PROCESSING OF tRNA IN PROKARYOTES AND EUKARYOTES

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I. INTRODUCTION

The primary product of transcription of a tRNA gene, in all systems so far examined, is a precursor molecule that must undergo processing to generate the mature, functional tRNA. The structure of a generalized tRNA precursor, incorporating features of these molecules from various sources, is depicted in Figure 1.

tRNA precursors generally contain extra nucleotides at both their 5′ and 3′ ends and in some instances also possess intervening sequences in the middle of the molecule. Included as part of the extra nucleotide sequences may also be additional tRNAs if the precursor happens to be multimeric. In addition, precursors are often devoid of the 3′ terminal –CCA sequence present in all functioning tRNAs, and they also lack the full complement of modified nucleotides that are a hallmark of tRNA structure. Knowledge of the structure of tRNA precursors and of tRNA genes has, of course, provided the most important information as to the type of processing events that are required to convert a tRNA precursor to a mature tRNA. It is now clear that tRNA processing involves a series of enzymatic reactions which cleave, trim, and splice the precursors, as well as reactions which add terminal nucleotides and which modify specific nucleotide residues.

In recent years considerable progress has been made in our understanding of tRNA processing. Intermediates in the process have been identified and characterized and enzymes which catalyze a number of the processing steps *en route* from a precursor to a mature tRNA have been isolated. These studies have suggested some order to processing events and have helped to define "processing pathways" in some instances. In addition, the more difficult question of processing enzyme-substrate recognition that underlies the specificity and order of the processing system has begun to be analyzed.

This review will concentrate on those aspects of tRNA processing in which nucleotides are removed from or added to tRNA precursors. Since a number of other earlier reviews dealing with tRNA biosynthesis, processing, and nucleotide modification have appeared,[1-5] this article will deal in more detail with newer information and will attempt to incorporate this new material into a general framework for tRNA processing. In addition, rather than examining all the fine points of tRNA processing in detail, most of which have been covered adequately by others, an attempt will be made to critically examine some of the more general features that are important to the study of tRNA processing.

II. APPROACHES AND PROBLEMS IN THE STUDY OF tRNA PROCESSING

In common with the elucidation of other biosynthetic pathways, a complete understanding of tRNA processing requires the identification and characterization of all the intermediates

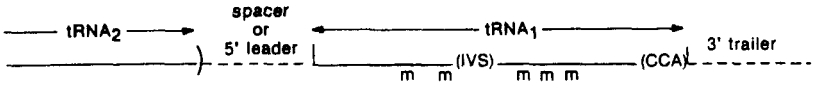

FIGURE 1. Structure of a generalized tRNA precursor. This schematic diagram incorporates features found in tRNA precursors from different sources. The dashed lines represent extra 5′ and 3′ sequences that will be removed during processing. The sequences in parentheses, i.e., intervening sequence (IVS) and CCA are not found in all precursors. The second tRNA, $tRNA_2$, is present if the precursor is multimeric. The letters, m, below the tRNA, represent nucleotide modifications that are added during processing.

and the isolation and study of the enzymes needed to convert a primary transcript to a functional tRNA. The difficulties in the analysis of tRNA processing pathways are that the intermediates are structurally complex molecules, present in small amounts, whose characterization requires considerable effort. This also complicates the identification and purification of the relevant processing enzymes since specific assays generally are quite time-consuming. Nevertheless, progress has been made, more so in the case of tRNAs than of other RNA molecules because their relatively small size has made sequence analyses possible.

Our current knowledge of tRNA processing pathways, as with other metabolic processes, comes largely from the isolation of mutants which are affected in a particular processing step. These mutants may affect a specific processing enzyme leading to the accumulation of intermediates at the processing block[3] or they may affect a tRNA gene itself leading to a precursor which cannot be processed efficiently or accurately.[6] In many cases, these altered precursors do not accumulate since their apparently abnormal conformation often leads to degradation.[7] The role of mutant isolation for studies of tRNA processing has, of necessity, been confined to those systems in which genetic procedures are readily available, namely, bacteriophage (mainly T4), bacteria (mainly *Escherichia coli*), and yeast.

An alternate approach has been employed for studying tRNA processing in higher and lower eukaryotes. The ability to clone specific eukaryotic tRNA genes[8] has allowed the development of in vitro transcription and processing systems which frequently accumulate sufficient levels of processing intermediates to permit structural analysis. The system of most use has been the *Xenopus laevis* germinal vesicle extract,[9] but studies have also been carried out with extracts from other sources as well.[10,11] In addition, as a complement to these experiments, cloned tRNA genes have been microinjected into *X. laevis* oocytes to identify the in vivo intermediates and to determine the subcellular localization of the processing events.[12-14] However, despite these approaches, our understanding of tRNA processing in higher organisms continues to be hampered by a lack of mutants, since without the appropriate mutants it is not possible to conclusively establish whether an apparent intermediate actually is on the processing pathway and whether an isolated enzyme really plays a role in tRNA processing in vivo. This is particularly true when, as seems to be the case, there are multiple enzyme activities in extracts able to catalyze particular processing reactions,[15] or when an enzyme's action in vitro may not reflect the actual function of an enzyme in the cell.[16]

Even the use of mutants may present certain inherent pitfalls. Enzymes of macromolecular metabolism are often present in cells at levels far in excess of their requirements.[17] Thus, the effects of a mutation in a processing enzyme may be masked by small amounts of residual enzyme present within the cell, although in vitro assays may suggest that the enzyme is absent. Likewise, mutants affected in processing may accumulate certain intermediates that ordinarily do not exist in the cell. For example, an *E. coli* mutant lacking RNase III accumulates a 30S precursor to rRNA,[18,19] whereas this molecule generally does not exist intact in the cell since it is cleaved by RNase III before its synthesis is completed.[20] One

additional problem that may be encountered in the use of mutants to study processing, especially in the case of brute force isolations in which heavy mutagenesis has been employed, is the occurrence of multiple or closely linked mutations, each of which alone, or in combination, affects the processing step under study. Detailed genetic studies may be required to sort out such a problem.

Coupled with the isolation of tRNA processing intermediates, either through the use of mutants or by accumulation in processing systems, is the structural characterization of these molecules. It was only with the advent of RNA sequencing procedures that studies of processing became feasible.[21] Without knowledge of the structure of the processing intermediates it is, of course, not possible to define a processing pathway or to identify a processing enzyme. The more recent introduction of gene cloning and DNA sequencing procedures has provided information on the probable structure of the primary transcripts.[22,23] Since the structures of mature tRNAs are known, the two ends of the processing pathway are defined and it is possible to infer what kinds of processing events would be required to convert the initial transcript to the final product. The availability of this sequence information for tRNA genes has led directly to our knowledge of which precursors have intervening sequences[24,25] and which ones encode the –CCA sequence.[26] Likewise, identification of transcription initiation and termination sites has indicated the amount of extra sequence which has to be removed during processing to generate the mature tRNA.

Once the identity of the processing intermediates has been determined and the general outline of the processing pathway elucidated, further understanding of the process becomes an enzymological problem, i.e., what enzymes are catalyzing the various processing steps, how do they work, and how are they regulated? The study of tRNA processing is now at this stage. Enzymes involved in tRNA processing are being identified, purified, and studied.[27] Most progress is being made with *E. coli* since in this system it has been possible to correlate in vivo processing defects in various mutant strains with the lack of specific enzymes.[3] In eukaryotes, on the other hand, the identification of certain potential processing enzymes on the basis of in vitro action has not yet led to conclusive involvement of these activities in tRNA processing.

Purification of individual tRNA processing enzymes has been a formidable task. Two main approaches have been used. In one, natural tRNA precursors have been isolated and used as substrates for conversion to the next intermediate in the processing pathway.[28,29] This procedure has the advantage of using the natural precursor, but it suffers from the difficulty of isolating large amounts of substrate and from the necessity of using gel electrophoretic analysis as the assay procedure. In addition, since highly radioactive, ^{32}P-labeled precursors are used, substrates are present in the assay much below K_m, large percentages of the substrates are converted, and initial rates are not being measured. These kinds of assay conditions are particularly troublesome when comparing the activity of different substrates with one enzyme or one substrate with different enzymes, since it is not possible to differentiate whether different levels of activity are due to effects on substrate binding or to actual differences in the rates of reaction. Nevertheless, despite these problems, this approach has proven successful for the isolation of *E. coli* RNase P,[29] RNase E,[30] RNase F,[31] and RNase PC,[32] for some eukaryotic tRNA splicing enzymes[33] and for the identification of several other potential processing activities.[34,35]

The second approach is the use of artificial tRNA precursors which have had radioactive residues enzymatically added to the 5′ or 3′ terminus of tRNA molecules to generate a substrate similar in structure to a natural precursor.[36] These substrates can be prepared in large amounts, can be added to assays at saturating concentrations, and can be used for simple "acid-soluble" assays. However, once an enzyme has been purified by this type of procedure, it must be tested against natural precursors. This approach has been used to isolate *E. coli* RNase D[37] and RNase BN[38] and *Schizosaccharomyces pombe* RNase P.[39] A

similar procedure has been used for identifying some eukaryotic tRNA ligases.[40] In a few cases, namely *E. coli* RNase III[41] and tRNA nucleotidyltransferase,[26] enzymes that had already been purified were shown by mutant analysis to be involved in tRNA processing. The catalog of tRNA processing enzymes has been expanding to the point that in *E. coli* the known activities may account for all the processing events known to occur.

A major difficulty in studying the properties and substrate specificity of processing enzymes is in knowing when they have been sufficiently purified free of other ribonucleases so that meaningful characterization can be made. Generally, this is not a concern when an enzyme has been purified to apparent homogeneity, but even in these cases contamination with small amounts of another active ribonuclease could distort the properties of the enzyme under study. Of more concern, however, is that in many instances relatively impure preparations have been used to implicate certain activities in tRNA processing events. If a number of nucleases are present, which is often the case, the spectrum of cleavages observed may not be a consequence of the action of a single enzyme, but of several, which could be working independently or in concert.[15] In addition, since different RNases may have overlapping specificities (this is particularly true for exoribonucleases), even the conclusions drawn from studies with a single substrate may be in error. This type of problem may have led to the early suggestion of an involvement of RNase II in tRNA processing, whereas it is now believed that RNase II may have merely been a contaminant in the enzyme preparation.[35]

The main solution to this problem, of course, is extensive purification of the enzyme under study. However, the problem can be alleviated to some degree by the use of extracts from mutant strains which are devoid of possible interfering activities. Also, comparison of the in vivo and in vitro effects of a mutation known to affect tRNA processing and the use of multiple substrates in vitro often can help to sort out multiple activities in an extract.[38]

One further problem that has complicated our understanding of the multiplicity of tRNA processing enzymes is that different laboratories have used different substrates for their isolation procedures. Since individual enzymes have the capacity to act on many different precursors, the same activities have probably been rediscovered on different occasions and given different names. Where possible, an attempt will be made here to identify what appears to be the same enzyme, although in many instances there is not enough information available to clarify the situation.

III. PROCESSING EVENTS

tRNA genes are found in a variety of different contexts in different organisms. In various systems they are found as single genes,[8] in clusters containing several tRNA sequences (as many as 21 in *Bacillus subtilis*),[42-44] in spacer[45] and distal[46] regions of rRNA operons, and in association with protein genes.[47,48] In *E. coli*, for example, each of these gene arrangements has been identified in one or another region of the chromosome.[49] As a consequence of these distinct types of gene organization, a variety of tRNA gene transcripts have been observed. Thus, tRNA precursors which represent transcripts from a single tRNA gene or from clustered tRNA genes have been observed.[35,50] In eukaryotic systems, tRNA precursors tend to be of the former type,[51] although some dimeric precursors have been found.[51,53] In contrast, tRNA precursors containing as many as seven tRNA sequences have been synthesized from restriction fragments of the *E. coli* genome,[54] and a transcript containing all eight phage-specified tRNAs, as well as two other low molecular weight RNAs, has been obtained in the phage T4 system.[55] In addition, tRNA sequences are present in the transcripts of the rRNA operons[56] and in polycistronic transcripts containing mRNA.[47,57]

Maturation of individual tRNAs from this diversity of transcripts involves a number of different processing events including endonucleolytic cleavages, 3′ terminal trimming, end-addition reactions, and intervening sequence removal. Depending on the initial transcript, all, or only some of these events may be required to generate the functional tRNA molecule.

A. Endonucleolytic Cleavages

The primary processing events in the maturation of tRNAs from all types of precursors are endonucleolytic cleavages which serve to separate the tRNA sequences from extraneous nucleotide residues or from other RNAs. If the primary cleavage is carried out by RNase P, the 5′ terminus of the mature tRNA is generated directly.[29] This is usually the case with monomeric precursors. In contrast, multimeric and polycistronic precursors containing tRNA sequences are often first cleaved by other endoribonucleases to generate smaller fragments which are then further processed to give the mature 5′ and 3′ termini of the tRNA.[34,35,50] Studies with mutant strains of *E. coli* have suggested that only a few endoribonucleases are involved in the tRNA processing from all types of tRNA precursors.[3] This finding implies that only a few different structural features, each recognized by a different nuclease, are sufficient to extract the tRNA sequence from the larger precursor.

Intact primary transcripts are not observed in normally growing, wild type cells, suggesting that endonucleolytic cleavages occur rapidly during or immediately following transcription. However, under certain circumstances, such as when a processing system is overwhelmed by a large number of transcripts or when a transcript is a poor substrate, tRNA precursors can be identified even in normal cells. This situation can occur both in bacteria[28] and eukaryotic cells[12] when a tRNA gene cloned in a phage or plasmid is introduced. Likewise, during normal phage T4 infection, dimeric tRNA precursors can be observed, presumably because RNase P cleavage cannot keep up with the large number of precursors being synthesized.[58] However, the initial cleavages that generate the dimeric precursors from the putative primary transcript must still occur sufficiently rapidly to prevent accumulation of the larger molecule. As noted above, tRNA precursors also can be made to accumulate in mutant cells either by inactivation of a processing enzyme[35,50,51] or by alteration of the tRNA precursor.[6] Each of these situations has been used to help identify endonucleolytic processing events and the enzymes involved in catalysis of these processes.

1. 5′ Terminal Processing

The 5′ phosphoryl terminus of mature tRNAs can be generated from tRNA precursors by the action of the endonuclease, RNase P.[59] This enzyme was first detected and purified by its ability to specifically remove a 41-nucleotide fragment from the 5′ terminus of the precursor to *E. coli Su3*$^+$ $tRNA^{Tyr}$.[29] Further studies of its action in vitro on other tRNA precursors, and analyses of tRNA precursors accumulating in mutant strains containing a temperature-sensitive RNase P,[60,61] have suggested that this enzyme is probably required for processing of all tRNA precursors in *E. coli* and phage T4-infected *E. coli*.[35,50,62] Likewise, since RNase P-type activities have now been identified in a variety of other prokaryotic[63] and eukaryotic organisms,[39,64,65] the enzyme may serve a similar function in a wide range of species.

The ability of RNase P to act on a wide spectrum of natural tRNA precursors,[35,50] as well as artificial precursors containing only a few extra nucleotides at the 5′ terminus,[39] suggests that features of the tRNA domain itself, rather than of the precursor-specific regions, are important for enzyme recognition. This was confirmed by the observation that oligonucleotides which bridge the normal cleavage site of a dimeric tRNA precursor are not substrates for RNase P,[66] demonstrating a role for the tRNA structure on the 3′ side of the cleavage site for enzyme action. It has also been shown that the actual secondary and tertiary structure of the adjacent tRNA domain is of prime importance since mutations which alter base-pairing in stems and which affect overall tRNA conformation can have profound effects on cleavage by RNase P.[6,67] For some precursors the presence of the 3′ terminal –CCA sequence also can influence the rate of RNase P action both in vitro and in vivo.[16,68]

As noted above, one problem with these types of experiments is that they do not distinguish between binding of the substrate and the actual rate of catalysis in assessing the overall

suitability of a mutant substrate. However, in one recent study it was shown that a mutant Su_3^+ $tRNA^{Tyr}$, containing a G43-to-U43 transversion in the anticodon stem, is cleaved more slowly than the wild-type precursor by RNase P and also forms a much less stable enzyme-substrate complex.[69] It is not known from these experiments whether V_{max} also is affected. At first glance it seems surprising that the change in a single nucleotide residue in a macromolecular tRNA precursor should have such a profound effect on binding of the substrate since it might be expected that many of the other contact sites would still be preserved. On the other hand, if one considers that RNase P must cleave a single phosphodiester bond from among the more than 100 present in the precursor, even incorrect binding of the substrate could be deleterious since one of the other phosphodiester bonds might then be positioned near the active site and be cleaved. One possible explanation may be that for this enzyme, stable binding occurs only when many specific interactions between substrate and enzyme are made and that binding and correct positioning are one and the same event. Alteration of the conformation of the substrate by a single nucleotide modification may prevent so many specific interactions that there are not sufficient contacts for stable binding to occur. If this idea is correct, then the binding between substrate and enzyme cannot be due to an array of nonspecific electrostatic and hydrophobic interactions since under such circumstances binding with incorrect positioning probably would take place. Rather, the enzyme and substrate must each provide specific, complementary surfaces in which binding automatically results in correct positioning.

The striking feature of RNase P which appears to support these speculations is that this enzyme is a ribonucleoprotein consisting of a small protein of about 18,000 mol wt and an RNA of 375 or 377 nucleotides (M1 RNA).[70,71] Genetic and biochemical analyses have shown that both the RNA and protein components are necessary for RNase P activity,[72-76] and since the protein by itself apparently has no nuclease activity,[72] it appears that the RNA does not simply confer specificity on an otherwise nonspecific enzyme. The simplest explanation to account for these findings and the points made above is that binding and correct positioning of a tRNA precursor on the enzyme is determined largely by base pairing of a precursor with the RNA component.

Further evidence consistent with this idea comes from the observation that the RNase P cleavage site in the phage T4 $tRNA^{Gln}$-$tRNA^{Leu}$ dimeric precursor is inaccessible to nuclease S1, presumably because the site is buried in the RNA structure, whereas it is accessible to the larger RNase P.[77] Perhaps, binding of the precursor to the enzyme opens up the structure of the dimer because more stable intermolecular interactions with M1 RNA substitute for the intramolecular interactions within the precursor. One recent finding that does not fit with this model is that the purified protein component of *B. subtilis* RNase P can bind to tRNA precursors by itself.[78] Whether this binding on nitrocellulose filters is physiologically relevant remains to be determined. Also, binding of both a tRNA precursor and M1 RNA directly to the small RNase P protein would appear to present some steric difficulties.

If the ideas presented above are valid it would be expected that M1 RNA would contain sequences complementary to invariant nucleotides in all tRNAs. In fact, sequence analysis of M1 RNA has shown that this molecule contains a five-nucleotide sequence, 5′-UGAAU-3′, in a loop region, which is complementary to the sequence 5′GTΨCPu-3′ found in all *E. coli* tRNAs.[79] In addition, a second sequence was found which could interact with a common sequence in the dihydrouridine loop of tRNAs.[79] One test of these ideas will come from comparison of the sequences of the RNA component of RNase P from other organisms with their homologous tRNAs, as well as from studies of mutant tRNAs and mutant M1 RNA molecules.

An additional feature related to RNase P which has recently become apparent is that the RNA component itself is subject to processing. In vitro and in vivo transcription of clones containing the M1 RNA gene indicate that it is synthesized with 36 or 37 additional residues

at the 3′ terminus.[79-81] Incubation of the transcript in an *E. coli* extract leads to its conversion to a molecule the size of M1 RNA.[80,81] The identity of the enzyme responsible for M1 RNA processing is not known. Although the M1 precursor accumulates in a temperature-sensitive RNase E$^-$ mutant at the nonpermissive temperature, the precursor cannot be processed by purified RNase E.[80] Perhaps the M1 RNA precursor requires association with some protein for correct processing by RNase E to occur. Alternatively, a more complicated explanation may be required. Nevertheless, these findings raise the possibility of regulation of RNase P synthesis and of a mechanism for maintaining the proper stoichiometry of synthesis of the two components of RNase P.

Another enzyme that plays a role, although somewhat indirectly, in the maturation of the 5′ terminus of certain tRNAs is RNase III.[82] Two situations have been identified in which this enzyme cleaves a polycistronic RNA, and in the process, participates in the release of a precursor to tRNA. In one, spacer tRNAs present in the region between the 16S and 23S rRNA portions of the ribosomal transcript are separated from the rRNA sequences by RNase III cuts.[83] Further processing by RNase P then generates the mature 5′ terminus of these tRNAs.[84] In the second example, RNase III cleaves the transcript of the phage T4 tRNA region six nucleotides before the first tRNA, that of tRNA^{Gln}.[85,86] This cut serves to separate the tRNAs from about a 1-kb leader sequence which is thought to represent a mRNA sequence. In mutant strains lacking RNase III this cleavage does not occur and the level of mature tRNA^{Gln} made is greatly decreased.[41,87] It has recently been shown that the decreased synthesis of T4 tRNA^{Gln} results from an alternate cleavage, by an unknown enzyme, in the absence of RNase III, only two nucleotides away from the 5′ terminus of tRNA^{Gln}. Cleavage at the 5′ terminus of tRNA^{Gln} by RNase P takes place much more slowly in this altered precursor, and consequently, much of it is degraded rather than converted to the mature tRNA.[88]

Studies with *rnc* (RNase III$^-$) mutants suggest that the overall role of this enzyme in tRNA processing is minor.[3] However, if this mutation is introduced into a strain already lacking both RNase P and another endonuclease, RNase E, tRNA maturation is more severely affected.[89] One possible explanation for this finding is that RNase III only participates in tRNA processing when the other two enzymes are missing, and under normal circumstances, i.e., in a wild-type cell, RNase P and RNase E have the primary role.

2. *Intercistronic Processing*

In addition to cotranscription with rRNA and mRNA, tRNAs are often transcribed together with other tRNAs, especially in prokaryotes, to give larger polycistronic or multimeric precursors.[49] Our understanding of the processing events involved in cleaving multimeric precursors, and of the enzymes which participate in these cleavages, is still quite minimal. The large majority of molecules which accumulate in *E. coli* RNase P$^-$ (*rnp*) mutant cells at a nonpermissive temperature are the monomeric and dimeric precursors which are the immediate substrates for RNase P.[35,50] Since many of these smaller precursors do not contain 5′ triphosphate termini, it is thought that they have been derived by cleavage of larger transcripts. In the case of phage T4-infected *E. coli*, the monomeric and dimeric precursors which accumulate in *rnp* strains are undoubtedly cleavage products of a larger molecule.[58,62,90] Likewise, in uninfected *rnp* cells, larger precursors transiently accumulate that contain sequences also found in the smaller precursors, and presumably, are converted to the smaller forms by endonucleolytic cleavages.[50]

The fact that smaller precursors can be found in *rnp* strains has led to the suggestion that other endonucleases, besides RNase P, also participate in tRNA processing.[34,35] The few attempts to identify these other activities has led to considerable confusion because each group that has found a potential processing activity has used different substrates, has given the activity a different name, and has not purified the activity sufficiently to characterize it

or to determine whether it is a single enzyme. What is clear is that activities are present in *E. coli* that can cleave in the spacer regions of multimeric transcripts to generate products that can be further processed by RNAse P.[34,35] For some precursors the presence of RNase P facilitates these cleavages, possibly by removing 5′ leader regions. This could explain why multimeric precursors transiently accumulate in *rnp* strains.

One enzyme, termed RNase P2, was partially purified on the basis of its ability to cleave in the 5′ leader sequence of the monomeric precursors to $tRNA_1^{Tyr}$ and $tRNA_3^{Gly}$.[35] Although these particular cleavage events are not normally observed in wild-type extracts or cells, they can be detected in extracts of the *rnp* mutant, A49, or in vivo with these mutant cells. Partially purified RNase P2 also cleaves multimeric precursors in the spacer regions between tRNAs.[35] In each of these examples the product still contains extra residues at the 5′ terminus and is a substrate for subsequent action by RNase P. RNase P2 has also been reported to cleave the large, 600-nucleotide precursor to $tRNA_1^{Tyr}$ synthesized in vitro from $\phi 80\ psu_3^+$ DNA to generate the intermediate-size $tRNA_1^{Tyr}$ precursor normally found in RNase P^- cells.[91]

A second activity implicated in the intercistronic processing of multimeric precursors has been called RNase O.[34] This enzyme was identified and partially purified on the basis of its ability to cleave certain multimeric precursors that are present in the *rnp* strain, TS241, and its inability to act on certain other small monomeric and dimeric precursors which are immediate substrates for RNase P.[92] RNase O is also able to cleave the double-stranded RNA, poly(A)·poly(U), quite efficiently. RNase O is stimulated by divalent cations and inhibited by monovalent cations at a concentration of 0.14 *M*. Gel filtration analysis suggested that the active form of the enzyme has a molecular weight of approximately 41,000.[92]

A third enzyme, called RNase PC, was isolated on the basis of its ability to cleave in vitro transcripts of the phage T4 tRNA gene cluster to monomeric and dimeric precursors that are similar to those which accumulate in *rnp* strains after T4 infection.[32] The cleavages that are made by this enzyme on the T4 transcript are adjacent to A residues.[93] However, it is not clear whether this is an intrinsic specificity of the enzyme or whether it simply reflects the large number of A residues in the intercistronic regions of the T4 tRNA transcript.

Another enzyme possibly involved in intercistronic tRNA processing is RNase E.[30] This enzyme has already been shown to play a role in the maturation of 5S RNA.[94,95] A temperature-sensitive *E. coli* mutant which contains a heat-labile RNase E has been shown to accumulate tRNA precursors at a nonpermissive temperature.[95] One of these precursors has been identified as a $tRNA_1^{Leu}$-$tRNA_1^{His}$ dimer. The precursor is not cleaved by a heated extract from an *rne* mutant, whereas an extract from wild-type cells is active. Surprisingly, purified RNase E cannot cleave this precursor, but in combination with the heated *rne* extract, cleavage takes place.[96] RNase E is known to facilitate RNase P cleavages with certain precursors, and *rne, rnp* double mutants accumulate more tRNA precursors than *rnp* mutants alone.[97] These findings suggest that some RNase E cleavages may precede, and be required, for subsequent RNase P action. It has also been suggested that these data might reflect the existence of a tRNA processing complex, and that a mutation in RNase E might affect the complex in such a way as to alter the activity of other enzymes associated with it.[98] Although the idea of a processing complex is interesting, as yet there is no direct evidence for its existence.[99]

The relationship among the various activities that can cut multimeric precursors in intercistronic regions is not understood. Functionally, RNase P2 and RNase O appear very similar. Yet, RNase O is able to cleave double-stranded RNAs,[92] whereas RNase P2 was reported to be devoid of RNase III activity.[35] Likewise, RNase PC preparations apparently do not contain RNase III activity.[32] Since RNase III involvement in tRNA processing is thought to be minimal, it is possible that the RNase III activity of the RNase O preparation simply represents contamination by the former enzyme. The two other enzymes, RNase PC and

RNase E, bear a resemblance to each other in that both activities favor cuts adjacent to A residues in a variety of molecules.[3,93,97] On the other hand, RNase PC seems to differ from RNase P2 since the latter activity can cleave the large $tRNA_1^{Tyr}$ precursor, whereas the former cannot.[91,93] Also, RNase E differs from RNase O since the former has a molecular weight of 70,000[30] and the latter has a molecular weight of about 40,000.[92] In view of the points raised, it is highly unlikely that RNases P2, O, PC, and E are four distinct enzymes. One possibility would be that RNases P2 and O, and RNases PC and E represent the same activity. However, the absence of appropriate mutants and the lack of highly purified enzymes indicate that considerably more work will be required to sort out this multiplicity of endonucleases.

3. 3′ Terminal Processing

Monomeric tRNA transcripts, as well as multimeric ones, can contain long extra sequences at their 3′ terminus. These are thought to be removed by an endonucleolytic event that generates a shortened molecule containing only a few extra 3′ residues, or one which requires –CCA addition to mature the tRNA. In the case of multimeric precursors, a 3′ endonucleolytic cleavage of an inner tRNA is the same as the intercistronic cleavages that have just been discussed. However, in the case of monomeric precursors, or of the 3′ terminal tRNA in a multimeric precursor, a distinct 3′ processing cleavage may occur which may involve a distinct endonuclease.

Studies of the transcription of the $tRNA_1^{Tyr}$ gene have shown that the precursor may contain hundreds of nucleotides following the 3′ terminal of this tRNA.[100-102] Yet, the precursor of $tRNA_1^{Tyr}$ that accumulates in vivo in *rnp* strains contains only three extra residues following the –CCA sequence.[35] An activity was detected in *E. coli*, termed RNase PIV, which could shorten a long in vitro synthesized transcript from this gene.[103,104] Using a synthetic $tRNA_1^{Tyr}$ gene, this endonucleolytic cleavage was shown to occur in a hairpin structure between the 7th and 8th nucleotides following the –CCA terminus.[105] The activity which converts this precursor, containing seven extra nucleotides, to the precursor that contains only three extra residues is not known. However, it has been shown for some other monomeric precursors that an RNase E cleavage near the 3′ terminus is required for further processing by RNase P.[97] The same sequence of events may occur here. Interestingly, the sequences of many tRNA genes display hairpin structures near the 3′ terminus of the tRNA.[54] These sequences may serve as processing sites for many tRNA precursors. The relation of RNase PIV to the other endonucleases discussed previously is not known, but the fact that RNase P2 also can cleave the long $tRNA_1^{Tyr}$ precursor near the 3′ end of the tRNA may be important in this regard.[91]

Recently, another *E. coli* activity which cleaves near the 3′ end of a tRNA-like structure was identified in extracts devoid of RNase III, RNase P, and RNase E.[31] This enzyme, termed RNase F, was partially purified on the basis of its ability to introduce a cleavage near the 3′ terminus of T4 species 1 RNA, the last RNA of the T4 tRNA gene cluster. Since species 1 has a structure similar to a tRNA, it has been suggested that the cleavage by RNase F is related to the endonucleolytic cleavages near the 3′ end of tRNAs. Interestingly, this cleavage also occurs in a stem-loop structure. Neither RNase III, RNase P, nor RNase E can affect a similar cleavage. The properties of RNase F differ from those of other known processing nucleases in that the enzyme does not require a divalent cation, is extremely heat stable, and produces a 3′ phosphoryl rather than a 3′ hydroxyl terminus.[31,106] Inasmuch as these properties are usually associated with degradative nucleases, further studies of RNase F are in order. It is also not clear whether other activities, such as RNase P2 or PIV, might also cleave in the stem-loop structure near the 3′ terminus of species 1 RNA. These latter enzymes would generate a 3′ hydroxyl terminus, rather than a 3′ phosphoryl, and 3′ hydroxyl termini would appear to be processed more directly by the known 3′ exonucleases (see below).

A summary of all the *E. coli* endonuclease activities that have been implicated in tRNA processing is presented in Table 1.

In contrast to the findings in *E. coli*, a different type of 3′ endonucleolytic cleavage has been observed in *Xenopus laevis* germinal vesicle extracts during the processing of eukaryotic tRNA precursors. These precursors lack the –CCA sequence normally found in *E. coli* tRNA precursors (see below), and 3′ processing, therefore, requires removal of extraneous 3′ residues and addition of the –CCA terminus. Processing of the transcription product of a cloned *Bombyx mori* $tRNA_2^{Ala}$ gene in this system revealed that the entire 3′ trailer of this transcript is removed as a single 22-nucleotide fragment.[107,108] Also, an endonuclease activity capable of removing extraneous 3′ oligonucleotides from artificial tRNA precursors has been identified in *X. laevis* oocyte nuclei.[15] Whether this mode of 3′ processing applies to all eukaryotic tRNA precursors and all systems remains to be determined. However, the identification of 3′ exonuclease activities in several eukaryotic systems suggests that both exonucleolytic and endonucleolytic 3′ processing may take place (see below).[15,109]

B. Exonucleolytic Trimming

The endonucleolytic cleavage by RNase P at the 5′ side of tRNA precursors directly generates the mature 5′ terminus of tRNA. In contrast, the endonucleolytic cleavages that have been observed in the 3′ region of tRNA precursors generally leave additional residues that must be removed by further processing. This processing is thought to involve an exonucleolytic trimming reaction.[35,50] Two types of tRNA precursors have been identified in various systems which differ with respect to their 3′ terminal structure and in their mode of 3′ terminal processing.[36] In one type of precursor that has been found exclusively in prokaryotes, the –CCA sequence is already present and is followed by a small number of additional residues (type I precursor). Final processing of the 3′ terminus of this type of precursor requires a nuclease which will remove the extra residues, but not enter the –CCA sequence, since studies with tRNA nucleotidyltransferase (*cca*) mutants have indicated that the latter enzyme is not required for tRNA biosynthesis in *E. coli*.[110] In the second type of precursor that has been identified in both prokaryotic and eukaryotic systems, all or part of the –CCA sequence is absent, and other residues are present instead (type II precursor). Processing of the 3′ terminus of this precursor requires a nuclease which will remove only the extraneous residues and leave a product that can be repaired with tRNA nucleotidyltransferase to generate the mature –CCA sequence. The specificity of tRNA nucleotidyltransferase requires that the nuclease involved in this processing event not remove nucleotides beyond the position that would be occupied by the –CCA residues.[111] Of course, tRNA precursors have also been identified that require no further nucleolytic action at their 3′ terminus[112] (because they already have the mature –CCA terminus or because they simply require –CCA addition). These precursors are not relevant to this discussion since the 3′ terminal processing event presumably has already occurred.

The different specificity requirements for 3′ processing of the two types of precursors described above have suggested that two distinct nucleases would be involved in these processes.[37] In fact, a mutant strain of *E. coli*, strain BN, has been isolated[113] which is unable to process the type II precursors specified by phage T4,[114] but matures the type I precursors normally.[114,115] Attempts to identify and purify the enzyme(s) responsible for 3′ terminal trimming of tRNA precursors have resorted to the use of two types of substrates: (1) natural precursors synthesized in in vitro transcription-processing systems[103] or that accumulate in *rnp*^{ts} strains at a nonpermissive temperature[35,92] and (2) artificial tRNA precursors synthesized from mature tRNA with the aid of tRNA nucleotidyltransferase.[36] Natural precursors from RNase P^- cells generally contain extra residues at the 3′ terminus,[35,50] presumably because RNase P action at the 5′ terminus of the precursor is a prerequisite to final 3′ trimming. The artificial tRNA precursors that have been used are tRNA-CCA-

Table 1
ESCHERICHIA COLI ENDORIBONUCLEASES IMPLICATED IN tRNA PROCESSING

Enzyme	Substrate and cleavage site	Molecular weight	Remarks
RNase P[a]	Mature 5′ terminus in tRNA precursors	Protein, ~18,000; RNA, ~375 nucleotides	Probably required for all tRNAs
RNase III[a]	Double-stranded regions in rRNA precursors; near $tRNA^{Gln}$ in T4 tRNA transcript	50,000	Minor role in tRNA processing
RNase P2	Intercistronic regions of multimeric precursors	—	May be the same enzyme as RNase O
RNase O	Intercistronic regions of multimeric precursors	~41,000	RNase O probably contaminated with RNase III
RNase PC	Intercistronic regions of T4 tRNA transcript adjacent to A residues	—	
RNase E	Intercistronic regions of multimeric precursors; between 5S and 4S in rRNA operon, favors cuts at A residues	~70,000	May be the same enzyme as RNase O
RNase PIV	Near 3′ terminus of $tRNA_1^{Tyr}$ in stem-loop	—	May be related to RNase P2
RNase F	Near 3′terminus of T4 species 1	—	Heat stable, makes 3′P, no Mg^{2+} requirement

[a] Has been highly purified.

[^{14}C]$C_{2\text{-}3}$, as an analog of the type I molecules, and tRNA-C-[^{14}C]U for type II precursors.[36] The advantages and disadvantages of each type of substrate have been discussed earlier.

It was originally suggested that the enzyme involved in 3′ processing of type I precursors was the known 3′ exonuclease, RNase II.[35,102] Using the $tRNA_I^{Tyr}$ precursor that accumulated in the *rnp* strain, A49, as substrate, a 3′ processing activity was partially purified which also retained RNase II activity against synthetic polynucleotides.[35] It was also shown that purified RNase II could remove extra nucleotides from the RNA_I^{Tyr} precursor.[102] On the other hand, RNase II was not active for the synthesis of functional Su_{III}^+ $tRNA_I^{Tyr}$ in an in vitro transcription-processing system.[104] Rather, an enzyme termed RNase PIII, separable from RNase II, was required.[103,104] Likewise, studies with artificial tRNA precursors and a homogeneous RNase II preparation indicated that although this enzyme can remove extra residues from type I precursor analogs, it does not stop at the –CCA sequence and does not regenerate amino acid acceptor activity for these molecules.[116] These findings are not at all surprising since RNase II is a processive exonuclease which would be expected to continue hydrolysis until it reached a resistant secondary structure in the tRNA.[116] These results make it extremely unlikely that RNase II is involved in tRNA processing. Rather, this enzyme is thought to be a degradative enzyme which may play a role in removal of mRNA in vivo.[117]

Using the total RNAs accumulated in another *rnp*^{ts} mutant (TS241) as substrate, two 3′ exonucleases, termed RNase Q and RNase Y, were identified and partially purified.[92] RNase Q could remove extra 3′ nucleotides from the monomeric precursors to $tRNA_I^{Asp}$, $tRNA_{III}^{Ser}$ and $tRNA_I^{Tyr}$ in the presence of RNase P to generate products with the electrophoretic mobility of mature tRNAs.[92] RNase Q, as purified, was also active against the synthetic polynucleotides, poly(A) and poly(U), and with these substrates had a pH optimum of 7.4 to 8.0 and required a divalent cation for activity.[92] The second activity, RNase Y, was less well characterized, but it also acted on the $tRNA_I^{Asp}$ precursor in the presence of RNase P to generate a product the size of mature tRNA. However, in the absence of RNase P, RNase Y digested this precursor to an acid-soluble form.[92]

An exonuclease, termed RNase D, was partially purified from *E. coli* on the basis of its ability to hydrolyze tRNA molecules with altered structures.[118] These substances were generated either by heat denaturation of tRNA or by removal of the 3′ terminal –CCA sequence which also serves to denature certain molecules in the tRNA population. Examination of the substrate specificity of RNase D indicated that it was also extremely active against the type I artificial tRNA precursor, but relatively inactive (about 40-fold less) against tRNA-C-U, the type II precursor analog.[37] Further purification of the enzyme to homogeneity indicated that RNase D is a monomer of about 40,000 mol wt.[119] It requires a divalent cation for activity and has a pH optimum at about pH 9.[120] RNase D is highly specific for tRNA substrates terminating with a 3′ hydroxyl group, with essentially no activity against rRNA and synthetic polynucleotides.[118] These latter features clearly distinguish RNase D from RNase II.

The aspect of RNase D specificity which suggests that it is involved in 3′ processing of type I precursors is its ability to remove the extra residues following the –CCA sequence in a random mode of attack, dissociating after each cleavage.[116] When the –CCA terminus is reached, the rate of hydrolysis drops about 30-fold,[37] and in the presence of aminoacyl-tRNA synthetases the tRNA product can be aminoacylated.[116] Presumably, in vivo, once the –CCA sequence is exposed rapid aminoacylation would prevent any further degradation especially since the –CCA sequence is hydrolyzed so slowly. The relative resistance of mature tRNA to hydrolysis by RNase D is not a simple consequence of the enzyme not being able to digest a –CCA sequence, since a second –CCA terminus added to tRNA is rapidly degraded.[120] Thus, the three-dimensional structure of native tRNA must somehow prevent accessibility of RNase D to the normal 3′ terminus. This feature of RNase D specificity is surprising since the 3′ terminus of tRNA is thought to be exposed in solution.[121]

Despite the aforementioned results certain discrepancies about a processing function for RNase D still remain. For example, although RNase D can act efficiently on a synthetic precursor to $tRNA^{Tyr}$ to regenerate tyrosine acceptor activity, in its action on a natural $tRNA_1^{Tyr}$ precursor in the presence of RNase P it does not stop hydrolysis at the –CCA terminus, but also removes the terminal two 3′ residues of the mature molecule.[121a] One possibility to explain this discrepancy is that the natural $tRNA^{Tyr}$ is not in its native conformation because it has been denatured in the course of its preparation.

A second and more serious discrepancy related to the function of RNase D is the recent isolation of an *E. coli* mutant strain containing a deletion of the *rnd* locus,[122] which is located at 40 min on the genetic map.[123] This strain has no detectable RNase D activity, yet it grows normally and processes tRNA precursors normally.[122] At a minimum, this finding indicates that RNase D is not essential for tRNA processing. On the other hand, the substrate specificity of RNase D strongly suggests that it must be involved in some aspect of tRNA metabolism. One possible explanation for this paradox is that in the absence of RNase D another exonuclease can take over its function. A likely candidate for this other enzyme is the recently discovered RNase BN (see below).[38]

The chromatographic and catalytic properties of RNase D suggest that it is identical to RNase PIII, although the latter enzyme was not sufficiently characterized for a detailed comparison.[104] It is also likely that RNase Q and/or RNase Y contained RNase D activity.[92] However, the RNase Q preparation undoubtedly also was contaminated with RNase II since its properties and action on synthetic polynucleotides were identical to those attributed to RNase II.[116] This again points out the difficulties in determining substrate specificities with less than pure ribonucleases.

Two mutants of *E. coli* have been isolated, strains BN and CAN, that restrict the growth of certain T4 phage which are dependent on the suppressor function of T4 $tRNA^{Ser}$.[113,115] Detailed analysis of strain BN revealed that this mutant accumulates precursors to the phage-coded $tRNA^{Ser}$, $tRNA^{Pro}$, and $tRNA^{Ile}$.[114] These precursors lack all or part of the –CCA sequence, and instead, contain other residues which normally are removed prior to –CCA addition.[62] The five other tRNAs specified by phage T4, which either are synthesized via type I precursors or require no 3′ trimming for further processing,[62] are made in normal amounts.[114] These findings led to the suggestion that strain BN is defective in an activity, termed RNase BN, that is required for 3′ processing of the phage type II precursors.[114]

Until recently, attempts to establish the existence of a distinct RNase BN have given inconclusive results. An initial report on the detection and partial purification of this activity most likely was due to the isolation of RNase D since the molecular weight and catalytic properties of the isolated RNase were similar to those determined for RNase D.[124] Furthermore, no enzyme distinct from RNase D and RNase II, and active on the artificial type II precursor, tRNA-C-[^{14}C]U, could be detected in *E. coli* strain B, the parent of strain BN.[116] The inability to identify RNase BN led to the suggestion that perhaps this activity was a manifestation of RNase D, and that the selection procedure for the BN mutant resulted in a RNase D which retained its activity against type I precursors, but which was defective against type II precursors.[116] This idea was strengthened by the finding that RNase D is altered after phage T4 infection (see below).[125]

Nevertheless, genetic studies showed that this idea could not be correct. Introduction of the *rnd*$^+$ gene into strain BN by P1-mediated transduction did not correct the mutant phenotype.[126] Likewise, a strain lacking RNase D plated T4 *psu*$_1^+$-amber phage normally.[122] These data indicated that the BN mutation could not be in the *rnd* gene or in a gene that affected the activity of RNase D. These findings, coupled with the observation that heated extracts of *E. coli* B that had lost RNase D and RNase II activity, still retained substantial activity against tRNA-C-[^{14}C]U,[38] led to a renewed search for RNase BN. The search was facilitated by the construction of RNase II$^-$, RNase D^{ts} derivatives of *E. coli* B and BN.[38]

Chromatography of extracts of these strains clearly demonstrated the existence of an enzyme in the parental strain, active against the artificial type II precursor, that was missing in the tRNA processing mutant, strain BN.[38] Similar studies with derivatives of the other mutant strain, CAN, and its parent, CA265, showed that it also was missing this particular RNase.[127] Undoubtedly, the large amounts of RNase II and RNase D in cells, and the similar chromatographic properties of these RNases, prevented the earlier identification of RNase BN.

RNase BN is a 3′ exoribonuclease. It is stimulated by low concentrations of divalent and monovalent cations, and has a pH optimum about 6.5. The enzyme has a molecular weight of about 80,000 based on gel filtration.[38] Preliminary characterization of the substrate specificity of RNase BN indicates that the enzyme favors type II precursors, but is still quite active against an artificial type I substrate.[127] This contrasts with RNase D which favors type I precursors by as much as 30- to 40-fold.[127] Thus, RNase BN is a distinct enzyme both by its biochemical and its genetic properties. RNase BN is the first *exo*ribonuclease to be implicated in tRNA processing in vivo.

The substrate specificities of RNase BN and RNase D may explain the in vivo properties of mutants defective in these activities. Since RNase BN can act relatively efficiently on type I precursors,[127] it could substitute for RNase D in the *rnd* deletion strain to allow normal growth. In addition, since type II precursors have not been detected in *E. coli*,[26] the absence of RNase BN would not be expected to have any deleterious effect on cell growth. In contrast, the relative inability of RNase D to act on type II precursors[37,120] would preclude substitution of this enzyme for RNase BN in phage T4-infected cells and would explain the phenotype of BN mutants. These explanations, unfortunately, still leave a big gap in our understanding of the involvement of exoribonucleases in tRNA processing in vivo since they give no role to RNase BN in the uninfected cell. Presumably, *E. coli* does not contain a RNase solely for action during phage infection!

A summary of the known *E. coli* exoribonucleases, their possible relationships, and possible involvement in tRNA processing is presented in Table 2.

The importance of exonucleolytic trimming for tRNA processing in eukaryotes is not known. Exoribonucleases capable of removing residues from the 3′ terminus of tRNA precursors have been detected in several eukaryotic systems, but at this time it is not clear that they play any role in tRNA processing in vivo.[15,109]

C. Terminal Addition Reactions

The existence of an enzyme, tRNA nucleotidyltransferase, that could accurately synthesize the –CCA sequence of tRNA in vitro naturally led to the conclusion that these nucleotides were added posttranscriptionally during tRNA maturation.[111] In recent years, the ease of cloning of tRNA genes has resulted in the sequencing of a large number of these genes from a variety of organisms. The sequence information has shown that there are two routes for –CCA incorporation into the 3′ terminus of tRNA. In prokaryotes, at least as exemplified by *E. coli, Bacillus subtilis,* and T-even phages, the information for the complete –CCA sequence of many tRNAs is encoded in the sequence of the tRNA genes.[42-44] No exception to this generalization has yet been found in *E. coli*,[26] but some *B. subtilis* and phage tRNA genes lack all or part of the –CCA sequence.[42,44,128] In contrast, all eukaryotic tRNA genes examined to date, including those of organelles, lack information for the –CCA sequence, and it must be added posttranscriptionally, presumably by tRNA nucleotidyltransferase.[26]

The physiological significance of the differences in 3′ terminal structure between tRNA genes of prokaryotes and eukaryotes is not known. It may represent an example of an evolving nucleotide sequence such that random mutations in the extraneous 3′ residues may have over time converted these nucleotides to –CCA in some systems. If encoding of the –CCA sequence in the gene conferred some advantage to the cells, such as more rapid tRNA processing[129] or lower energy requirements, these genetic changes could be fixed. Why

Table 2
ESCHERICHIA COLI EXORIBONUCLEASES[a]

Enzyme	Substrate	Molecular weight	Remarks
RNase II[b]	All RNAs, synthetic polynucleotides	~75,000	Probably degradative enzyme, not involved in tRNA processing; processive action
RNase D[b]	tRNAs with altered structure, type I precursors	40,000	Probably involved in tRNA metabolism, but deletion strain viable; random action
RNase PIII	$tRNA_1^{Tyr}$ precursor	—	Probably RNase D
RNase Q	$tRNA_1^{Asp}$, $tRNA_{III}^{Ser}$, $tRNA_1^{Tyr}$, synthetic polynucleotides	—	Probably contaminated with RNase II, RNase D also may be present
RNase Y	$tRNA_1^{Asp}$	—	—
RNase BN	tRNA-C-U, type II precursors	~80,000	Required for certain T4 tRNAs; function in host unknown
RNase R[127]	rRNA, mRNA	~80,000	Function unknown

[a] All exoribonucleases release 5′ mononucleotides from 3′ terminus of RNA.
[b] Has been highly purified.

laboratory strains of *E. coli* have attained such a dramatic change to complete encoding of –CCA, whereas this has not occurred in other systems, is not clear. Perhaps it is a consequence of the many years of growth under laboratory conditions. Examination of tRNA genes from newly isolated *E. coli* populations would be interesting in this regard.

Direct evidence for the involvement of tRNA nucleotidyltransferase in the biosynthesis of tRNAs has come from studies of *E. coli* mutants deficient in this enzyme.[17,52,130] As expected, the mutant strains were not affected in tRNA biosynthesis, except possibly for $tRNA^{Cys}$.[110,131] However, tRNA nucleotidyltransferase is essential for the normal growth of *E. coli* because the enzyme is required for repair of defective tRNAs which arise by end turnover.[110] In contrast to the situation in uninfected *E. coli*, considerable information has accumulated which indicates that tRNA nucleotidyltransferase is required for the biosynthesis of certain tRNAs specified by the T-even bacteriophages.[62] Detailed examination of tRNA synthesis after infection of a *cca* mutant has shown that two of the eight T2 tRNAs, four of the eight T4 tRNAs, and one of the six T6 tRNAs lack an intact –CCA sequence, and that purified tRNA nucleotidyltransferase can add the –CCA in vitro to a dimeric T4 precursor that accumulates in the *cca* mutant.[122,133] In addition, expression of the T4 serin-inserting psu_1^+-amber suppressor tRNA and the gluatmine-inserting psu_2^+-ochre suppressor tRNA are greatly depressed in *cca* mutant strains, and the level of suppressor activity remaining is related to the level of tRNA nucleotidyltransferase remaining in the mutant strains.[17]

Since eukaryotic mutants lacking tRNA nucleotidyltransferase have not yet been obtained, evidence for the involvement of this enzyme in eukaryotic tRNA biosynthesis has, of necessity, been indirect. However, the presence of this enzyme in all eukaryotic systems that synthesize tRNA,[111] including mitochondria[134] and chloroplasts,[135] and the high degree of specificity of the reaction carried out by this enzyme,[111] make it extremely likely that tRNA nucleotidyltransferase is required for tRNA biosynthesis in eukaryotes.

Recently, another type of addition reaction involving the 5′ terminus of $tRNA^{His}$ has been identified.[136] It has been known for some time that $tRNA^{His}$ from prokaryotic and eukaryotic organisms is one nucleotide longer at the 5′ terminus compared to other tRNAs.[136,137] In eukaryotes this extra nucleotide is not encoded in the gene, but is added posttranscriptionally.[136] The reaction, which involves the addition of a guanylate residue in normal phosphodiester linkage, is catalyzed by a *Drosophila* cell extract. The reaction requires a guanosine substrate and ATP. In this system a portion of the added pG is modified in an unknown fashion.[137] In mouse and human, the 5′ pG is methylated.[137] The physiological significance of this interesting terminal addition reaction is not known.

D. Intervening Sequence Removal

A number of eukaryotic tRNA genes, particularly those from yeast, have been shown to contain intervening sequences, or introns, that are transcribed and must be removed during processing of the tRNA precursors.[24,25,138-140] Recently, intervening sequences have also been identified in at least two tRNA genes from the archaebacterium *Sulfobolus solfataricus*.[141] The physiological significance of having intervening sequences in some tRNA genes, but not others, is not known.[142] It has been estimated that only about one fifth of the tRNA genes of yeast contain introns,[143] so it is clear that this sequence is not obligatory for global tRNA precursor processing or tRNA gene expression. Nevertheless, it has been shown that precise removal of the intervening sequence from the *SUP6* $tRNA^{Tyr}$ gene of yeast converts it to a less efficient suppressor in vivo, probably because a pseudouridine modification normally present in the anticodon is not made in the tRNA precursor synthesized from the deleted gene.[143] Since intervening sequences are always found near the 3′ side of the anticodon and contain a sequence complementary to the anticodon,[24,25,13] they undoubtedly affect the conformation of tRNA precursors in this region of their structure and may influence processing of certain tRNAs.

Despite the fact that our understanding of the physiological role of intervening sequences is still unclear, considerable progress has been made in clarifying what are the structural determinants of the tRNA precursors important for efficient splicing of the intron, and what are the enzymes involved in the splicing reaction. Alteration of the intervening sequence itself by introduction of a 21-base-pair DNA fragment into the yeast $tRNA_3^{Leu}$ gene did not affect its transcription and processing in a *Xenopus* germinal vesicle extract.[144] Likewise, incubation of these tRNA precursor molecules with a yeast-splicing system led to efficient excision of the intervening sequence and ligation of the half molecules.[144] These results suggest that the size and structure of the intron may not be important for accurate recognition by the endonuclease responsible for excision. This idea is consistent with the finding that probably only a single endoribonuclease is required for splicing yeast tRNA precursors with different intervening sequences.[145]

In contrast to the change within the intron,[144] mutations at the splice junction[146] or near the intervening sequence of *SUP4* $tRNA^{Tyr}$ [147] or in the D stem of $tRNA_3^{Leu}$ [148] have a profound effect on splicing. These studies suggest that the overall structure of the tRNA precursor directly influences intron removal or affects a prior processing step which leads to inhibition of splicing. In addition to these intragenic lesions, two other distinct yeast mutations affect intervening sequence removal from a whole class of tRNA precursors. These mutants, *rna1*[51] and *los1*,[149] both accumulate tRNA precursors which are mature at their termini, but which still contain the intervening sequence. The molecular basis for the lesions in the mutants has not been identified, but both apparently still retain tRNA splicing activity,[150] suggesting that the enzymes themselves are not affected. Besides their usefulness for studying the physiology of processing, these mutant strains have provided a source of substrates for identifying and purifying splicing enzymes.

Splicing of yeast precursors in vitro has been shown to take place in crude fractions of yeast[151,152] and in germinal vesicle extracts of *Xenopus*.[153] The reaction can be separated into two stages.[154] In the first, the precursor is cleaved at the splice junctions to generate half molecules with the release of the intervening sequence. In the second step the half molecules are ligated in an ATP-dependent reaction to give the mature tRNA. In the absence of ATP, or in the presence of mature tRNA which inhibits the ligation step, the half-tRNA molecules accumulate.[154] Analysis of the termini of all the cleavage products indicated that the endonucleolytic scissions on either side of the intron generated 5′-hydroxyl termini and phosphorylated 3′ termini, in contrast to other endonucleolytic cleavages that occur during RNA processing which generate 3′-hydroxyl termini.[145,154]

Using as an assay the generation of half molecules or the ligation of half molecules to mature tRNA, it has been possible to separately purify the tRNA splicing endonuclease and splicing ligase.[145,155-157] The endonuclease has been partially purified from *X. laevis* oocyte nuclear extracts[156] and extensively purified from yeast, although it still is not pure.[145] Surprisingly, the yeast-splicing endonuclease behaves as an integral membrane protein, but its in vivo location has not been ascertained.[145] The purified yeast endonuclease cleaves a variety of tRNA precursors at two sites, excising the intervening sequence, and giving products that possess 5′-hydroxyl and 2′,3′-cyclic phosphodiester termini. The enzymes require no cofactors although spermidine stimulates the extent and accuracy of the cleavage. It appears that a single endonuclease acts on most, if not all, yeast precursors containing introns.[145] Presumably, as suggested from the mutant studies,[144] the enzyme recognizes a common tertiary structure in the mature domain of the precursors since the size and sequences of the introns and the sequences of the intron boundaries are not conserved.[143]

tRNA ligase activities have been purified from yeast[155] and wheat germ,[157] and studied in extracts of HeLa cells[40] and *Chlamydomonas*[40] and in *Xenopus* oocytes.[158] The yeast[155] and wheat germ ligases,[159] and probably also the one from *Chlamydomonas*,[40] appear to work by a similar mechanism. The ligase requires a divalent cation and a nucleoside tri-

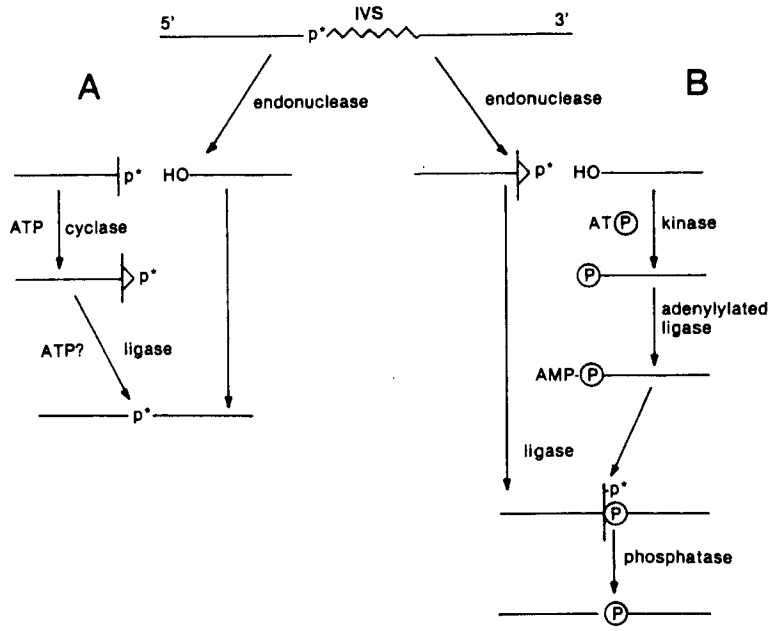

FIGURE 2. Pathways of tRNA splicing in different cells. In pathway A, which has been observed in extracts of HeLa cells and *Xenopus* oocyte nuclei,[40,160] the phosphate in the splice junction is derived from the 3′ terminal phosphate of the 5′ half molecule. In pathway B, which has been found in yeast, wheat germ, and Chlamydomonas,[155,157,160] the splice junction phosphate is derived from ATP by phosphorylation of the 5′ hydroxyl of the 3′ half molecule. The 3′ terminal phosphate of the 5′ half molecule is found in the 2′ position of the splice junction and subsequently hydrolyzed.

phosphate. The enzyme catalyzes ligation of the 5′ half molecule containing a 2′,3′ cyclic phosphate terminus and the 3′ half molecule containing a 5′ hydroxyl terminus to give a product containing a 2′ phosphoryl group and a normal 3′,5′-phosphodiester linkage.[155,159] Examination of the reaction mechanism indicates that the 5′ hydroxyl is first phosphorylated by a polynucleotide kinase activity and ATP and the resulting 5′ phosphate is then activated by adenylylation from an adenylylated ligase intermediate. Condensation of the activated 5′ terminus and the 2′,3′ cyclic phosphate terminus releases AMP, forms a phosphodiester linkage and leaves a 2′ phosphate which originated from the cyclic phosphate. The phosphate in the new phosphodiester linkage is derived from ATP. Associated with the yeast and wheat germ ligases throughout purification are polynucleotide kinase and cyclic phosphodiesterase activities which would be expected from the reaction mechanism.[155,159] However, it is not exactly clear how these activities are related.

The pathway of tRNA ligation in HeLa cells and, probably, also, in *Xenopus* extracts appears to proceed by a different route from that in the plant systems.[40,160] In this case the product contains no 2′ phosphoryl group, and the phosphate in the new phosphodiester linkage is derived from the 3′ phosphate of the 5′ half molecule.[40] HeLa cell and *Xenopus* oocyte nuclear extracts contain an ATP-dependent RNA 3′-terminal cyclase and it is thought that ligation requires prior conversion of the 3′ phosphoryl group to the 2′,3′ cyclic phosphate,[160] although this latter point has not yet been demonstrated with tRNA half molecules. A summary of the two splicing mechanisms as currently envisaged is shown in Figure 2.

E. Order and Location of Processing Events

A great deal of attention has been given to elucidation of the sequence of processing

events that occur during the maturation of a tRNA precursor in vivo. These studies have relied on the structural analysis of tRNA precursors that accumulate in *E. coli*[35,50] or yeast tRNA processing mutants,[51] or that can be identified in *Xenopus* oocytes upon injection of a cloned tRNA gene.[12] By comparison of the structures of the intermediates present, it has been possible to infer the sequence of reactions leading to mature tRNA. The picture that has emerged from these studies is that for each precursor there is probably a preferred order of processing in vivo. It is also clear that this order may vary with different precursors, so that there does not appear to be an obligatory order of processing applicable to all tRNAs.[35,50,112] Furthermore, it has become evident that the order of processing events in vitro may differ from that in vivo,[16] and also may differ depending on the processing system used.[10,33] This is not surprising since the sequence of processing events is most likely a function of the conformation of a particular precursor in a given system, and of the relative levels of the various processing enzymes, which also may differ in different systems. However, in general, it appears that the coarse endonucleolytic cleavages that separate transcripts of different cistrons or that remove long leader or trailer sequences occur first. It is mainly in the final cleavages and addition reactions that generate the mature 5′ and 3′ termini that different orders of processing have been found.

For example, in *E. coli* RNase P mutants a variety of monomeric precursors are found to accumulate.[35,50] The fact that most of the precursors have short 5′ leader segments with monophosphates at the 5′ ends and short 3′ extra sequences suggests that coarse processing can occur in the absence of RNase P cleavages. However, since many of these precursors, such as $tRNA_1^{Tyr}$, $tRNA_1^{Leu}$, $tRNA_1^{Asp}$, and $tRNA_3^{Ser}$, still contain extra 3′ residues, in these molecules RNase P cleavage at the 5′ terminus must precede final 3′ maturation.[35,50] However, in others such as $tRNA_3^{Gly}$, $tRNA^{Phe}$, and $tRNA^{Glu}$, extra residues are still present at the 5′ end, but the 3′ terminus is mature.[35,112] Thus, these findings indicate that in *E. coli*, RNase P cleavages may precede or follow final 3′ processing in vivo.

A similar situation obtains in phage T4-infected *E. coli*. In detailed studies of tRNA processing in this system it was shown that prior maturation of the 3′ –CCA terminus of the $tRNA^{Pro}$-$tRNA^{Ser}$ dimeric precursor was a prerequisite for RNase P cleavage between the two tRNAs.[62] However, RNase P cleavage of the 5′ leader sequence from the $tRNA^{Pro}$ product proceeded prior to 3′ trimming and –CCA addition. Studies with this particular precursor also have demonstrated differences between processing in vivo and in vitro.[16] Whereas in vivo RNase P cleavage between the two tRNAs always takes place before cleavage at the 5′ leader sequence, in vitro these two RNase P cleavages occur at similar rates.

Studies of the order of tRNA processing in eukaryotic cells have suggested that processing at the 5′ and 3′ termini precedes removal of the intervening sequence. First of all, tRNA precursors that accumulate in the *rna1* and *los1* yeast mutants in vivo still contain their intervening sequence but possess mature termini,[51,149] indicating that terminal maturation is unaffected by a block in removal of the intron. Secondly, a detailed analysis of the processing pathway of the cloned yeast *SUP8*$^+$ $tRNA^{Tyr}$ gene in *Xenopus* oocytes has shown that removal of the intervening sequence is the last processing step other than modification of two nucleotides in the anticodon loop.[14] Processing of this precursor proceeded through a series of steps in which precursors of 108, 104, and 92 nucleotides could be detected, as well as the mature tRNA containing 78 nucleotides. These precursors correspond to one containing an intact 5′ leader, intron and 3′ trailer (108 long), one containing a shortened 5′ leader (104 long), and one with mature 5′ and 3′ termini, but still containing the intron (92 long).[14] Since processing of the termini took place very rapidly in this system, it was not possible to conclusively determine which end was matured first. However, in vivo in a yeast system in which a $tRNA^{Tyr}$ gene subcloned into phage M13 was used to hybrid select processing intermediates, removal of the 5′ leader was shown to precede maturation at the 3′ terminus.[161]

In contrast, in a HeLa cell system, the 3′ trailer is processed prior to removal of the 5′ leader.[162]

In vitro, processing of eukaryotic precursors generally is in good agreement with the order observed in vivo. Thus, in extracts from *Xenopus* germinal vesicles or yeast the intervening sequence appears to be the last segment to be removed.[33,152] However, in one exception to these findings it was observed that a cloned yeast $tRNA_3^{Leu}$ gene could be processed in a heterologous HeLa cell extract to give a product in which the intron had been removed, but in which the 5′ and 3′ termini were not yet mature.[10] This same precursor processed in *Xenopus* oocytes follows a pathway in which splicing occurs last.[10] At present, the explanation for the differences with the HeLa system is not known, but the results again show that there is not necessarily one obligatory order for processing tRNA precursors.

Another interesting question related to tRNA processing in eukaryotes is the intracellular location of the various processing steps. This often is a difficult question to answer because of problems with nuclear leakage. However, using nonaqueous microdissection techniques, tRNA precursors in Dipteran salivary glands were shown to be exclusively in the nucleus.[163] Likewise, in studies with *Xenopus* oocytes, which contain a large, easily isolatable nucleus, it was demonstrated that all processing steps, other than some nucleotide modifications, take place in the nucleus.[14] Also, the enzymes, tRNA nucleotidyltransferase[164] and those involved in tRNA splicing,[165] were shown to be present in oocyte nuclei. Whether nuclear processing of tRNA precursors is a general phenomenon clearly is an important question that requires further study. In addition, an understanding of the role of nuclear-cytoplasmic transport in tRNA maturation is of major importance since it has already been shown that a point mutation in human $tRNA_i^{Met}$ can prevent its transport out of *X. laevis* oocyte nuclei.[166]

IV. FUTURE DIRECTIONS AND QUESTIONS

Research in the field of tRNA processing has reached a point at which emphasis is shifting away from identifying processing intermediates and elucidating processing pathways to studying the enzymes involved in the process and understanding their mechanism of action. In prokaryotes, at least, it is possible that all the ribonucleases that play a role in tRNA processing have already been identified. However, many of these enzymes still need to be purified so that their specificity and mechanism can be studied in the absence of other interfering activities.

Once the enzymes that catalyze a processing pathway have been identified, the primary question becomes one of understanding how these enzymes recognize their substrates, and what features of the substrate and the enzyme determine the sequence of events in the maturation of the tRNA.[167] It is already evident from studies of mutants affected in tRNA structure that the conformation of a tRNA intermediate is a primary determinant for proper processing. In fact, it is likely that the order in which enzymes act on a tRNA precursor during processing is determined solely by the changing structure of the precursor as it proceeds through the pathway. Thus, at each step, presumably only one enzyme from the whole complement of processing activities would be best able to recognize the precursor and rapidly catalyze the next step in the pathway. However, since enzyme specificities are not absolute, especially with these macromolecular substrates, alternate pathways may develop in the absence of the primary processing enzyme. This is what occurs in mutant strains lacking a particular enzyme. It might also be expected that changing the ratio of processing enzymes by increasing the level of one of these proteins could alter the order of processing events. For example, an enzyme with a low rate of activity on a particular intermediate, and that normally would not have a significant effect on the precursor, might by an increase in its level become the major catalyst at that step of processing.

Related to these considerations is the question of whether tRNA precursors are processed in association with protein. It is already known that processing of rRNA and mRNA takes place while these molecules are in ribonucleoprotein complexes. A similar situation might obtain for tRNA, although studies of tRNA processing enzymes generally do not take this possibility into account. Most likely, tRNA molecules are always bound to protein in vivo. These could be the aminoacyl-tRNA synthetases or elongation factor Tu, both of which are close to stoichiometric with tRNA in cells. The role these proteins might play in tRNA processing has not been considered. In this regard it is of considerable interest that tRNA precursors in mammalian cells are found in association with a protein that is reactive with anti-La lupus antibodies.[168]

Finally, and most importantly, there are the questions, "Why is RNA processed?" and "Is this process subject to regulation?" Although many suggestions have been made, it is not at all clear why RNA molecules are synthesized as precursors which must then be processed to the mature, functional molecules. If this process has some regulatory significance in controlling levels of functional RNA in cells, it might be expected that various steps in the RNA processing pathway would be controlled. To date, no conclusive evidence for regulation of tRNA processing pathways or enzymes has been obtained, although some hints that this might be the case have appeared. For example, a phage-T4 gene, termed *mb* or *Ml*, has been shown to affect the processing of certain T4-specified tRNAs, although the enzymes involved in the process are all thought to be products of the host. It has also been shown that the chromatographic properties of RNase D change after T4 infection due to association with a low molecular weight phage-specified protein, although no change in function was detected.[125] Perhaps, the best evidence for the regulation of a processing enzyme is the recent demonstration that RNase III is positively regulated by phosphorylation by the phage T7-coded protein kinase.[171] Although this modification is not related to tRNA processing, it sets the precedent for direct regulation of nuclease activities. All of these possible regulatory processes take place after phage infection when there is a major change in gene expression. Whether similar regulation also exists in the uninfected cell remains to be determined.

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