complexes may have an overall topological similarity, with individual structural variations superimposed on the general features.

Concluding Remarks

The results of these various analyses have given considerable insight into the architectural features of synthetase–tRNA complexes. However, these approaches have not revealed the molecular basis for specificity, although they do indicate regions on the tRNA on which to focus further attention. It should also be pointed out that many other approaches have also been employed to explore synthetase–tRNA interactions, such as studies of mutant tRNAs, chemically modified tRNAs, and fragments of tRNAs. References 2–4 review much of the literature in this area.

To consider the specificity and strength of the interaction, let us return to the thermodynamics. As shown in Table I, the association constant under optimal conditions for cognate synthetase–tRNA pairs is on the order of 10^8 M^{-1}. Interestingly enough, a stability constant of this magnitude can readily be achieved if the enzyme only binds to two or three nucleotide units in the entire chain. Thus, Michaelis constants for the interaction of small ligands with enzymes commonly fall in the range of 10^3–10^5 M^{-1}.[48] For example, the complex of Ile-tRNA synthetase with ATP has association constant of around 10^4 M^{-1} at pH 8, 25 °C.[49] Hence twice the free energy of association of ATP with synthetase gives rise to a stability constant on the order of 10^8 M^{-1}. These considerations simply show that subtle effects in the tRNA structure—for example, the presence or absence of a particular base at a particular point—can have large effects on the stability of the complex and also on the probability of forming a particular transition state in the aminoacylation reaction. These facts serve as a reminder that, although our understanding of the complexes has proceeded a great way, the remaining objectives present a vigorous challenge.

As mentioned earlier, tRNAs are believed to complex with a variety of other proteins. Data on these systems may be expected to be forthcoming in due course. It will be interesting to see, for example, if each general class of interactions involves a different area of the tRNA molecule. For instance, it may be that the extra loop, a region that varies in length among different tRNA species, serves as a particular type of receptor (see discussion in ref 6). These kinds of questions will obviously be prime issues in future investigations, and the approaches described above may go a long way toward yielding the answers.

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(49) F. X. Cole and P. R. Schimmel, *Biochemistry*, **9**, 480 (1970).

Seven Terminal Steps in a Biosynthetic Pathway Leading from DNA to Transfer RNA

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Much of the work of science takes the form of pathway elucidation. As the tools of exploration become sharper, they reveal in finer and finer detail the object under investigation. In an accompanying article, Alexander Rich traces the backbone of the polynucleotide chain in yeast phenylalanine transfer RNA ($tRNA^{Phe}$), revealing a fantastically detailed architecture.[1] Living organisms manufacture dozens of different tRNAs, each to precise specifications of nucleotide sequence and chain length. The nucleotide sequence is determined by the DNA, but the chain length is specified by a complex series of steps that form a biosynthetic pathway. In this Account we shall see how it is possible to elucidate such a pathway, with the revelation of detail right down to the molecular level.

The transfer of primary sequence information from DNA to RNA is referred to as transcription. In this process, ribonucleoside 5′-triphosphates pair with their Watson–Crick complements in the DNA. Incoming residues are added as nucleoside 5′-monophosphates by the formation of a covalent sugar–phosphate bond, with the release of pyrophosphate; this reaction is catalized by the enzyme RNA polymerase. But the complete process leading to tRNA is far more complex, since the initial product of transcription is a longer RNA molecule (precursor RNA) containing extra nucleotide residues not present in the tRNA. The presence of these extraneous nucleotide residues means that one or more enzymatic cleavages of the precursor RNA are

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(1) A. Rich, *Acc. Chem. Res.*, **10**, second paper in this issue.

required to excise the functional tRNA.

The discovery that tRNAs are synthesized via larger precursor RNAs has raised several questions related to the process by which these molecules are converted into tRNAs. Of special interest are the identities of the participating enzymes (processing enzymes) and the nucleotide sequences of the initial precursor RNA and all precursor RNA intermediates generated in the production of the tRNAs. It becomes of interest to enquire about the metabolic fate of the precursor RNA-specific residues subsequent to the production of tRNAs. An appreciation of these details should provide a framework for asking how a precursor RNA is enzymatically recognized and handled so that its accurate conversion into tRNA is guaranteed. Perhaps the most intriguing question, but also the one we have the least expectation of answering in the near future, relates to the biological significance of the entire process: Why has nature chosen to utilize precursor RNAs in the production of tRNAs?

The involvement of precursor RNAs in the synthesis of tRNAs was first demonstrated in mammalian cells.[2] However, most of our knowledge about this process has come from studies with microorganisms, where nucleotide sequence determination and mutant isolation are possible. Two such organisms are a virus, bacteriophage T4, and its host cell, *Escherichia coli*.

Following bacteriophage infection, T4 DNA is transcribed to yield several precursor RNAs which are then processed by the host enzyme system into eight tRNAs. Since *E. coli* DNA is not transcribed during infection, one can selectively label with ^{32}P bacteriophage-specific precursor RNAs and tRNAs. The labeled RNAs can be purified to homogeneity by polyacrylamide gel electrophoresis,[3] and the sequences of these molecules can then be determined using the methods of Sanger.[4] Furthermore, there is a collection of *E. coli* mutant strains deficient in specific enzymes of RNA metabolism, and some of these enzyme activities participate in the conversion of the bacteriophage precursor RNAs into tRNAs. Infection of such strains allows the recovery of incompletely processed precursor RNAs that are intermediates in the production of tRNAs. Analysis of these intermediates indicates the nature and order of enzymatic reactions leading to tRNAs.

Precursor RNA Structure and Processing Pathway

Thus far our studies have focused on a T4 precursor RNA chain that contains the sequences of two tRNA species, $tRNA^{Pro}$ and $tRNA^{Ser}$. The top panel of Figure 1 shows the sequence of the precursor RNA (Pro-Ser precursor RNA)[4] while the bottom panel shows the sequences of the tRNAs.[4,5] The Pro-Ser precursor RNA is characterized by the following feature: it contains the sequences of two tRNA species that are arranged in the linear order 5′-$tRNA^{Pro}$-$tRNA^{Ser}$3′; it contains 13 precursor-specific nucleotides that are located at the 5′ and 3′ termini, and in the region between the two tRNA sequences; it lacks a 5′-triphosphate group that is characteristic of the immediate product of transcription, indicating that some precursor RNA processing occurred prior to isolation of the molecule; it contains all of the modified nucleotides of the tRNAs except for the Gm^2 of serine tRNA, indicating that prior to its isolation the residues of the precursor RNA had substitutions and rearrangements that yielded the modified nucleotides (an accompanying article lists the abbreviations used for modified nucleotides[6]); and, it lacks CCA_{OH} sequences found at the 3′ ends of both tRNA chains. (The symbol CCA_{OH} represents a normal trinucleotide with 3′-OH on the A unit.)

Altman and Smith elucidated the first precursor RNA sequence.[7] They worked with an *E. coli* precursor RNA that was somewhat smaller than the molecule of the T4 Pro-Ser precursor RNA, since it contained only a single tRNA, that of $tRNA^{Tyr}$. More recently, two similar small precursor RNAs in *E. coli* have been sequenced.[8] Sequences have also been reported for precursor RNAs that contain two tRNA sequences each, one in *E. coli* and another in T4.[9,10] In *E. coli* there is indirect evidence indicating that precursor RNAs can contain as many as seven tRNA species.[11,12] In the case of precursor RNAs containing multiple tRNA species, there appears to be no obvious structural or functional relationship between the tRNA members of a given molecule. In cells of more complex organisms, such as mammals, silk worm, and yeast, only precursor RNAs containing a single tRNA species have been reported.[2,13] As the analysis of precursor RNAs in these organisms is still in its infancy, there remains the possibility that these precursor RNAs also contain multiple tRNA sequences.

In returning to the structure of the T4 Pro-Ser precursor RNA, we can compile a catalogue of alterations that must be involved in its conversion into $tRNA^{Pro}$ and $tRNA^{Ser}$: a pair of endonucleolytic cleavages indicated by the arrows in Figure 1, thus generating two smaller precursor RNAs which contain the 5′ termini of the tRNAs; nucleolytic removal of residues from the 3′ termini of the resultant smaller precursor RNA chains, a U_{OH} leading to the formation of $tRNA^{Pro}$, and UAA_{OH} leading to the formation of $tRNA^{Ser}$; formation of CCA_{OH} at the 3′ termini of the newly generated precursor RNA chains; and formation of Gm^2 in $tRNA^{Ser}$.

To identify the host enzymes that catalyze these reactions we used *E. coli* mutant strains that lack specific enzymes associated with RNA metabolism. Using a genetic technique, we showed that T4 $tRNA^{Ser}$ is not produced in cells that lack any one of three enzymes: ribonuclease P, tRNA nucleotidyltransferase,

(2) D. Bernhardt and J. E. Darnell, Jr., *J. Mol. Biol.*, **42**, 43 (1969).
(3) W. H. McClain, C. Guthrie, and B. G. Barrell, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 3703 (1972).
(4) J. G. Seidman, B. G. Barrell, and W. H. McClain, *J. Mol. Biol.*, **99**, 733 (1975).
(5) W. H. McClain, B. G. Barrell, and J. G. Seidman, *J. Mol. Biol.*, **99**, 717 (1975).
(6) J. McCloskey and S. Nishimura, *Acc. Chem. Res.*, **10**, fourth paper in this issue.
(7) S. Altman and J. D. Smith, *Nature (London), New Biol.*, **233**, 35 (1971).
(8) G. Vogeli, H. Grosjean, and D. Söll, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 4790 (1975).
(9) S. Chang and J. Carbon, *J. Biol. Chem.*, **250**, 5542 (1975).
(10) C. Guthrie, *J. Mol. Biol.*, **95**, 529 (1975).
(11) P. Schedl and P. Primakoff, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2091 (1973).
(12) H. Sakano, S. Yamada, T. Ikemura, Y. Shimura, and H. Ozeki, *Nucleic Acid Res.*, **1**, 355 (1974).
(13) G. S. Chen and M. A. Q. Siddiqui, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2610 (1973).

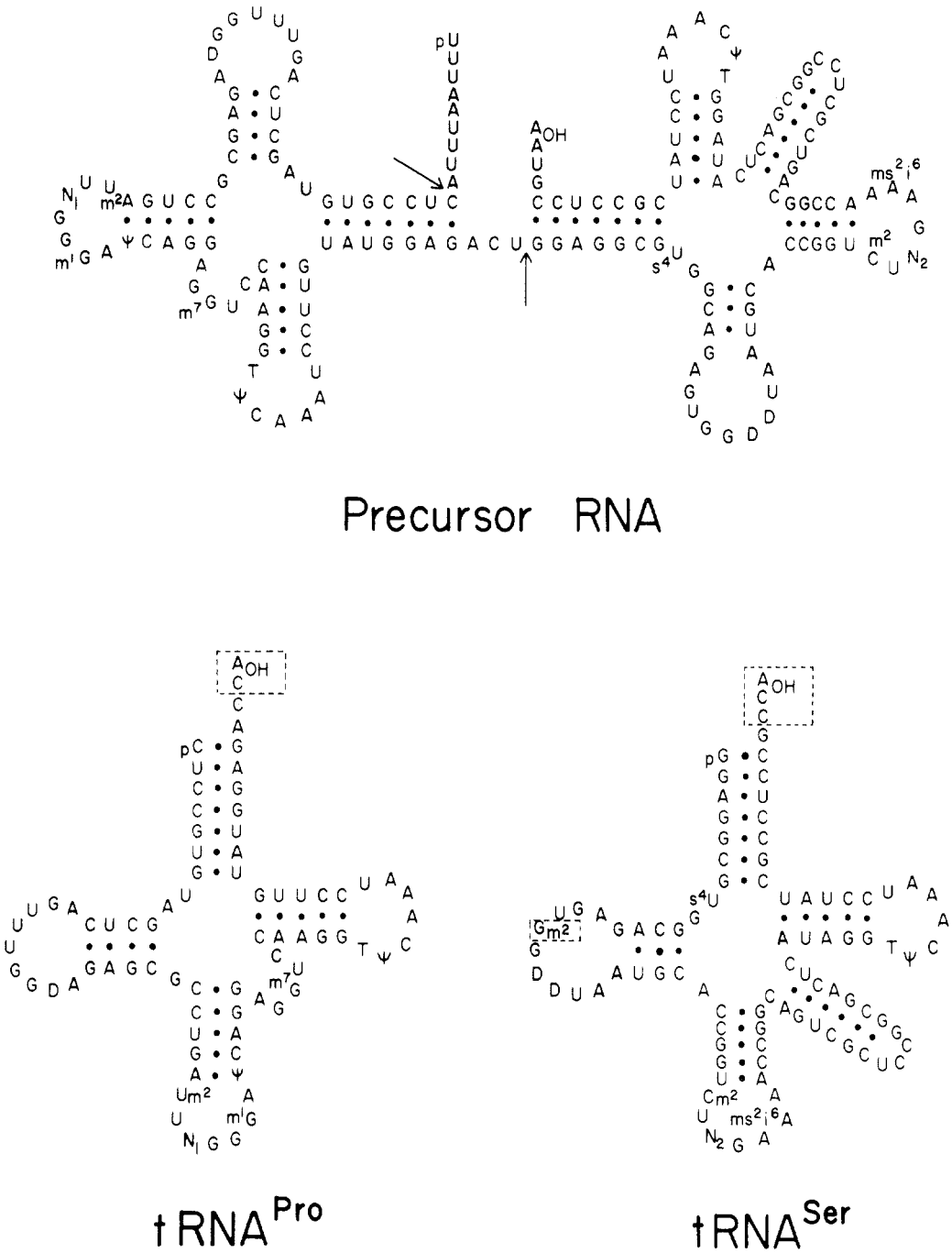

Figure 1. Nucleotide sequences of bacteriophage T4 Pro-Ser precursor RNA, $tRNA^{Pro}$, and $tRNA^{Ser}$. The tRNA sequences are presented in the cloverleaf form. At the top, arrows indicate the 5′ termini of the tRNA sequences. At the bottom, broken boxes denote the differences between tRNAs and the precursor RNA. N_1 and N_2 are unidentified modified residues of U; other abbreviations are given in McCloskey and Nishimura.[6] The 3′,5′ residues of internucleotide phosphates are omitted for brevity.

and an exonuclease.[4,14,15] Thus, these enzymes are tentatively identified as processing enzymes associated with the production of T4 $tRNA^{Ser}$. By contrast, T4 $tRNA^{Ser}$ is produced in mutant cells that lack ribonuclease I, ribonuclease II, ribonuclease III, and polynucleotide phosphorylase, indicating that these activities are not involved in T4 $tRNA^{Ser}$ biosynthesis. The latter conclusion must be stated with reservation, however, since it is possible that residual enzyme activity persists in the mutant cells and is sufficient to sustain the production of T4 $tRNA^{Ser}$. Too, enzyme activity lost by mutation might be replaced by an auxiliary enzyme system. Aside from such uncertainties, these studies have allowed the identification of three host cell enzymes that are involved in the maturation of T4 Pro-Ser precursor RNA. Additional studies must be carried out to determine how these enzymes alter the precursor RNA and the order in which they function.

A classic approach for elucidating the order of steps in a biosynthetic pathway involves the use of mutant strains whose defects in specific enzymes result in accumulation of metabolic intermediates. Cells that lack one of the processing enzymes block the appear-

(14) M. P. Deutscher, J. Foulds, and W. H. McClain, *J. Biol. Chem.*, **249**, 6696 (1974).
(15) J. G. Seidman, F. J. Schmidt, K. Foss, and W. H. McClain, *Cell*, **5**, 389 (1975).

ance of tRNA and a precursor RNA accumulates; normally, this precursor RNA is the substrate of the missing enzyme. The nucleotide sequence of the accumulated precursor RNA then indicates the kinds of enzymatic alterations to it that have occurred in previous steps. The availability of a series of mutant strains, each deficient in a different processing enzyme, should allow elucidation of the entire stepwise process by which the Pro-Ser precursor RNA is converted into $tRNA^{Pro}$ and $tRNA^{Ser}$.

Figure 2 is a schematic representation of how the Pro-Ser precursor RNA is altered in the various reactions that lead to the formation of $tRNA^{Pro}$ and $tRNA^{Ser}$.[4,15,16] Only those nucleotide residues affected by these reactions are shown, and a continuous line is used to represent the remainder of the sequence. Dots represent the hydrogen-bonded interactions between the bases. The structure at the top of Figure 2 is in the same orientation as the Pro-Ser precursor RNA in Figure 1. Figure 2 shows that processing is initiated by the replacement of UAA_{OH} by CCA_{OH} at the 3′ terminus of the Pro-Ser precursor RNA chain through the combined action of exonuclease and tRNA nucleotidyltransferase. The intermediate thus generated is then cleaved twice by ribonuclease P to yield two tRNA-size products that contain the 5′ termini of the tRNA species. The cleavage product derived from the 3′ terminus of the molecule containing the newly added CCA_{OH} residues is immature $tRNA^{Ser}$; addition of a 2-*O*-methyl group to this tRNA species represents the terminal step in the biosynthesis of T4 $tRNA^{Ser}$. The second ribonuclease P cleavage product is a species of immature T4 $tRNA^{Pro}$ that lacks part of the 3′-CCA_{OH} residues; the combined action of exonuclease and tRNA nucleotidyltransferase bring about the formation of T4 $tRNA^{Pro}$.

A surprising feature of the pathway is that the synthesis of CCA_{OH} at the 3′ terminus of the Pro-Ser precursor RNA is a prerequisite to its cleavage by ribonuclease P. This is reflected by the finding that the Pro-Ser precursor RNA accumulates at the expense of the tRNAs in cells that lack either exonuclease or tRNA nucleotidyltransferase. There seems to be no alternative route to the formation to $tRNA^{Ser}$. Such a route would involve the production of $tRNA^{Ser}$ terminating in $CCUCCGUAA_{OH}$ or $CCUCCG_{OH}$, and neither of these tRNA species are observed in appreciable amounts in normal or mutant cells. With regard to the ribonuclease P cleavage that generates the 5′ terminus of $tRNA^{Pro}$, we note that immature $tRNA^{Pro}$ from exonuclease-deficient cells contains the 5′ terminus of the tRNA, even though the 3′ terminus is CU_{OH}. Thus, synthesis of an intact CCA_{OH} sequence with this molecule is not a prerequisite to RNase P cleavage. This result contrasts that obtained with the larger Pro-Ser precursor RNA, where ribonuclease P cleavage is observed only after the formation of CCA_{OH}. At present, we do not appreciate the reason for this difference.

Greater confidence in the validity of the pathway shown in Figure 2 has come from a reconstitution of the reactions *in vitro* from purified precursor RNAs and processing enzymes. As expected, in reactions with

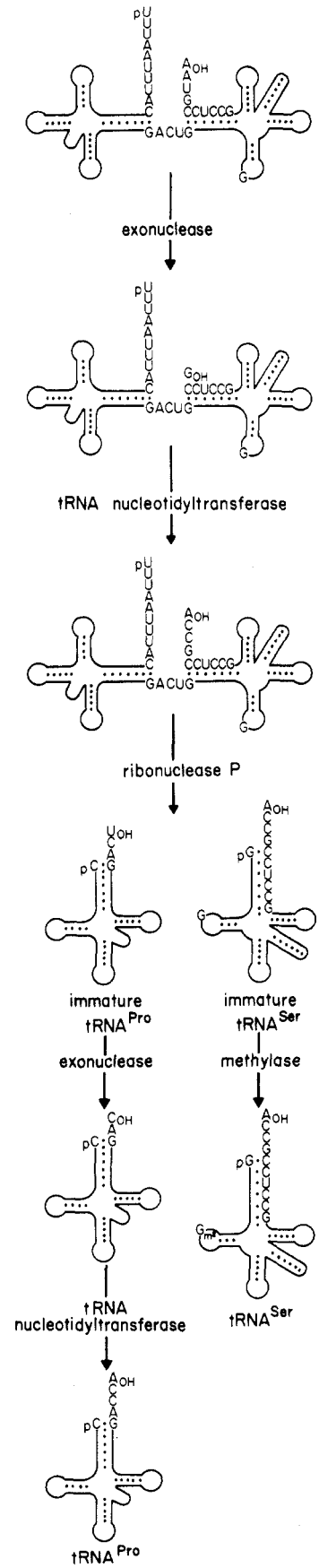

Figure 2. Seven terminal steps in the biosynthetic pathway leading from Pro-Ser precursor RNA to $tRNA^{Pro}$ and $tRNA^{Ser}$. Nucleotide residues not associated with the reactions are represented by a continuous line. Dots represent the hydrogen-bonded base pairs of the cloverleaf forms. Precursor RNA represented at the top is in the same orientation as the molecule pictured in Figure 1. Note that two changes occur in the third reaction; ribonuclease P cleaves the precursor RNA twice, but the order of these cleavages has not yet been determined.

(16) J. G. Seidman and W. H. McClain, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 1491 (1975).

ribonuclease P, Pro-Ser precursor RNA terminating CCA_{OH} was a better substrate than the corresponding RNA terminating UAA_{OH}. Also, the Pro-Ser precursor RNA was cleaved twice, as indicated in Figure 2.[17] The oligonucleotide $pUUUAAUUUA_{OH}$ was recovered as an additional product of this reaction. Since a similar product is not found in infected cells, this oligonucleotide is presumed to be nucleolytically degraded to mononucleotides once it is produced.

Both exonuclease[15] and tRNA nucleotidyltransferase[18] activities have been purified and shown to be capable of catalyzing the reactions shown in the pathway. In these in vitro studies all three enzyme activities indicated in Figure 2 were purified from uninfected *E. coli* cells. We have not tested if any or all of these enzyme activities undergo bacteriophage-induced modification during infection, although this seems possible. Despite this uncertainty, the results obtained in the reconstituted reactions are as expected from the Figure 2 pathway, which was derived by the classic method of mutant methodology.

What is known about the processing of other precursor RNAs? In bacteriophage T4, preliminary evidence indicates that processing of the Thr-Ile precursor RNA is initiated by the removal of residues from the 3′ terminus of the molecule. In overall structure, this precursor RNA is similar to that of the Pro-Ser precursor RNA, since residues at the 3′ terminus of the molecule must be removed and replaced by CCA_{OH}.[15] Therefore, it would not be surprising if the Thr-Ile precursor RNA pathway is similar in several respects to that shown in Figure 2.

Studies with uninfected *E. coli* cells show that many precursor RNAs accumulate in the absence of ribonuclease P. Since this situation is lethal to the cell, a special type of mutant strain is used for these studies. In this strain the ribonuclease P molecule contains an amino acid change that abolishes enzyme activity at high but not at low temperature. Thus, at high temperature, precursor RNA molecules accumulate, and, as with bacteriophage T4, these RNA chains contain the nucleotide sequences of one or multiple tRNA species.[19,20] These precursor RNA chains often contain residues beyond the terminal CCA_{OH}, a finding that indicates removal of 3′ residues occurs after (or with) ribonuclease P cleavage. Further work has led to the claim that sequential cleavage is necessary for normal processing of precursor RNAs. In these studies, precursor RNAs isolated from ribonuclease P-deficient cells were cleaved *in vitro* by partially purified preparations of ribonuclease P, or another enzyme activity (or activities) provisionally termed ribonuclease P_2 or ribonuclease O. The products of the reactions were characterized for reduction to tRNA-sized molecules by polyacrylamide gel electrophoresis; nucleotide sequences were not determined. These studied showed that complete cleavage to tRNA-sized molecules could only be achieved by a combination of the two enzyme activities. This observation was interpreted as evidence for sequential processing, whereby cleavage by one enzyme exposed a site in the precursor RNA for the second enzyme. However, there is the possibility that precursor RNA processing might normally occur sequentially during the process of transcription. According to this interpretation, some of the large precursor RNAs isolated from ribonuclease P-deficient cells would never exist under normal physiological conditions. Therefore, the sequential processing observed *in vitro* would have no counterpart *in vivo*. A similar critism does not apply to the T4 Pro-Ser precursor RNA, because processing of this molecule begins at the 3′ terminus, only subsequent to the completion of transcription.

Rudimentary studies indicate that all *E. coli* tRNAs, with the possible exception of $tRNA^{Cys}$, receive their 3′-CCA_{OH} residues via transcription rather than by tRNA nucleotidyltransferase addition.[21] This is in contrast to the situation in bacteriophage T4 where half of the tRNA chains receive CCA_{OH} by enzymatic addition.[22] Clearly, the different modes of CCA_{OH} synthesis reflect tRNA gene evolution, but any selective pressures which produce these differences are unclear. One provocative speculation is that primitive tRNAs did not require 3′-CCA_{OH} residues to function, and that the requirement for these residues evolved as the protein-synthetic machinery was refined. Initially, this requirement was met in the total tRNA population by tRNA nucleotidyltransferase; subsequently, individual tRNA genes mutated to forms that encoded the 3′-CCA_{OH} residues in their DNA sequences, thereby releasing the biosynthesis of that tRNA from a requirement for one or more enzymes. By decreasing the number of biosynthetic steps involved, hence enabling more rapid tRNA biosynthesis, this feature contributed to selection for tRNA genes encoding 3′-CCA_{OH} residues.[22]

Specificity of Processing Enzymes

One of the striking features of the synthetic pathway of the Pro-Ser precursor RNA is the highly specific nature of the reactions that are involved in the production of the tRNAs. Eventually, we would like to understand how the various processing enzymes achieve such precision, although it is unlikely that this goal will soon be realized. This is because specificity is a reflection of the molecular mechanisms underlying enzyme–RNA interaction, and at present biochemists have little appreciation of what these mechanisms might be. As Paul Schimmel describes in the accompanying article, biochemists are presently laboring to define architectural features associated with enzyme–RNA complexes;[23] only after such information is available can molecular mechanisms be considered. Unfortunately, we cannot use the sophisticated experiments of Schimmel to study processing enzyme–precursor RNA complexes, because neither enzymes nor RNAs are available in sufficient quantity or purity. Nevertheless, we have been able to gain some appreciation of the grosser features associated with the interaction between several of the processing enzymes and precursor RNAs.

(17) F. J. Schmidt, J. G. Seidman, and R. M. Bock, *J. Biol. Chem.*, **251**, 2440 (1976).
(18) F. J. Schmidt, *J. Biol. Chem.*, **250**, 8399 (1975).
(19) P. Schedl, J. Roberts, and P. Primakoff, *Cell*, **8**, 581 (1976).
(20) H. Sakano and Y. Shimura, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 3369 (1975).
(21) M. P. Deutscher, J. J.-C. Lin, and J. A. Evans, *J. Mol. Biol.*, in press.
(22) W. H. McClain, J. G. Seidman, and F. J. Schmidt, *J. Mol. Biol.*, submitted for publication.
(23) P. R. Schimmel, *Acc. Chem. Res.*, **10**, fifth paper in this issue.

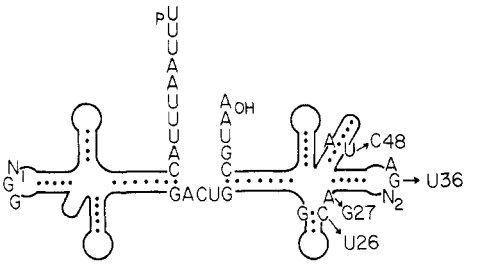

Figure 3. Nucleotide substitutions of Pro-Ser precursor RNA that affect the function of $tRNA^{Ser}$. Precursor RNA is diagrammed as in Figure 2. Substitutions are designated according to nucleotide residue and position from the 5′ terminus of $tRNA^{Ser}$. These nucleotide substitutions allow normal tRNA synthesis, except in C48 where synthesis is partially reduced.

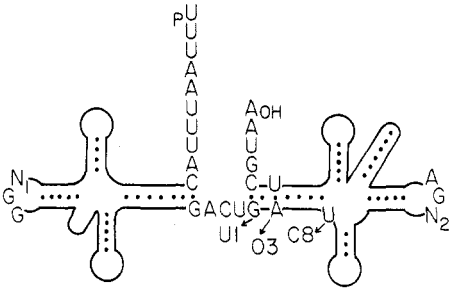

Figure 4. Nucleotide substitutions of Pro-Ser precursor RNA that affect ribonuclease P cleavage. These nucleotide substitutions allow the formation of precursor RNA terminating CCA_{OH}. Precursor RNAs of strains O3 (a deletion of residue A3) and C8 are partially defective in the enzymatic conversion of UAA_{OH} to CCA_{OH}.

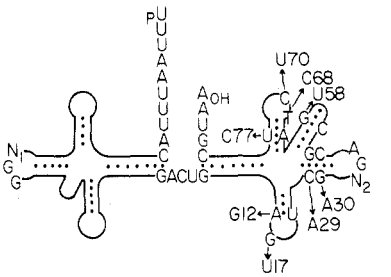

Figure 5. Nucleotide substitutions of Pro-Ser precursor RNA that affect the removal of UAA_{OH} residues. These nucleotide substitutions cause an accumulation of precursor RNA terminating UAA_{OH}. Twelve additional nucleotide substitution mutants with these effects have been characterized thus far.[26]

With regard to the specificity of ribonuclease P, purified preparations of this enzyme have been shown to accurately cleave the Pro-Ser precursor RNA[17] and several other precursor RNAs.[9,10,24] In every case, ribonuclease P generates the 5′ termini of the tRNAs. It has emerged from these studies that ribonuclease P cleaves a variety of nucleotide bonds. Therefore, enzyme specificity cannot be determined by nucleotide sequence alone. Moreover, the results shown in Figure 2 mean that the correct nucleotide sequence at the cleavage site is not in itself sufficient for ribonuclease P action. Precursor RNA molecules terminating $CCUCCGUAA_{OH}$ and $CCUCCG_{OH}$ are much poorer substrates for ribonuclease P than molecules terminating $CCUCCGCCA_{OH}$. These observations point to the ability of the enzyme to somehow distinguish between potential substrates according to their 3′-terminal residues. How is this accomplished? In the Pro-Ser precursor RNA, it is likely that the tRNA sequences are in their final conformations (see below). Such a structure brings the ribonuclease P cleavage site in the central portion of the precursor RNA in close proximity to the 3′ terminus of the molecule (see Figure 1). This allows even the smallest of enzyme molecules to simultaneously interact with both the 3′ terminus and the cleavage site. Perhaps the CCA_{OH} sequence of the Pro-Ser precursor RNA must be bound by ribonuclease P for cleavage to occur, but we do not have enough data at present to evaluate this idea. Note that if such binding did occur, this in itself would determine the order of the initial steps shown in Figure 2.

Additional information concerning processing enzyme specificity has been obtained through the use of a genetic technique that allows the isolation and characterization of mutant precursor RNAs.[25] In these mutant molecules, either the biosynthesis or function of $tRNA^{Ser}$ is affected by a nucleotide substitution. By characterizing the mutant precursor RNAs for their sequence alterations and their defects in enzymatic processing, we have gained a greater knowledge of the individual processing reactions.

Mutations that block the production of $tRNA^{Ser}$ fall into three classes. In one, neither the Pro-Ser precursor RNA nor the tRNAs are produced.[25] These T4 strains are defective in an earlier biosynthetic step, one that normally precedes the production of Pro-Ser precursor RNA. Thus, these strains are not relevant to the present studies, although they will prove useful in analyzing the large precursor RNA that gives rise to the Pro-Ser precursor RNA.

In the second class, a specific nucleotide is substituted in $tRNA^{Ser}$, but this does not prevent the synthesis of $tRNA^{Ser}$-sized molecules. These tRNAs are presumed to be altered in a step of protein synthesis, such as codon recognition,[5] aminoacylation, or ribosome interaction. Figure 3 shows the members of this class. In Figure 3, much of the sequence of the precursor RNA is abbreviated as in Figure 2, though nucleotides are indicated in the tRNA anticodons and at the sites of the various nucleotide substitutions. In designating a particular nucleotide substitution, we refer to the identity of the new nucleotide and its location in the sequence, counting from the 5′ terminus of $tRNA^{Ser}$. Thus U36 refers to $tRNA^{Ser}$ which contains a U in the anticodon at position 36. When consulting Figure 3 and subsequent ones similar to it, the reader should remember that, because of the genetic technique used, all mutant strains were derived from one in which $tRNA^{Ser}$ contained an N_2UA anticodon, so that all subsequent $tRNA^{Ser}$ molecules contained this anticodon sequence, as well as a newly substituted nucleotide. With some of the mutant strains, $tRNA^{Ser}$ of the starting T4 bacteriophage had a CUA anticodon.

Of the nucleotide substitutions pictured in Figure 3, only C48 causes a reduction in the amount of $tRNA^{Ser}$, and this reduction is to about 25% that characteristic of normal $tRNA^{Ser}$. As will be seen presently, mutations shown in Figure 3 are unusual because the vast majority of nucleotide substitions prevent the production of any $tRNA^{Ser}$. Thus, the results of Figure 3 identify two of the regions of the Pro-Ser precursor RNA that can be

(24) H. D. Robertson, S. Altman, and J. D. Smith, *J. Biol. Chem.*, 247, 5243 (1972).
(25) W. H. McClain, C. Guthrie, and B. G. Barrell, *J. Mol. Biol.*, 81, 157 (1973).

altered without affecting $tRNA^{Ser}$ biosynthesis; these regions are the anticodon loop and the junction between the dihydrouridine stem and the anticodon stem.

Figures 4 and 5 show examples of the third class of mutations.[5,26] In normal cells infected with a bacteriophage carrying one of these nucleotide substitutions, the production of $tRNA^{Pro}$ and $tRNA^{Ser}$ is blocked, and the Pro-Ser precursor RNA accumulates. In some cases accumulation is not extensive, apparently because nucleolytic degradation of the precursor RNA is also occurring.

Figure 4 shows nucleotide substitutions that block only the ribonuclease P reaction. In these precursor RNAs, UAA_{OH} residues are removed and replaced by CCA_{OH}. However, the UAA_{OH} to CCA_{OH} transformation is not always complete; with respect to the normal Pro-Ser precursor RNA, mutant C8 is about 20% as complete, mutant O3 (a deletion of residue A3) is about 30% as complete, whereas mutant U1 is essentially normal. These results indicate that the integrity of the amino acid stem of $tRNA^{Ser}$ is not necessary for the actions of exonuclease and tRNA nucleotidyltransferase.

One might have speculated that the terminus of the amino acid stem would provide a reference point to guide the tailoring at the 3′ terminus of the molecule. However, the present results demonstrate that this part of the molecule neither limits nor directs the action of exonuclease and tRNA nucleotidyltransferase. This is most obvious in the case of the U1 precursor RNA, where the terminal base pair is absent. How exonuclease removes UAA_{OH} residues while preserving the remainder of the sequence is an interesting question for future study.

Pro-Ser precursor RNAs with nucleotide substitutions pictured in Figure 5 are defective in the first step of the biosynthetic pathway, the removal of UAA_{OH} residues. Of the nucleotide substitution mutants isolated thus far, approximately 95% are of this type. Possibly, these precursor RNA molecules are not recognized by exonuclease, or, if they are recognized, the action of the enzyme is not limited to UAA_{OH} residues so that continued degradation of the molecule occurs.

Certainly, not all residues pictured in Figure 5 participate directly in the exonuclease–precursor RNA complex. This is because sufficient specificity and strength in an enzyme–RNA complex might well be attained through formation of only a few bonds.[23] What we are probably witnessing in Figure 5 is an expression of the fact that tRNA molecules exhibit long-range order, so that alterations in one part of the molecule may be observed by a change in a property at another part of the molecule. With this interpretation comes an appreciation of the importance of a normal conformation of the precursor RNA for it to successfully interact with processing enzymes.

One of the more obvious features of the nucleotide substitutions in Figures 4 and 5 that block the synthesis of $tRNA^{Ser}$ is that they are confined to one part of the precursor RNA molecule, that containing the sequence of $tRNA^{Ser}$. This result seems to indicate that the nucleotide sequence of $tRNA^{Pro}$ is unimportant for the reactions leading to $tRNA^{Ser}$; if this sequence was important, it would have displayed nucleotide substitutions. Thus, processing enzymes that catalyze the first three steps shown in Figure 2 primarily interact with the residues of $tRNA^{Ser}$, and for these interactions to take place, $tRNA^{Ser}$ must be in its normal conformation.

Two further examples demonstrate the importance of conformation for processing of the precursor RNA. Frequently, the adverse effects of nucleotide substitutions in the stem of the cloverleaf form can be reversed by a further nucleotide substitution that replaces the nucleotide opposite the initial one. For example, the effects of the A30 substitution (Figure 5) are reversed by a further substitution giving U42, with the overall consequence of changing a base pair of G·C to A·U.[26]

Pro-Ser precursor RNAs with nucleotide substitutions like those in Figures 4 and 5 lack many modified nucleotides, in addition to Gm^2. However, these deficiencies are confined to the residues of $tRNA^{Ser}$, for all modified nucleotides are present in $tRNA^{Pro}$.[27] This result demonstrates the critical importance of $tRNA^{Ser}$ conformation when precursor RNA is acted on by the nucleotide modification enzymes. There seem to be no significant interactions between the residues of the two tRNA sequences, for such interactions should have been observed as a reduction in nucleotide modifications in $tRNA^{Pro}$. Thus, it is likely that the precursor RNA contains two independent entities in the form of the tRNA sequences folded in their respective three-dimensional conformations.

We obtained analogous results with the T4 Gln-Leu precursor RNA. This molecule is quite similar to the Pro-Ser precursor RNA in several respects. The sequence arrangement in the precursor RNA is 5′-$tRNA^{Gln}$-$tRNA^{Leu}$-3′.[10] The CCA_{OH} residues of $tRNA^{Leu}$ are at the 3′ terminus of the molecule,[22] and at the 5′ terminus there are only six precursor RNA-specific residues. With the exception of Gm^2, all modified nucleotides of the tRNAs are in the precursor RNA molecule.[27] Mutants defective in production of $tRNA^{Gln}$ were obtained as with $tRNA^{Ser}$.[28] In these mutant strains, nucleotide substitutions occurred in the sequences of both $tRNA^{Gln}$ and $tRNA^{Leu}$.[27,29] This distribution contrasts that observed with mutants defective in the synthesis of $tRNA^{Ser}$. Two facts explain the difference. First, $tRNA^{Gln}$ is situated in the 5′ half of its precursor RNA, whereas $tRNA^{Ser}$ is in the 3′ half of its precursor RNA molecule. Second, with both precursor RNAs, the ribonuclease P cleavage in the central portion of the molecule is dependent on the normal conformation of the 3′-terminal tRNA sequence. Therefore, $tRNA^{Gln}$ is not produced when the conformation of $tRNA^{Leu}$ is blemished by nucleotide substitution.[27] Nucleotide substitutions within the sequence of $tRNA^{Gln}$ are without effect on the ribonuclease P cleavage in the central portion of the precursor RNA, although many such substitutions do inhibit ribonuclease P cleavage at the 5′ terminus of the molecule.[29] The failure of nucleotide substitutions in the sequence of $tRNA^{Gln}$ to affect the central ribo-

(26) W. H. McClain, unpublished data.

(27) W. H. McClain and J. G. Seidman, *Nature (London)*, **257**, 106 (1975).

(28) M. M. Comer, C. Guthrie, and W. H. McClain, *J. Mol. Biol.*, **90**, 665 (1974).

(29) J. G. Seidman, M. M. Comer, and W. H. McClain, *J. Mol. Biol.*, **90**, 677 (1974).

nuclease P cleavage corresponds to the previous observation that nucleotide substitutions blocking the production of $tRNA^{Ser}$ were not found in the sequences of $tRNA^{Pro}$ (Figures 4 and 5).

What has emerged from observing the effects of nucleotide substitutions in these different precursor RNAs is an appreciation of the mode of action of ribonuclease P. As ribonuclease P seizes a precursor RNA molecule in an interaction leading to bond scissions, the enzyme is interacting with the native conformation of the tRNA sequence located at the 3′ terminus of the precursor RNA chain. When the conformation of the 3′-terminal tRNA species is blemished by nucleotide substitution, a successful interaction does not occur.

In contrast to the ribonuclease P reaction, mutant substitutions in the Gln-Leu precursor RNA affect nucleotide modifications in only one tRNA sequence, that which contains the mutant nucleotide. This result again indicates that each tRNA sequence is in its final conformation while still part of the precursor RNA chain.

The proposal that normal precursor RNA conformation is defined by the conformations of its member tRNA species gives us some perspective on the manner in which processing and nucleotide-modifying enzymes achieve specific recognition of precursor RNAs of diverse sizes and sequences. In interacting with precursor RNAs, processing enzymes are looking for the common feature shared by these molecules, namely their three-dimensional, tRNA-like structures. This property of processing and modifying enzymes offers an interesting contrast to the aminoacyl-tRNA synthetases with regard to their specificity. Enzymes of the latter group utilize as substrates only a small number of tRNA chains. In contrast, processing and nucleotide-modifying enzymes appear to act on a great number of precursor RNA chains. Thus, aminoacyl-tRNA synthetases recognize the differences between tRNA molecules, whereas processing and modifying enzymes recognize the similarities. Enzymes of the latter group nevertheless seem to achieve a high degree of specificity in catalyzing their reactions. But it is important to point out that we do not know if this specificity is comparable in magnitude to that of aminoacyl-tRNA synthetases, for the absence in normal cells of inaccurately processed precursor RNAs may in part reflect the action of degradative nucleases.

Biological Significance

Over the past several years, the principle has emerged that in all cell types molecules of tRNA arise by sequential cleavage of precursor RNAs of longer nucleotide sequences than the tRNAs. The biological role of precursor RNAs is a mystery. However, knowledge of the details by which a few precursor RNAs are converted into tRNAs has give us some insight into this mystery.

In precursor RNAs containing two tRNAs, the region between the tRNA sequences is short. Perhaps the residues in this region allow the tRNA sequences to fold into their three-dimensional structures, since these conformations are necessary for enzymes to act on the precursor RNA. With regard to the requirement of a specific conformation for conversion of precursor RNA into tRNA, this might reflect the operation of a cellular editing system that helps guarantee the overall fidelity of RNA transcription. The consequences of nucleotide substitutions in precursor RNAs certainly indicate this. Also, to enable processing enzymes to recognize those features common to precursor RNAs containing different tRNA chains, the evolution of the processing enzyme–precursor RNA system might have occurred in such a manner as to require that specific conformation.

Perhaps the regions at the termini of precursor RNAs contain part of the initiation and termination signals used by RNA polymerase in synthesizing these molecules. Incorporation of these sequences into RNA means they must be removed subsequently, for they would otherwise limit tRNA sequence diversity, thereby restricting the range of specific interactions available to the RNA molecule. If precursor RNA-specific residues do indeed represent remnants of transcription signals, then synthesizing multiple tRNA species from one precursor RNA molecule represents an economy in this process.

Conclusions

The production of T4 $tRNA^{Pro}$ and $tRNA^{Ser}$ represents the best understood example of a biosynthetic pathway leading to tRNA. The seven terminal steps involved in the biosynthesis of these tRNAs have been defined by mutant methodology and confirmed by a reconstruction of the reactions *in vitro* from purified precursor RNA intermediates and enzymes. In this work, we have gained some appreciation of the interactions between a precursor RNA and its processing and modifying enzymes.

The next phase of this work will be directed at obtaining a more comprehensive understanding of the molecular features underlying the specificity of the interactions. This effort is likely to be assisted by the availability of mutants altered in specific interactions, together with a knowledge of the three-dimensional features of the relevant parts of the precursor RNA molecule. An additional effort will be made to extend the biosynthetic pathway back toward the DNA to include a definition of the steps responsible for nucleotide modifications and possible cleavage reactions that lead to the Pro-Ser precursor RNA.

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