of tRNA to carry amino acids. Not only does tRNA regulate its own production, but through such regulation, it influences protein synthesis.

Since our research has established a working transcription system which transcribes a substrate used in studying 3' terminal processing, questions can now be asked about this system and the goal of this research. Is the

action of the 3' processing nuclease, "3' pre-tRNase", similar to RNase P? Does the 3' processing endonuclease require an RNA component? What role do the 5' leading sequence and the 3' trailing sequence play in the control of processing?

The unique structure of some tRNAs raises questions relating to universal processing. The configuration of tRNAs can vary due to extended trailers, modifications of base within a sequence and additional length on the variable loop. Yet studies reveal that RNase P accurately processes tRNAs at the 5' terminus and it is assumed that "3' pre-tRNase" will accurately process all tRNAs at the 3' terminus. What unique sequence or structure do these enzymes recognize to assure accurate processing?

In order to answer these questions, the purification and detailed characterization of RNase P and "3' pre-tRNase" must be completed. The research conclusions reached in this report are a beginning step toward such a goal.

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