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Nucleolytic Processing of Ribonucleic Acid Transcripts in Procaryotes

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INTRODUCTION

Numerous messenger ribonucleic acid (mRNA) and stable RNA species are found in procaryotes. In *Escherichia coli*, at least 70 species of stable RNA are present, coded within at least 27 transcription units. Each of these primary transcripts contains "extra" spacer sequences which are "processed" away and degraded as mature RNA species are formed. During this processing, cotranscribed species are also separated from one another. On average, these extra sequences comprise 20% of each primary transcript. In addition, a number of mRNA species are processed posttranscriptionally. Since previous reviews of procaryotic RNA processing (2, 4, 39, 54), a number of advances have been made in this field. Most *E. coli* ribosomal RNA (rRNA) and transfer RNA (tRNA) genes have been cloned, characterized, and sequenced. RNA has been shown to act catalytically (ribonuclease [RNase] P), and introns have been identified in genes of bacteriophage T4 and archaebacteria. In addition, considerably more information about different RNA species in other procaryotes (*Bacillus subtilis*, archaebacteria) is now available, allowing direct interspecies comparison of processing reactions and of some precursor RNA structures.

Some processing reactions have an unequivocal impact on the structure and function of the RNA species processed. For example, as we detail below, processing is required to form active tRNAs from precursors. Previous reviews have attempted to categorize processing reactions in terms of such ultimate functions in vivo. This approach works well in some instances, but not so well when the function of processing reactions is subtle or unclear; for example, cleavages of the leader sequences of certain lambda phage tran-

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scripts may mature an RNA species, initiate turnover, or both. To avoid forcing some processing events into arbitrary categories, this review is divided into discussions of the nature of processing events and separate discussions of possible functions of processing. This format fragments the treatment of individual RNA species, but facilitates both comparative discussions and treatments of complex cases.

The first major section of the review details information about different RNA species, their function, abundance, and gene organization. The second considers the nature of the processing reactions which form or destroy these species or do both. The final section considers the possible functions of these processing reactions in vivo. In most cases, *E. coli* is the paradigm for the discussion with comparisons drawn to other species. In some instances, however, other organisms will be considered primarily, because some reactions have been better characterized in them than in *E. coli*, and in some cases a reaction of potential interest has not been shown to occur in *E. coli* as yet.

RNA SPECIES

The RNA species in *E. coli* have been categorized in numerous ways. A distinction between stable and unstable RNA is often made based on both theoretical and empirical considerations. Operationally, an RNA species which disappears rapidly in a pulse-chase experiment is "unstable," while a species which persists is "stable." Theoretically the distinction is between structural RNA (stable) and mRNA (unstable). In general, theoretical and empirical distinctions agree for a given RNA species (i.e., mRNA is empirically unstable), but this type of categorization is not easily applied to some RNA species. In particular, it is now clear that some RNA species can serve both structural and regulatory roles (e.g., the *divE* gene encodes a serine tRNA which controls gene expression and cell division as well as functioning as a tRNA; see below).

Another common distinction is made between genes under stringent and relaxed control. Stringent control is associated with classical structural RNAs (rRNA and tRNA) and with mRNAs coding for ribosomal proteins and RNA polymerase components. Stringent control is characteristic of stable or "structural" RNA (except for a specific subclass of mRNA), whereas relaxed control is characteristic of unstable or "nonstructural" RNA. However, both 6S RNA, a metabolically stable small RNA, and spot 42 RNA, a metabolically unstable small RNA, are transcribed under relaxed control. Clearly then, metabolic stability of an RNA species does not always imply that the species has a structural role or that its expression is controlled by the stringent response. In this discussion we consider four categories of RNA species: tRNA, rRNA, mRNA, and small RNAs. As will be seen, a few RNA species defy this simple categorization and will appear under multiple headings. Of these groups, the small RNAs are by far the most structurally and functionally heterogeneous, as they are classified as such by their length only: arbitrarily, less than 200 nucleotides.

tRNA

tRNA genes are distributed throughout the *E. coli* chromosome and are found in operons containing rRNA genes, protein genes, and other tRNA genes. The amount of each isoacceptor species has been measured in *E. coli* (79), and the linkage map locations of 53 tRNA genes in *E. coli* have been determined (49, 75). These map locations are indicated in Fig. 1. The tRNA genes reside in 27 separate operons with diverse organization. A total of 26% of the tRNA genes occur in rRNA operons, 42% occur in multicistronic operons containing only tRNA genes, 19% occur in monocistronic operons, and at least 13% occur in three multifunction operons containing protein-coding genes as well as tRNA genes (3, 74, 77, 152). This latter type of organization is important in assessing the type and number of processing reactions required to generate mature tRNA species. If a protein-coding sequence follows a tRNA gene, some type of endonucleolytic processing reaction is required to separate the two cistrons prior to 3′ maturation of the tRNA by an exonuclease (see below). In no case is a tRNA gene transcribed to produce a mature species as a primary transcript: all tRNAs require 5′ and 3′ processing to generate their mature termini.

About 60 tRNA genes have been identified in *B. subtilis* (more may be present and function during sporulation). These tRNA genes are arranged in much the same manner as in *E. coli*; however, clustering is more pronounced. Most of the tRNA genes are associated with rRNA operons. Some are found between 16S and 23S rRNA and are cotranscribed with the rRNA genes (104, 136). Some are found as clusters located between adjacent rRNA operons. One of these clusters, containing six tRNA genes, has been sequenced and appears to consist of two separate transcription units (188). Two other clusters of this type have 2 to 3 and 8 to 12 tRNA genes, but have been less well characterized (188). Finally, the largest clusters (16 and 21 tRNA genes) are found distal to rRNA operons (59, 189). These clusters have their own promotors and terminators. There are few repeats of amino acid acceptor types within a given cluster, and some of the tRNA genes in clusters do not encode a 3′-terminal CCA sequence. Genes lacking a CCA sequence seem to be randomly distributed among other tRNA genes in these clusters. Finally, few tRNA genes are found singly, unlinked to rRNA operons (193).

Bacteriophage T4 codes for eight tRNA species. The genes for these tRNAs are located in a single transcription unit located between genes "e" and "57" (51). Within this operon, the tRNA genes are arranged in two clusters. Seven of the eight tRNA genes are located in the 5′ region of the transcript. Six of these are present as closely spaced pairs, with the tRNA Gly gene somewhat separate from the rest. The last tRNA gene is located approximately 500 nucleotides downstream from the other tRNA genes, paired with an RNA species of unknown function (species II). Another RNA of unknown function (species I) is located more distally (51). Some of these phage genes do not encode a 3′-terminal CCA sequence.

In archaebacteria, tRNAs are again found in the spacer regions between rRNA genes (110) as well as in other locations. A cluster of two tRNA genes has been identified (84). The archaebacterial tRNA genes studied thus far do not encode a 3′-terminal CCA sequence (84). In addition, at least three tRNA genes from archaebacteria have been shown to contain introns ranging from 15 to 105 nucleotides in length (38, 84). These introns occur in the anticodon loop, one to two nucleotides from the anticodon, and are excised post-transcriptionally.

rRNA

Seven rRNA operons exist in *E. coli* and their distribution in the chromosome is indicated in Fig. 1. All seven operons have very similar organization (20), and nearly all of the

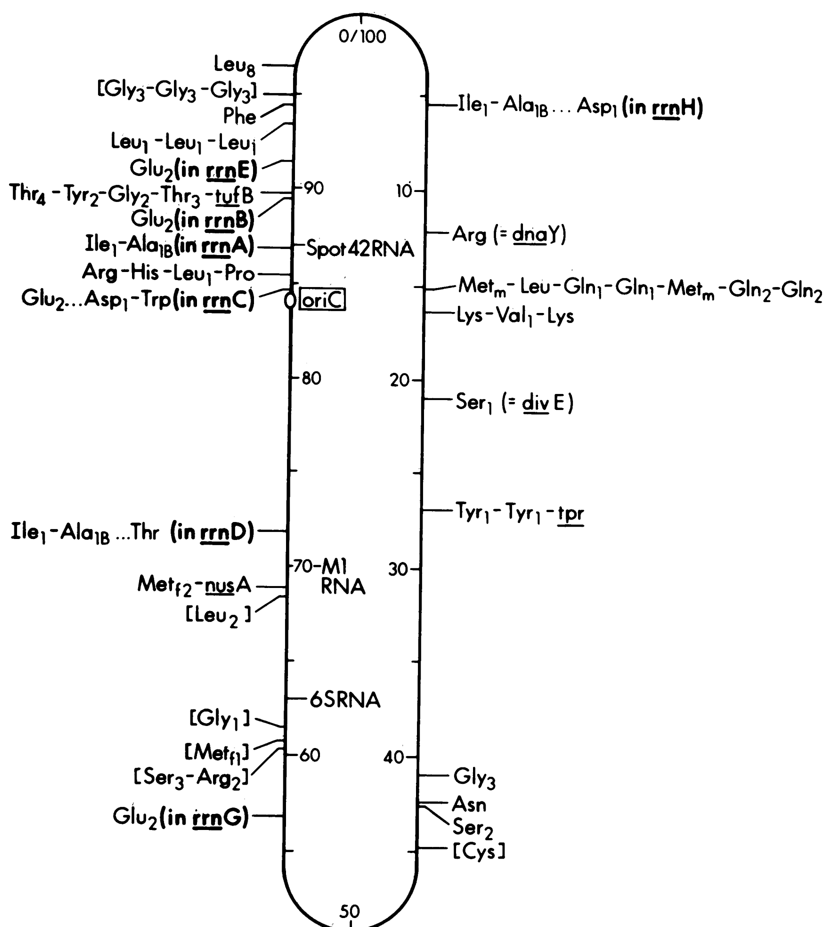

FIG. 1. *E. coli* linkage map. The seven rRNA operons (*rrnA*, *rrnB*, *rrnC*, *rrnD*, *rrnE*, *rrnG*, and *rrnH*) are marked with heavy letters. tRNA genes are indicated; those for which DNA sequence data have not been obtained are bracketed (see reference 49 for details). *dnaY* and *divE*, corresponding to an arginine and a serine tRNA, are indicated, as are 6S RNA, spot 42 RNA, and M1 RNA.

nucleotide sequences are known. In each, the small subunit RNA gene (16S rRNA) is located nearest the 5′ end of the operon. The gene for the large subunit RNA (23S rRNA) is located distal to the 16S rRNA gene and the 5S rRNA gene is nearest the 3′ end (see Fig. 2 and reference 20). In one case (*rrnD*) two 5S rRNA genes are located at the 3′ end of the operon. The three mature rRNA sequences are separated by spacer sequences which are removed during processing. All rRNA operons contain tRNA genes in the spacer between 16S and 23S rRNA, and some contain tRNA genes distal to 5S rRNA.

The spacer regions flanking the rRNA cistrons are highly conserved between operons. Long inverted repeats, flanking both 16S and 23S rRNA sequences, have the potential to form double-stranded stems at the bases of 16S and 23S rRNA (19, 195). These double-stranded regions are predicted to be stable in vivo (195) and have been observed directly by electron microscopy (96). Each operon contains two tandem promoters which are responsive to stringent control. Initial cleavages separate 16S and 23S RNA, usually before transcription of the operon is complete (4, 92). As with tRNA, no mature rRNA species are formed as primary transcripts.

The *B. subtilis* genome contains 10 rRNA operons, 9 of them mapped to date (99), each with an organization similar to that in *E. coli*. Long inverted repeats are present flanking both 16S and 23S rRNA as in *E. coli*, suggesting a similar secondary structure for nascent transcripts (105) (Fig. 2 and 3). Some of these operons are located very close to one another in a head-to-tail orientation (188). The significance of their proximity is not clear, but these rRNA operons are less genetically stable than those of *E. coli*, as variants with deletions of an operon are observed (58).

Archaebacteria contain rRNA operons with an organization again very similar to that found in *E. coli* (71, 80, 132). 16S rRNA is most 5′ proximal, followed by tRNA genes, 23S rRNA, and finally 5S rRNA. The potential to form a large stable RNA stem from precursor sequences exists, at least around 16S rRNA in *Halobacterium halobium* (111). Sequences surrounding 23S rRNA have not yet been reported. Hypothetical secondary structures of the precursor rRNA sequences surrounding 16S rRNA from *E. coli*, *B. subtilis*, and *H. halobium* are shown in Fig. 3. Although the primary sequences of the precursors differ, the similarity of these secondary structures is striking and suggestive (see "Conclusion").

Unlike eubacteria, which contain multiple RNA operons, some archaebacteria contain only one rRNA operon (132). Some archaebacteria also contain an additional 5S rRNA gene which is not associated with an rRNA operon at all (132). At least one archaebacterial 23S rRNA gene contains an intron (95). As this is the only 23S rRNA gene in the

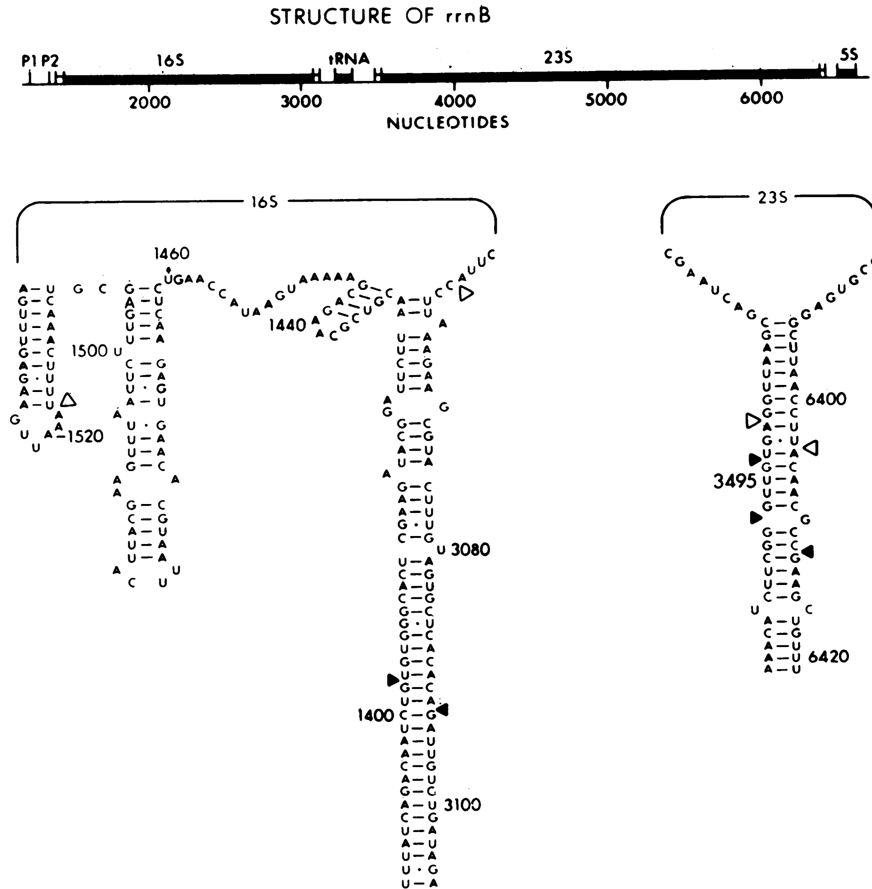

FIG. 2. Typical rRNA operon, shown schematically. The secondary structure of the spacer sequences bordering 16S and 23S rRNA are shown, corresponding to the lowest energy state predicted by computer analyses (see reference 96). Open triangles show the positions of mature termini; closed triangles show the positions of RNase III cleavage sites.

organism, it cannot be a pseudogene. The intron sequence is not present in the mature 23S rRNA of this organism, indicating that it is spliced out. The intron is 622 nucleotides long and is located in domain IV of 23S rRNA. It shares features of both the class I introns found in some eucaryotic rRNA transcripts and the class III introns found in some eucaryotic tRNA transcripts (95 and references 14 to 19 therein).

A 5S rRNA gene from *Halococcus morrhuae* has been shown to contain an insertion of 108 nucleotides relative to 5S rRNA of closely related species (106). This intronlike sequence is retained in the mature, functional 5S rRNA, however. No other archaebacterial 5S rRNA genes have been found to contain introns or similar insertions, suggesting that this is an unusual feature of this particular organism.

mRNA

mRNAs are heterogeneous in size and organization. Approximately 950 mRNA genes have been assigned on the *E. coli* linkage map at present; of these, 25% occur in multicistronic operons, composed of genes with related functions (6). As noted above, a small number occur in operons with tRNA and other small RNA genes (3, 74, 77). Organizationally, ribosomal protein (r-protein) genes are somewhat distinct. They are under stringent control, unlike most other mRNA genes, and 75% of them occur in multicistronic operons composed mainly of r-protein genes. This has regulatory significance, since the translation of many if not all r-protein mRNAs is controlled by feedback inhibition by one of the r-protein products of a given operon (47, 135).

Unlike primary rRNA and tRNA transcripts, mRNA "processing" has only been shown to occur in a few instances and has not been shown to be of functional significance in any case in *E. coli*, though some examples of regulation at this level have been observed in phage and transposons (see below). Highly conserved sequence elements 35 nucleotides in length which may control mRNA expression or stability or both have been identified at the 3′ ends of mRNA operons and in intercistronic regions (70). These REP (repetitive extragenic palindromic) sequences tend to occur as inverted repeats with the potential to form a single, energetically stable double-stranded stem. More than 500 copies reside in the *E. coli* genome, but none have been identified in its phages (176). These sequences are clearly distinct from transcription termination sites, although both contain double-stranded structures. The significance of these sequences will be considered later.

Small RNAs

Small RNAs comprise the most heterogeneous group of RNA species considered here. They include regulatory RNAs, enzymatic RNAs, RNA primers, RNA fragments derived from RNA processing reactions, and small RNAs of

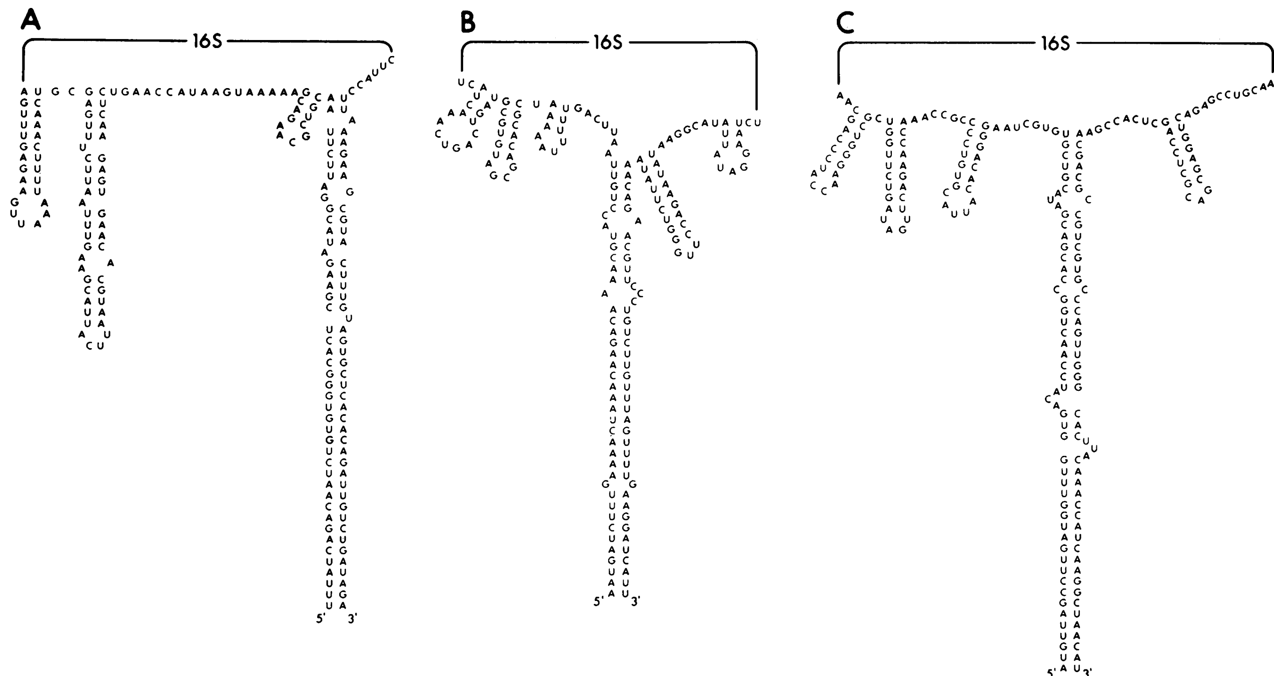

FIG. 3. Precursor rRNA sequences surrounding 16S rRNA from (A) *E. coli*, (B) *B. subtilis* (104, 105, 188), and (C) *H. halobium* (111), shown in hypothetical secondary structures.

unknown function, as well as two tRNAs which apparently subserve functions in addition to their isoacceptor function during translation.

RI RNA has a regulatory function in the control of plasmid copy number for ColE1-derived plasmids. It is a 108-nucleotide RNA, synthesized from the L-strand of ColEI DNA, which hybridizes to and prevents RNase H processing of primer RNA by displacing the primer from H strand. Failure to process the primer RNA to the proper length results in failure of initiation of DNA replication (183). No comparable regulatory system has been identified in *E. coli*, although RNase H is required for *E. coli* viability (85).

mRNA interfering complementary (MIC) RNA is a small regulatory RNA involved in regulation of *ompF* expression (which codes for an outer membrane protein) (124). *ompC* (producing another outer membrane protein) gene expression is coordinately regulated with *ompF* expression in such a way that the sum of *ompF* and *ompC* gene products is a constant. MIC RNA is transcribed from its own operon in a direction opposite to that of *ompC*. The MIC RNA sequence is complementary to the leader region of the *ompF* message and apparently hybridizes to it, inhibiting its translation. It is hypothesized that MIC RNA transcription is coordinate with *ompC* transcription, thus down-regulating *ompF* expression when more *ompC* is produced (124).

M1 RNA is the only RNA in *E. coli* known to have an enzymatic function. It is the catalytically active component of RNase P, a ribonucleoprotein complex composed of M1 RNA (375 nucleotides; located at 70 min on the *E. coli* map [148]) and a protein of 18.5 kilodaltons whose gene is located at 83 min (2). Both RNA and protein moieties are required for full RNase P activity, but the primary transcript of M1 RNA is catalytically active on tRNA precursors under certain experimental conditions (61; see next section). An RNA species with a sequence nearly identical to that of *E. coli* M1 RNA has been isolated from *Salmonella typhimurium* (a corresponding RNA species has been isolated from *B. subtilis*; see below). This RNA species also catalyzes the 5′ maturation of tRNA precursors in vitro in the absence of protein (7). M1 RNA is transcribed from a single operon under stringent control. Termination occurs approximately 40 nucleotides distal to the mature M1 RNA 3′ terminus, but the 5′ ends of the primary transcript and mature M1 RNA are identical (148). The 3′-flanking region of this operon contains a 113-base-pair sequence reiterated 3.5 times as well as multiple short open reading frames. The significance of this unusual organization is not clear, but it resembles the arrangement at the 3′ end of the TyrT operon (147, 152).

RNA primers are required for the initiation of DNA replication in *E. coli* and for discontinuous replication of the lagging strand. Primers for discontinuous DNA replication average two to three nucleotides in length in *E. coli* but may be up to 10 nucleotides long in some of its phages (98). These very small RNAs probably never exist as free RNA molecules but remain covalently linked to the newly synthesized deoxyribonucleic acid (DNA) until they are degraded. Primers for the initiation of *E. coli* DNA replication at OriC are expected to have specific nucleotide sequences, but these have not been identified as yet and their lengths are unknown (137). In *B. subtilis*, RNA primers for discontinuous DNA replication are similarly short-lived, with none detectable in full-length Okazaki fragments (ca. 750 nucleotides in length) (8). Many of these primers share a common sequence in *B. subtilis*, of the form (p)ppApG(pC)pC.

Small RNA fragments derived from the processing of larger RNA transcripts are likely very rapidly degraded to mononucleotides. They have not been extensively studied and are not known to serve any function after they are excised from precursor RNAs. Most of these RNAs probably exist as single-stranded species prior to degradation, but in at least one case, double-stranded RNA fragments are

predicted. RNase III cleavage of the double-stranded stems at the base of 16S and 23S rRNA precursors should generate these double-stranded fragments.

6S RNA, of unknown function, is 180 nucleotides in length. It is transcribed from a single site located at 63 min on the *E. coli* chromosome. Its transcription is under relaxed control. The transcript contains an open reading frame 75 nucleotides distal to the mature 3′ end of 6S RNA, which could code for a single protein of 180 amino acids. 6S RNA is cotranscribed with this open reading frame and so requires 3′ processing. In addition, its transcription initiation site is located six to eight nucleotides proximal to the mature 5′ terminus, implying 5′ processing as well. 6S RNA is rather stable (although its half-life is not known precisely) and exists as a ribonucleoprotein complex sedimenting at 10S. It seems to be dispensible (73, 100).

In archaebacteria, a 7S RNA species has been identified (126). It is a relatively abundant RNA species transcribed from a single-copy gene. Its sequence indicates the potential to form a long double-stranded stem. Its sequence shows no homology to *E. coli* 6S RNA, but its structure is somewhat similar to the RNA component of the eucaryotic signal recognition particle. No precursor species has been observed (127). Its gene is not linked to an rRNA operon.

Spot 42 RNA is a small unstable RNA composed of 109 unmodified nucleotides. It is transcribed from a single-copy gene located at 86 min on the *E. coli* chromosome. The gene includes a rho-independent termination site, and the mature species is apparently identical to the primary transcript. The RNA contains an open reading frame which could code for 15 hydrophobic amino acids, including three leucines (149, 155). Its sequence shows marked homology to that of $tRNA_1^{Leu}$, and it may act as a tRNA analog during leucine starvation (R. M. Williamson and J. H. Jackson, Fed. Proc. **44**:1814, 1985). It is present at 150 to 200 copies per cell and its concentration is down-regulated by cyclic adenosine 3′,5′-monophosphate. Tenfold overproduction of this RNA by a multicopy plasmid results in cells with an increased generation time and retarded adaptation to changes in culture media (149, 156).

4.5S RNA is an RNA 114 nucleotides in length transcribed from a single-copy gene in the *E. coli* chromosome. Its map location is not known. It is cotranscribed with an open reading frame coding for 116 amino acids, located 111 nucleotides distal to the mature 3′ terminus of 4.5S RNA. Processing is required to generate the 3′ terminus; similarly, the mature 5′ terminus is located 24 nucleotides distal to the transcription start site. Its transcription is under stringent control. It is stable and present at approximately the level of an individual tRNA species (1,000 copies per cell). A secondary structure can be constructed for this RNA involving 75% of the nucleotides in a double-stranded stem with a calculated ΔG value of -60 kcal/mol (1 kcal = 4,186 J) (72). Its function is unknown, but it is essential, since cells cured of a prophage containing their only functional 4.5S RNA gene are nonviable (21).

divE is the name given to a temperature-sensitive mutation which results in the inability of cells to divide or synthesize several cell-cycle-specific proteins at nonpermissive temperatures. The mutation responsible for this phenotype is a change from A to G at position 10 in the D loop of $tRNA_1^{Ser}$. The mutant is suppressible by a normal $tRNA_1^{Ser}$ (180). This tRNA is located at approximately 22 min on the *E. coli* chromosome and is transcribed from an operon with an open reading frame coding for a 23.5-kilodalton protein located 3′ to the tRNA gene. It is not clear how this mutation produces the pleiotropic phenotype observed. Its effects may be mediated by changes in the translational function of the tRNA or by some direct regulatory mechanism unrelated to protein synthesis (180).

dnaY is another temperature-sensitive mutation in which DNA synthesis is blocked at nonpermissive temperatures. This mutation is located in an arginine tRNA gene located at 12 min on the *E. coli* chromosome (49, 130).

Small RNAs (species I and II) of unknown function are encoded by bacteriophage T4 and are cotranscribed with its tRNA species (51).

RNA PROCESSING AND TURNOVER REACTIONS

Processing reactions are understood in detail in a few instances. In most cases, the precursors and products have been characterized but the enzymatic activities involved, the abundance of various precursor species, and the rates of processing reactions are not known. Some reactions clearly represent processing events (e.g., maturation of the 3′ end of tRNA) in that they must occur before a molecule is functional. In other cases, processing is not clearly distinct from turnover (e.g., processing of the mRNA *rplJL-rpoBC* has no defined effect on translation, but it may affect turnover). The specificity of a given reaction does not distinguish turnover from processing (e.g., functional inactivation of mRNA may occur at very specific locations in *lac* mRNA). Furthermore, processing and turnover cannot be distinguished as endonucleolytic and exonucleolytic, since for example, maturation of the 3′ end of tRNA precursors and final chemical degradation of mRNA are both mediated by exonucleases. Therefore, the only real distinction between processing and turnover is operational (in terms of a reaction's functional significance). Since the purpose of many processing reactions is uncertain, and since it is conceivable that one reaction may serve to both mature and destabilize an RNA species, we will not attempt to distinguish processing reactions rigorously from turnover reactions. Processing and turnover reactions will be discussed together in this section, and a distinction between the two will only be made when the function of a particular reaction seems clear.

tRNA Processing

tRNA processing enzymes are among the best characterized in *E. coli*. Some have been assigned different names by different investigators; Table 1 includes all enzymes thought to be involved in tRNA processing and suggests which of them may represent equivalent activities.

As mentioned before, some tRNA precursors are multimeric and so might require endonucleolytic cleavage to separate individual tRNA molecules prior to final maturation. Since all tRNA molecules are eventually cleaved at their 5′ end by the endonuclease RNase P (39), it is formally possible that RNase P could also separate multicistronic tRNA precursors in *E. coli* (as well as generating mature 5′ ends). In some cases, RNase P clearly makes the initial cleavage which separates dimeric tRNA precursors (162). There is also evidence that tRNAs in some multicistronic precursors are separated prior to RNase P cleavage in vivo (159). As mentioned above, in transcripts in which a protein-coding sequence occurs distal to a tRNA gene, some type of endonucleolytic cleavage is required 3′ to the tRNA, since final maturation of the 3′ terminus is carried out by an exonuclease (see below). An *E. coli* endonuclease, RNase PC, is known to separate T4 phage tRNA precursors prior to

final processing (56), and it may be involved in the processing of *E. coli* tRNA precursors as well.

In archaebacteria, the structure of the introns and their location in the tRNA sequence are similar to eucaryotic tRNA introns, but their mode of excision has not been characterized and self-splicing remains a possibility. Similarly, the activities which generate the mature 5′ ends, remove 3′ nucleotides, and add the 3′ CCA sequence have not been characterized.

RNase P and 5′ maturation. RNase P is an endonuclease which generates the mature 5′ terminus of all tRNA species in *E. coli*. Similar activities have been identified in *B. subtilis* and *S. typhimurium* (7, 53). It must recognize mature tRNA sequences rather than precursor segments, since it correctly processes tRNAs with variable precursor sequences, including those from different species (2). Some modifications of the mature tRNA structure have been shown to result in delayed or inaccurate processing of the affected precursor (52, 169). RNase P is not obligately the first or last step in tRNA maturation, since species with either mature or immature 3′ ends are substrates for it. However, it has been shown that different substrates are cleaved more efficiently than others by RNase P. tRNA precursors lacking 3′-terminal CCA are poorer substrates for isolated M1 RNA (the catalytically active component of RNase P) than are species containing CCA (63). In a dimeric T4 tRNA precursor with two RNase P cleavage sites, the internal site is cleaved twice as rapidly in vitro and is greatly favored in vivo (162). (This preference may be due to the fact that the 5′ tRNA in the precursor dimer lacks a 3′ CCA sequence; see below.) That different substrates are cleaved more or less rapidly is consistent with the notion that the initial separation of multicistronic tRNA precursors may be mediated by an activity other than RNase P. For instance, in multicistronic transcripts which are a poor substrate for RNase P, another endonuclease might cleave more rapidly than RNase P, whether or not there is an absolute requirement for it.

The catalytic function of the M1 RNA component of RNase P is clearest at elevated levels of Mg^{2+} ions. The RNA itself base pairs with the substrate and can mature pre-tRNA, but the protein component of the RNase is required for the maturation of another substrate, p4.5S RNA (62). Because the sequences of M1 RNA from *E. coli* and *B. subtilis* show no cross-hybridization, the structure of the RNA rather than its sequence must be critical for its function (see "Efficiency of Processing Enzymes Made of Protein and RNA" below) (53). One third of the 3′ region of M1 RNA can be removed without destroying its enzymatic activity (1).

Precursor tRNA species with immature 5′ ends are not isolated from wild-type *E. coli* strains. Precursor tRNA species do accumulate when very high levels are transcribed from a multicopy plasmid. In addition, mutations in mature tRNA sequences which lead to slower rates of processing by RNase P (see above; 52, 169) cause abnormal tRNA precursors to accumulate. These findings suggest that normally RNase P cleavage must proceed with a short half-reaction time, due either to an abundance of enzyme or to a high enzyme turnover number. The number of molecules of RNase P per cell has not been measured.

3′ Maturation. The mature 3′ terminus of tRNA species is generated exonucleolytically, but the activity or activities involved have not been unambiguously defined. Some specificity for this processing reaction seems likely, since the exonuclease involved does not simply remove 3′ nucleotides processively until a double-stranded region in the tRNA is encountered. On the other hand, the tertiary structure of the tRNA and of the exonuclease could interact sterically to block degradation near the appropriate 3′ terminus. Deutscher and co-workers compared RNase II and RNase D in their ability to mature tRNA 3′ ends and found that RNase D, a nonprocessive nuclease, generated the correct mature terminus whereas RNase II, a processive nuclease, removed one to two additional nucleotides (35). The extra nucleotides removed by RNase II could be replaced by tRNA nucleotidyltransferase, but a mutant deficient in that enzyme shows no detectable alteration in tRNA processing (40). Since mature and precursor tRNAs are exposed to RNase II in the cell, at least some tRNA precursors may be processed by RNase II. Although RNase D would seem to be the major

TABLE 1. RNA processing nucleases

Activities	Function	Reference(s)
Exoribonucleases (all 3′ → 5′)		
RNase II	Single strand-specific nuclease involved in mRNA chemical decay	43
Polynucleotide phosphorylase	mRNA chemical decay	43
RNase R	Possible role in mRNA metabolism	89
RNase D	3′ Processing of tRNA precursors	35
RNase BN	3′ Processing of tRNA precursors in phage T4	5
RNase T	"End turnover" at 3′ end of mature tRNA species	41
Endoribonucleases		
RNase III	Initial processing of rRNA precursor, minor role in tRNA processing in T4, possible role in mRNA metabolism, etc.	13, 151
RNase H	Degrades RNA of RNA-DNA hybrids	34
RNase P	5′ Processing of tRNA precursors	61, 62
RNase E	Initial 3′ and 5′ processing of 5S rRNA precursor	123
RNase M5	3′ and 5′ processing of 5S rRNA in *B. subtilis*	140
RNase PC	Separation of multimeric T4 tRNA precursors	56
RNase I	Turnover of rRNA	131, 146
Uncharacterized		
RNase "M16S"	Final 5′ and 3′ processing of 16S rRNA; may be two distinct activities	37, 67
RNase "M23S"	Final 5′ and 3′ processing of 23S rRNA; likely two distinct activities	167
RNase "M5S"	Final 5′ and 3′ processing of 5S rRNA in *E. coli*	46
RNase P2 RNase O RNase F	Endonucleolytic separation of multimeric tRNA precursors; the three activities may or may not be distinct	39, 50
RNase N	Endonuclease	122

3′-processing activity, a deletion mutant (for the RNase D gene) is viable and has no noticeable alterations in tRNA processing (15). Clearly another enzyme can substitute (possibly RNase BN; see below).

Primary tRNA transcripts in *E. coli* contain the entire mature sequence of each tRNA molecule, but in *B. subtilis* and some *E. coli* phages (e.g., bacteriophage T4) a 3′ CCA must be added post-transcriptionally to some tRNA precursors (51, 189). An *E. coli* enzyme, RNase BN, is required for the 3′ trimming of these phage tRNA precursors prior to CCA addition (since a mutation in this enzyme greatly reduces the efficiency of phage infection [5]). The nature of this reaction is different from that for the 3′ maturation of *E. coli* precursors, in that three additional nucleotides must be removed from the 3′ end of the tRNA precursors. It has been suggested that RNase BN may substitute for RNase D in the RNase D deletion mutant described above. However, a mutant deficient in RNase D, RNase BN, RNase I, and RNase II apparently processes tRNA normally (197). (Since this mutant does not have a deletion of the RNase BN gene, it is possible that the allele was sufficiently leaky to allow complementation by residual RNase BN activity. This explanation seems unlikely, since the mutation results in failure of phage growth. Nevertheless, since a different reaction is required for phage tRNA maturation [i.e., the removal of three additional 3′ nucleotides], it is possible that residual RNase BN activity suffices to mature *E. coli* tRNA precursors.) which of these activities is dominant in wild-type *E. coli* and whether additional uncharacterized activities are involved in 3′ maturation of tRNA precursors remain unsettled.

tRNA end turnover. RNase T is a nonprocessive exonuclease which removes the 3′-terminal adenosine residue from mature tRNA species in vitro (41). This reaction has been called "end turnover," and its significance is uncertain, but it does not lead to tRNA degradation. As mentioned above, the adenosine removed can be replaced by tRNA nucleotidyl transferase. This RNase T reaction is at least somewhat specific, in that RNase T is much less active with tRNA species with sequences other than CCA at their 3′ end. RNase T is not essential in wild-type *E. coli*, since a mutant deficient in RNase T activity grows normally. In an *E. coli* strain deficient in tRNA nucleotidyltransferase activity, the abnormally high level of tRNA species lacking a 3′-adenosine residue is reduced when a mutant RNase T allele is introduced into the strain. This suggests that RNase T functions in vivo to remove 3′-terminal adenosine residues (42). Indeed, some second-site revertants of strains deficient in tRNA nucleotidyltransferase are low in RNase T activity. RNase T is not efficient at maturing normal tRNA precursors, and so it is doubtful that it could substitute for RNase D or BN. Other "nonspecific" nucleases may be capable of similar actions, for instance RNase II (see above).

tRNA processing in bacteriophage T4. tRNA processing in bacteriophage T4 is complex, involving at least five different processing activities (RNase P, RNase III, RNase BN, RNase PC, and tRNA nucleotidyltransferase) (161). As discussed above, the T4 tRNA gene cluster is transcribed as a single unit. In vivo, the transcript is quickly cleaved by an endonuclease (RNase PC) to yield three dimeric tRNA precursors (Gln-Leu, Pro-Ser, and Thr-Ile), a tRNA Gly precursor, a species I RNA precursor, and a tRNA Arg precursor attached to the species II RNA precursor. (Productive phage infection is dependent on RNase PC function [56].) These dimer species are observed during infection by wild-type phage, although some are more abundant (Pro-Ser) (see below). Each tRNA dimer is separated by RNase P cleavage which generates the mature 5′ terminus of all eight tRNAs (as well as those of species I and II RNAs).

3′-Processing is even more complex, as only the genes for tRNAs Leu, Gly, Thr, and Arg encode a 3′-terminal CCA sequence (51). These species are likely processed as are *E. coli* tRNA precursors (by RNase D or its substitute). The tRNA Gln gene encodes only the first C of its 3′ CCA sequence. This precursor species has no extraneous 3′ nucleotides (after tRNA Leu is removed by RNase P) so CA can be added directly by tRNA nucleotidyltransferase. The other three tRNA precursors lacking an encoded CCA sequence (Pro, Ser, and Ile) each have at least one extra nucleotide at their 3′ ends which must be removed prior to CCA addition (161). This removal is accomplished by RNase BN (5), one of the *E. coli* enzymes essential for efficient T4 infection (see above). (Similar types of processing reactions are likely involved in the maturation of *B. subtilis* tRNA precursors [which have multimeric precursors with some genes lacking the 3′ CCA sequences].)

Some of these processing reactions are very sensitive to the structure of their substrates. In some cases this dependence establishes an absolute order of processing. For example, in the case of the Pro-Ser tRNA dimer, RNase P cleavage at the 5′ end of tRNA Ser is dependent on prior processing and CCA addition at the 3′ end of the dimer. (The dimer accumulates in cells deficient in RNase BN activity.) RNase P processing of the tRNA Pro of this dimer is not dependent on its 3′ maturation, however. In the case of the Thy-Ile dimer ordering of processing is not as marked; 3′ maturation of tRNA Ile is not absolutely required for RNase P processing at the 5′ end. (RNase P cleavage is observed in RNase BN-deficient cells, but in wild-type cells all Thr-Ile dimers have mature 3′ termini.)

Another instance of an obligate order for processing reactions involves RNase P cleavage at the 5′ end of precursor tRNA Gln. This step is absolutely dependent on prior RNase III processing six nucleotides proximal to the mature 5′ terminus in a region of potential secondary structure. Although tRNA Gln formation fails in the absence of RNase III cleavage, tRNA Leu (part of the Gln-Leu dimer) is matured normally.

tRNA turnover. In no instance has bulk tRNA degradation been observed. Even after ultraviolet irradiation or during productive lambda infection which leads to cell death, tRNA molecules remain intact (14, 139). This suggests that tRNA structure is completely resistant to nucleases, at least in *E. coli*; the argument that structure is protective is strengthened by the findings that tRNA species with particular mutations which affect their secondary structure are degraded (169).

rRNA Processing

The processing of rRNA is complex, involving multiple cleavage reactions at the 5′ and 3′ ends of each species. Some reactions do occur (or at least can occur in vitro) with naked pre-rRNA, while others require that critical r-proteins be associated with the pre-rRNA. Some of the final processing reactions may even require that the preribosome engage in protein synthesis (23, 65, 109, 196).

RNase III and processing of 16S and 23S rRNA. In wild-type *E. coli*, the first cleavage to occur is the RNase III-mediated separation of 16S and 23S rRNAs from each other and from 5S rRNA and any tRNAs present in the operon. (RNase III is a dimer of 50,000 molecular weight [13]. A similar activity is found in *S. typhimurium* [177] and

possibly in *B. subtilis* [143].) The RNase III cleavage reactions occur in double-stranded regions formed by complementary sequences flanking both 16S and 23S rRNA (19, 195). The major cleavages occur at staggered positions in each double-stranded stem (Fig. 2) (19, 195), suggesting that cleavage involves separate reactions in each strand. This view is strengthened by the observation that at some RNase III cleavage sites in T7 mRNA (45, 162), at the λ *sib* cleavage site (30), and at several minor sites adjoining 23S rRNA (165, 166) only one strand of the stem is cleaved. RNase III cleavage occurs in vitro with naked precursor rRNA at the same sites as in vivo, so that there is no dependence on r-protein binding for this reaction (13).

The specificity of RNase III cleavage is critically dependent on the concentration of monovalent cations, and many additional cleavage sites appear at low ionic strength (13). Although no primary or secondary structural features completely define an RNase III cleavage site in any system, it has been suggested that the tertiary structure of the cleavage site is the critical factor in RNase III recognition (150, 151). Indeed, the precise structure of the RNase III cleavage site at the 5′ end of 23S rRNA is essential for proper processing by RNase III. When an rRNA operon with a small deletion in the stem surrounding 23S rRNA was expressed in a maxicell system, RNase III processing failed (174). In this case, the loss of only 3 base pairs from the 26-nucleotide-long double-stranded stem at the base of 23S rRNA completely blocked RNase III processing both in vivo and in vitro.

RNase III cleavage results in qualitatively different precursor species for 16S and 23S RNA. The pre-16S species produced retains long precursor RNA sequences at both its 5′ (115 nucleotides) and 3′ (33 nucleotides) ends, whereas the pre-23S species produced is much more similar to mature 23S rRNA, with only 7 nucleotides at its 5′ end and 7 to 9 at its 3′ end (Fig. 2). These differences may be related to the final secondary structure of these rRNA species (i.e., the 5′ and 3′ ends of mature 16S rRNA are widely separated in ribosomes, whereas those of 23S rRNA may be closely associated). The RNase III reactions occur quite rapidly, even before transcription of the entire operon is complete (4). The reaction half-time for RNase III cleavage in vivo in wild-type *E. coli* is 0.2 to 0.5 min in rich media at 37°C, consistent with cleavage occurring prior to the completion of transcription of the operon (92). For this reason, the unprocessed primary transcript encompassing the entire operon, "30S pre-rRNA," is not found in wild-type *E. coli*. (This 30S pre-rRNA can be isolated from an RNase III-deficient mutant [see below].) Kinetic experiments with rifampin to block transcription show that RNase III cleavage normally occurs at the 5′ end of a 23S rRNA precursor molecule before the 3′ end of that molecule is transcribed (92). This result implies that an intramolecular double-stranded stem does not serve as the recognition site for RNase III. The simplest explanation for this result is that intermolecular hybridization between the 5′ end of a 23S precursor undergoing transcription and the 3′ end of a completed 23S transcript forms a double-stranded recognition site for RNase III. The possibility that some other base-pairing scheme forms the RNase III site has not been excluded, however.

Much of the information about this cleavage reaction is derived from the study of a mutant deficient in RNase III activity, AB301/105 (44). To date, only one mutant allele of RNase III has been isolated. This allele is a point mutation, and its degree of leakiness in vivo is not fully understood. The gene has been cloned into a multicopy plasmid (187), and its sequence has been determined (113). Overproduction of RNase III in cells harboring this plasmid does not result in any obvious phenotypic changes, suggesting that the level of RNase III in wild-type *E. coli* cells is high enough to saturate any of its functions (187). Experiments to determine the effects of deletions in the RNase III gene (or of a temperature-sensitive lesion in RNase III activity, etc.) have not been reported. It seems likely that some minimum level of RNase III activity is absolutely required in *E. coli*, since the mutant allele has markedly pleiotropic effects on plasmid replication, on phage growth, and on the quantity of various *E. coli* proteins synthesized, as well as on rRNA processing (28, 55, 69).

Detailed studies of this mutant by S1 nuclease analysis (92) have shown that RNase III processing in the stem surrounding 16S RNA does not occur, but that 16S RNA is matured normally (at the same rate as if RNase III cleavage had occurred). The situation is quite different for 23S RNA. Again, the major RNase III cleavages found in wild-type *E. coli* do not occur, but the normal mature 5′ and 3′ termini are not generated. Instead, most of the functional 23S rRNA in the mutant is present as longer pre-rRNA species (though residual RNase III activity in the mutant cleaves the 23S precursor at one alternate 5′ site which is four nucleotides shorter than the normal 5′ terminus [93]). These results indicate that the normal maturation of *E. coli* 23S rRNA is absolutely dependent on processing by RNase III in vivo (and in vitro; see below).

Little information is available about the processing pathway for 23S and 16S rRNAs in *B. subtilis*. Initial processing cleavages around both 16S and 23S rRNAs occur in double-stranded regions and involve an RNase III-like activity (105, 143).

16S rRNA maturation. Enzymatic activities which mature the 5′ and 3′ ends of 16S rRNA have been partially purified (36, 37, 67), and a mutant with altered maturation at the 5′ end of 16S RNA has been isolated (36). The reaction half-times for maturation at the 5′ end range from 2 to 4 min in wild-type cells under various growth conditions (92), implying that 4 to 8% of 16S rRNA 5′ ends in growing cells are immature. The rate of this reaction does not depend on prior RNase III cleavage of the precursor.

A mutant, the "BUMMER" strain, which accumulates a novel 5′ precursor of 16S rRNA has been characterized (37). This novel precursor, 16.3S RNA, has 66 extra nucleotides at its 5′ end and accounts for up to 50% of 16S rRNA species in some growth conditions (36). The rate of 5′ maturation of 16S rRNA is three times slower in this strain than in wild-type *E. coli* under comparable growth conditions (92). Extracts from the parental strain can convert 16.3S RNA in vitro to mature 16S rRNA only if the 16.3S RNA is in the form of 30S subunits or 70S ribosomes, demonstrating that the reaction is dependent on ribosome assembly (see below). Analysis of the reaction products indicates that this processing reaction is endonucleolytic, and rifampin pulse-chase experiments suggested that 16.3S rRNA is a precursor of mature 16S rRNA in this strain (36, 92). This activity has been partially purified and has a molecular weight of approximately 70,000. The novel precursor species in the BUMMER strain could result from nonspecific cleavage (of a primary transcript already cleaved by RNase III) at an accessible (single-stranded) point in the precursor's secondary structure (Fig. 2). 23S rRNA processing is not altered in this strain (36, 92).

The enzyme which generates the mature 3′ terminus of 16S rRNA is less well characterized than the activity which

matures its 5′ end. This activity has a molecular weight of approximately 45,000 and can convert a ribonucleoprotein particle with an immature 3′ end to mature 16S RNA (67). It is thought to be an endonuclease, since intermediates with multiple discrete 3′ ends have not been detected. The reaction half-times for this cleavage range from 6 to 9 min in wild-type cells under various conditions (92), generally about twofold slower than for maturation of the 5′ end of 16S rRNA. Like the rate of 5′-end maturation, the rate of 3′-end maturation is not slowed in mutants deficient in RNase III. However, the rate of this reaction is markedly slowed (threefold) in the BUMMER mutant, which is deficient in 5′ maturation activity (92). There are at least two explanations for this observation. Some have suggested that the 5′ and 3′ activities are identical (141). (The molecular weights of crude preparations are not consistent [70,000 versus 45,000]; however, neither activity has been sufficiently purified to test this hypothesis rigorously.) Second, it is possible that there is an obligate (or at least markedly preferred) order of processing, the 5′ step occurring first. This ordering could result from the reaction's dependence of ribosome assembly. No test has been made of this hypothesis.

23S rRNA maturation. Maturation of 23S rRNA is complex. While there are predominant mature 5′ and 3′ termini, other termini exist in wild-type cells (165). The rates of conversion of RNase III-cleaved 23S pre-RNA to mature 23S RNA have not been measured, but as these precursors are rare in wild-type cells (constituting much less than 5% of the total number of 5′ and 3′ ends [92]), this reaction must proceed quite rapidly.

As noted above, "normal" maturation of 23S rRNA does not occur in cells deficient in RNase III activity. Instead, multiple discrete species with 20 to over 97 extra nucleotides at the 5′ end are found. One discrete species with 53 extra nucleotides at the 3′ end is present, in addition to a larger species. All of these species could be produced by nonspecific nuclease action at sites relatively exposed in the precursor RNA's secondary structure (see reference 93; Fig. 2). (In addition to these species, a 23S rRNA species lacking four nucleotides [compared to the predominant 5′ terminus in wild-type cells] at its 5′ end is prominent [93]. It is inferred that this unusual 5′ terminus results from RNase III cleavage because the same species is found in wild-type *E. coli* at low levels and can be formed by the action of purified RNase III in vitro [165]. Presumably the mutation in the RNase III allele in the mutant prevents it from cleaving at its major cleavage sites but not at some usually minor cleavage sites.)

Since the RNase III-deficient mutants are viable and contain only "immature" or "abnormal" 23S 5′ and 3′ ends, ribosomes composed of some, and probably most, of these species are capable of protein synthesis. This is consistent with observations that each of these species is present in roughly the same proportion in free ribosomes and in polysomes (166).

Addition of purified RNase III to these "immature ribosomes" results in the production of the major RNase III cleavage products observed in wild-type cells (species with three and seven extra nucleotides at the 5′ end and eight extra nucleotides at the 3′ end [167]). After RNase III processing, 23S rRNA retains a double-stranded stem about 15 base pairs long, and further processing reactions may occur in this remaining double-stranded portion (Fig. 2). If a wild-type cell extract is added to immature ribosomes from the mutant in conditions which support protein synthesis, the major mature 23S rRNA 5′ terminus is produced in addition to small amounts of the other species. When the same extract is added in Tris-Mg^{2+} buffer, only the RNase III cleavages occur (167), suggesting that protein synthesis (or at least conditions favorable for protein synthesis) is needed for maturation of the 5′ end of 23S rRNA.

Examination of 23S rRNA 3′ ends yields a different result. Addition of a wild-type extract results in the production of ragged 3′ termini ranging in length from the RNase III-cleaved species to two nucleotides shorter than the usual 3′ terminus (167). This result strongly suggests that an exonuclease acts on the RNase III-cleaved precursor to yield the mature 3′ terminus. This reaction is accelerated by, but is not completely dependent upon, protein synthetic conditions. All intermediate species formed in vitro are observed at low levels in wild-type cells.

The 5′ and 3′ termini of 23S RNA are thus somewhat heterogeneous in vivo, with several discrete 5′ species and multiple 3′ termini. An endonucleolytic activity which is dependent on both prior RNase III cleavage and protein synthetic conditions is likely responsible for generating the major 5′ terminus in wild-type cells. The 3′ maturation of 23S RNA appears to be mediated by an exonucleolytic activity, which is also dependent on prior RNase III cleavage. Further, this 3′ exonuclease is apparently dependent on prior 5′ maturation (167). One explanation for this dependence is that maturation at the 5′ end (leaving the 3′ precursor segment single stranded) may potentiate 3′-exonucleolytic activity (Fig. 2).

It is not known whether the double-stranded stems at the bases of pre-16S and pre-23S rRNA have similar functions. However, the removal of the stem at the base of pre-16S rRNA is essential to permit its 5′ and 3′ termini to be far apart, as they are in mature active 30S subunits. In accordance with this, *E. coli* mutants deficient in 16S rRNA processing are generally not viable (175). In contrast, *E. coli* 23S rRNA can apparently function in an incompletely processed form, with its termini base paired. Indeed, 23S rRNA termini are probably base paired in wild type mature 50S subunits (see discussion in reference 166).

5S rRNA processing. Processing of 5S rRNA also requires multiple steps in *E. coli*. A 5S rRNA precursor (9S RNA) has been found to accumulate in an RNase E-deficient mutant (123). This 9S precursor is composed of 5S rRNA with approximately 85 extra 5′ nucleotides extending to the RNase III cleavage site near the 3′ end of 23S rRNA and extra 3′ nucleotides extending to the operon's terminator. This 3′ segment would contain trailer tRNAs if they were present in the operon (and had not been removed by RNase P). This 9S precursor is not observed in wild-type cells, since it is rapidly cleaved by RNase E to yield 5S rRNA with three extra nucleotides at both its 5′ and 3′ ends. RNase E has been partially purified and has a molecular weight of about 70,000; it acts on 9S RNA in vitro to produce the same 5S RNA precursor found in vivo (123, 153, 154, 178). This RNase E mutation has rather pleiotropic effects (e.g., it inhibits processing of M1 RNA in addition to its effect on 5S RNA processing; see below).

It has been suggested that RNase E recognizes a particular RNA sequence rather than RNA secondary or tertiary structural features as the sequence surrounding the RNase E cleavage sites at the 5′ end of RI RNA (another possible substrate of RNase E involved in ColE1 replication; see below) and at the 5′ end of 5S RNA (ACAGU/AUUUG and ACAGA/AUUUG, respectively) are similar (181). However, the RNase E cleavage site at the 3′ end of 5S rRNA (AUCAA/AUAAA) does not conform to this consensus sequence. Furthermore, since RNase A can make an iden-

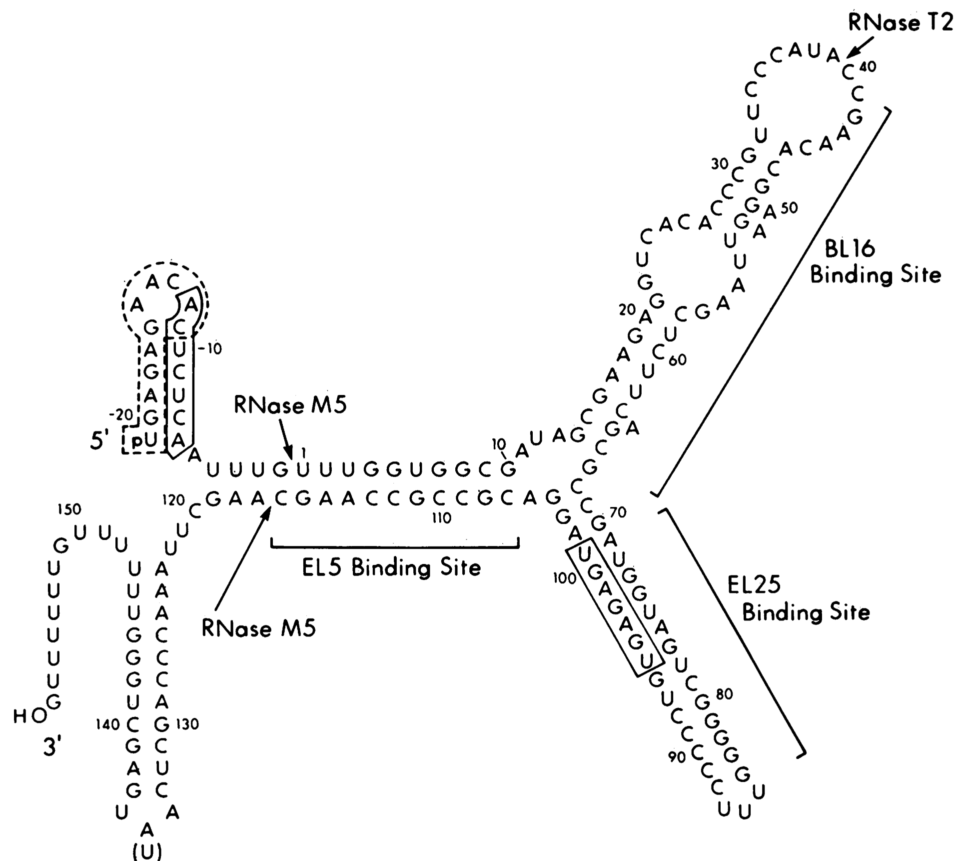

FIG. 4. 5S RNA from *B. subtilis* shown in a probable secondary structure. The two RNase M5 cleavage points and binding zones of BL16, EL25, and EL5 are shown. Boxed sequences −12 to −5 and 95 to 101 are complementary. The sequence boxed with dashed lines (−21 to −11) is complementary to precursor rRNA sequences located near the 5′ end of 23S rRNA (see text) (171, 172).

tical cleavage in RI RNA in vitro (182), it seems likely that higher-order structural features are important for RNase E cleavage.

Other activities evidently complete the processing of 5S RNA, but these have not been characterized in *E. coli*. Multiple 5′ species with one, two, or three extra nucleotides accumulate in the absence of protein synthesis (46). These species are also observed shortly after pulse-labeling cells and disappear thereafter (82), implying that they are true precursors of 5S RNA. 5S rRNA precursors accumulate during chloramphenicol treatment and in some ribosome assembly mutants, implying that final processing of 5S rRNA may depend on 50S subunit assembly. Since precursor species are found in polysomes, final 5S rRNA processing likely occurs in polysomes within functioning ribosomes (46). Also, 5S rRNA precursors must be capable of functioning in protein synthesis (at least to some extent). Further support for this idea comes from the study of the RNase III-deficient strain, in which some 30S pre-rRNA accumulates. Morphologically normal 50S subunits can be isolated from this strain which contain physically linked 5S and 23S rRNAs from the same unprocessed rRNA operon (27). Maturation of the 3′ end of 5S rRNA has not been investigated.

5S RNA processing is much better understood in *B. subtilis*, in which processing is mediated by a single enzyme, RNase M5, which generates both mature 3′ and 5′ termini (119). Both cleavages probably occur at the same time, since intermediates (with one end processed and the other unprocessed) are not found in vivo or in vitro. RNase M5 is composed of two subunits, only one of which is catalytically active (see below). The 5S rRNA precursor from *B. subtilis* is shown in Fig. 4. The 5′ and 3′ ends of 5S rRNA form a long base-paired stem in which the mature termini are staggered by one nucleotide.

Experiments in which precursor and mature 5S rRNA species were cleaved into 5′ and 3′ halves by RNase T2 and then reannealed to form species with mature 5′ and precursor 3′ or precursor 5′ and mature 3′ ends have been instrumental in defining the substrate requirements for processing (Fig. 4) (119). Species with mature 3′ and precursor 5′ ends were substrates for RNase M5, but species with mature 5′ and precursor 3′ ends were not. Addition of a guanosine residue to the 5′ end of a mature 5′ terminus with RNA ligase was sufficient to allow 3′ processing of the hybrid species, however (119). Indeed, the addition of different nucleotides to this "core" precursor species had no effect on RNase M5 recognition, suggesting that the only structural constraint on precursor sequences for efficient RNase M5 processing is the continuity of the double-stranded region at this cleavage site (120). This hypothesis was confirmed by substituting all possible combinations of nucleotides at positions −1 and 116 and testing these semisynthetic species as RNase M5 substrates. The combinations of nucleotides which could form Watson-Crick base pairs served as the efficient substrates for RNase M5 (170).

Since precursor sequences are relatively unimportant for RNase M5 recognition and since RNase M5 shows great substrate specificity (no substrate other than pre-5S rRNA has been identified for it), mature 5S rRNA sequences must provide much of its substrate specificity. Indeed, when precursor species lacking different regions of mature 5S rRNA sequences were prepared, species lacking any mature sequences except nucleotides 70 to 101 (Fig. 4) were not substrates for RNase M5 (118). The dependence of RNase M5 on mature 5S rRNA sequences may be due in part to the binding requirements of one of the two protein "subunits" of RNase M5 (which has been shown to be identical to ribosomal protein BL16 [equivalent to *E. coli* r-protein L18] [140]). This r-protein is thought to bind to p5S rRNA and promote a conformation which is recognized by the catalytic subunit of RNase M5 ("alpha subunit") in physiological conditions. The alpha subunit was shown to be the catalytically active subunit by demonstrating that it could specifically cleave p5S rRNA in the absence of BL16 under special reaction conditions (e.g., in 30% dimethyl sulfoxide [140]).

In all, three ribosomal proteins bind to p5S or 5S rRNA. Cleavage of p5S RNA is indifferent to the presence or absence of one of them (EL25), but the others have very strong effects on maturation. BL16 specifically binds to p5S rRNA and promotes its maturation in physiological conditions, but BL16 shows no preference for p5S as opposed to mature 5S rRNA (171). As the BL16-5S rRNA complex appears to be labile, it is not unexpected that mature 5S rRNA is a competitive inhibitor of p5S rRNA processing in vitro (140). This may not be relevant in vivo, however, because binding of the third r-protein (in these studies r-protein L5 derived from *E. coli*) inhibits processing by RNase M5 (171). The binding site for this r-protein encompasses the double-stranded stem containing the RNase M5 processing sites, suggesting that p5S rRNA once incorporated into 50S subunits would be a very poor substrate for RNase M5. In other words, processing seemingly occurs early in the life of a p5S molecule, when BL16 has already bound but EL5 has not. The mature 5S rRNA would then be sequestered in a 50S ribosome, unable to compete for BL16 with nascent p5S RNA. These results and model are in marked contrast with those obtained in *E. coli*, which suggest that the final step of p5S rRNA processing occurs in the assembled ribosome (see above). Also, neither RNase E cleavage nor the maturation cleavages for 5S rRNA in *E. coli* appear to occur simultaneously as is seen in *B. subtilis*.

rRNA processing and ribosome assembly. Considerable evidence suggests a close link between rRNA processing and ribosome assembly. Although some processing enzymes (e.g., RNase III) effectively cleave at the same sites in both free RNA and ribosomes, other processing activities show varying requirements for ribosome assembly. As discussed earlier, the activity which generates the mature 5′ terminus of 16S rRNA (37) requires that the p16S rRNA be in the form of 30S or 70S ribosomes (at least in vitro). Furthermore, while p16S rRNA in a 27S ribonucleoprotein particle served as a substrate for 3′ maturation in vitro, the precise maturation of the 5′ terminus depended on conditions which favor protein synthesis (67). A similar observation has recently been reported for the processing of 23S rRNA in vitro (166). The finding that incompletely processed rRNA species constitute a significant fraction of polysomes in vivo (109, 167) further supports the idea that a strong linkage exists between ribosome assembly and RNA processing in vivo as well as in vitro.

Considerable information about the linkage between ribosome assembly and rRNA processing has been derived from mutagenesis of a multicopy plasmid carrying an *rrnB* rRNA operon of *E. coli* followed by specific expression of the cloned rRNA genes in a maxicell system (173). This approach allows assessment of the effects of an rRNA mutation on processing and ribosome assembly in the absence of expression of the wild-type copies of the rRNA genes present in the *E. coli* chromosome.

A number of different mutants with small deletions in the central domain of 16S rRNA have been studied. All of these showed failure or marked slowing of 30S subunit assembly with a concomitant block in rRNA processing (175). While RNase III still functioned on these mutant rRNAs yielding 17S pre-rRNA, further maturation to 16S rRNA was greatly impaired, even though the deletions were located physically far away from either the 5′ or 3′ terminus of 16S rRNA. Conversely, point mutations in the 3′ region of 16S rRNA, which did not impair ribosome assembly, also did not impair rRNA processing (81). These experiments are all consistent with the idea that assembly of 30S subunits is closely linked to 16S rRNA maturation.

Expression of mutant 23S rRNA genes in a maxicell system leads to very different conclusions about the relationship between 50S subunit assembly and 23S rRNA processing than that for 16S rRNA. 23S rRNA processing occurs even in deletion mutants which block 50S ribosome assembly (168). 23S rRNA is similarly processed in mutants which allow 50S assembly (even though the subunits formed may not be functional) (199). These results strongly imply that 23S rRNA maturation is largely independent of 50S subunit assembly.

Although RNase III probably acts on the nascent rRNA transcripts before they associate with proteins, further maturation reactions probably occur when the rRNA has associated with specific r-proteins. These r-proteins may provide the characteristic conformation for the processing activity to make a specific cleavage. This requirement is evidently more stringent for 16S rRNA than for 23S rRNA (or possibly for 5S rRNA; see above). From the available data it is impossible to infer the degree of assembly required for different processing reactions. Some observations (discussed above) suggest that the final steps may occur only when a ribosome has bound to mRNA. No direct tests of this hypothesis have been made as yet.

rRNA turnover. Turnover of rRNA occurs to a very limited extent if at all in growing *E. coli* (12). Intact ribosomes are resistant to all nucleases present in the cell's cytoplasm. The only nuclease known to be capable of attacking intact ribosomes is RNase I, a periplasmic enzyme (131). This endonuclease is released into the cytoplasm only under adverse circumstances (12, 146). Its release is apparently controlled by several other genes (78). Presumably after initial attack by RNase I, other nucleases such as RNase II participate in bulk rRNA degradation. RNase I has been shown to cleave 50S subunits first at a particular site in the ribosome (at the central protuberance) (146). Recently it has been suggested that a specific conformational change in 50S ribosomal particles may trigger "ribosome collapse," resulting in the loss of a set of ribosomal proteins and 5S rRNA and in the degradation of 23S RNA (68). Although free rRNA is generally susceptible to nucleolytic attack by nucleases other than RNase I, when precursor rRNA species accumulate during amino acid starvation, many are conserved and converted into ribosomes if the cells are resupplemented with amino acids (107). This result suggests that, when mutant rRNA molecules are degraded (175), alter-

ations in the structure of rRNAs may be important independent of the presence of ribosomal proteins.

mRNA Processing

The only processing reactions known to exert a regulatory effect on procaryotic mRNAs are those which initiate mRNA turnover. Clearly, mRNA turnover has a major effect on gene expression in *E. coli*. Different mRNA species have very different half-lives and so produce very different yields of protein molecules per mRNA molecule (10, 186), and the half-lives of some mRNA species depend on cellular growth rate (134).

Even though processing reactions aside from turnover are not currently known to be important in *E. coli*, they are clearly of importance in some of its phages. Since the machinery for these reactions is coded for by the *E. coli* genome, it seems likely that some of these reactions have significance in *E. coli* as well (16).

mRNA processing in bacteriophage. Recently, an intervening sequence has been identified in the thymidylate synthase gene of bacteriophage T4 (26). This 1,017-nucleotide sequence separates the protein-coding sequence into a 5′ portion coding for 183 amino acids followed by a stop codon at the beginning of the intron and 102 3′ amino acids preceded by a methionine codon. The methionine codon at the beginning of the 3′ exon is not found in the protein products, and no evidence has been found of post-translational joining of two separate protein fragments.

Northern blotting analysis soon after T4 infection (3 min) shows a large, somewhat heterogeneous pre-mRNA which hybridizes to probes for both intron and thymidylate synthase-coding sequences. Later during infection (7 min), 1.3- and 1.0-kilobase (kb) bands begin to accumulate. The 1.3-kb band hybridizes only to thymidylate synthase-coding sequences, while the 1.0-kb band hybridizes only to intron sequences. These results strongly implicate post-transcriptional splicing as the mechanism of intron removal (11). Additional support for this idea was obtained by preparing a synthetic 24-mer oligonucleotide whose sequence spans the splice junction. This probe hybridized to the mature mRNA species (1.9 kb) but not to the pre-mRNA species or the intron (11).

During T4 infection, the appearance of the mature mRNA species (1.3 kb) coincides with the onset of production of thymidylate synthase protein, as expected. Interestingly, chloramphenicol treatment blocks the formation of the mature message but does not block intron excision, suggesting that intron excision and exon joining are separable processes. The excised intron sequence is quite stable relative to the mature mRNA species.

Bacteriophage T4 encodes an RNA ligase activity, so it would seem capable of carrying out an mRNA splicing reaction. However, the message is expressed appropriately when cloned in *E. coli*, suggesting that *E. coli* also contains the necessary machinery for splicing (26). Autocatalytic splicing, as characterized in *Tetrahymena* spp. (24), has not been excluded, however. Indeed, when *Tetrahymena* splice sites are cloned into *E. coli*, accurate and efficient splicing occurs (145). The most 5′ nucleotide of the thymidylate synthase intron is a thymidine and its most 3′ nucleotide is a guanosine. These same two nucleotides are invariant in the *Tetrahymena* self-splicing reaction. No other examples of introns interrupting mRNA-coding sequences are known in procaryotes.

During T7 infection, some phage mRNAs are synthesized as long single transcripts from a multicistronic operon. The individual mRNA species are usually separated by RNase III cleavage post-transcriptionally. The sites of cleavage are well characterized and consist of double-stranded regions with structural features usually associated with RNase III recognition sites. Although these cleavages are not required for translation of the majority of individual mRNAs (192), cleavage probably enhances the translational efficiency of some (44, 69).

The expression of one T7 gene does depend strongly on RNase III processing. Failure of processing at this RNase III site, located just proximal to the 5′ end of gene 0.3 (R1 site), in an RNase III-deficient host diminishes gene 0.3 expression. (Although the mechanism of inhibition is not fully understood [161], it has been suggested that the uncleaved RNase III stem masks the ribosome-binding site of this mRNA.)

This RNase III site has been cloned. When it is placed immediately distal to a human interferon-α 5 gene in a multicopy plasmid, expression of the interferon gene is increased, apparently due to an increase in the half-life of its mRNA (142). This transplanted RNase III site is efficiently cleaved in vivo, generating an mRNA species shortened at its 3′ end. This site was cleaved less efficiently in an RNase III-deficient host; however, the effect of RNase III cleavage on the expression of this mRNA was not specifically measured (142) (see below).

Although expression of gene 1.2 is apparently independent of RNase III processing in wild-type T7 phage, a mutation in an RNase III site distal to the gene affects its expression in a complicated manner. Two RNase III sites 29 nucleotides apart are located 3′ to gene 1.2 (R5a and R5b sites are located in a double-stranded stem, staggered by two nucleotides) which is transcribed as a polycistronic mRNA with gene 1.1 (157). A point mutation (HS9) which abolishes gene 1.2 function maps outside the structural gene and prevents RNase III cleavage at the proximal (R5a) but not at the distal (R5b) RNase III site. Expression of this mutant phenotype is dependent on RNase III, since gene 1.2 function is restored in an RNase III-deficient host. Second-site revertants of this mutation destroy the distal RNase III site, indicating that gene 1.2 expression is blocked only when the distal RNase III cleavage occurs in the absence of the proximal cleavage.

Considerable sequence homology exists between the ribosome-binding site for gene 1.1 and the region between the two RNase III sites, and it has been suggested that hybridization between these two sequences might cause translational repression of gene 1.1, leading to a polar effect on gene 1.2 (157). Whether or not this explanation is correct, RNase III cleavage events in this operon in wild-type phage do not exert any obvious regulatory control (although a more subtle form of control has not been excluded). The kinetics of these cleavage reactions have not been characterized.

In phage lambda, expression of the *int* gene is under the control of an RNA structural element, the *sib* site, located near the 3′ end of its gene (29). This element consists of a double-stranded stem which contains a cleavage site for RNase III. When the *int* gene is transcribed from the p_L promoter, the association of N protein with RNA polymerase allows transcription to proceed through this double-stranded structure, which would otherwise serve as a terminator of transcription. Transcription of the complete stem creates an RNase III site which is subsequently cleaved. The 3′ end generated by RNase III is apparently susceptible to attack by a 3′→5′ exonuclease which rapidly destroys the *int* message, decreasing *int* expression. The *int* gene can also be transcribed from the p_I promoter, in which case N protein is

not associated with the RNA polymerase and transcription terminates in the double-stranded *sib* site. Termination here results in an incompletely formed stem which is not a substrate for RNase III. Consequently, the mRNA is not cleaved by RNase III and is much less susceptible to exonucleolytic attack, allowing for increased *int* expression. This mechanism for controlling mRNA stability and therefore expression is thus far unique (30, 60), but suggestive.

An additional RNase III processing site is present in the lambda p_L transcript located just distal to the protein N gene. Cleavage at this site separates protein N-coding sequences from more distal sequences (which are transcribed only under the influence of protein N antitermination). This cleavage does not occur in an RNase III-deficient host, and the protein N mRNA is more stable in the absence of this processing event (190).

mRNA processing in *E. coli*. Several examples of mRNA processing have been defined in *E. coli*. One involves the cleavage of the *rplJL-rpoBC* transcript in the intercistronic region separating cistrons coding for ribosomal proteins from those coding for RNA polymerase subunits (9). This cleavage does not occur in RNase III-deficient cells, again implicating this endonuclease. A double-stranded stem consistent with an RNase III site can be drawn at the appropriate site in the primary sequence. This cleavage or the lack of it has no clear effect on expression of any of these messages (see below) (9).

A second example of mRNA processing by RNase III is found in the *rpsO-pnp* operon which codes for r-protein S15 and polynucleotide phosphorylase (179). The organization of this operon is complex. It contains only one strong promoter located proximal to the *rpsO* gene and has a transcriptional attenuator in the intercistronic region as well as two RNA processing sites. These processing sites have been mapped by S1 nuclease analysis, and both are found to occur in regions of potential secondary structure located approximately 100 nucleotides apart in the intercistronic region. These cleavages were found not to occur in an RNase III-deficient host (179). Any effect of these cleavages (or the lack of them) on the stability and expression of either cistron has not been reported.

In addition, there is a suggestion that intercistronic cleavages occur in the *trp* operon and that they are involved in turnover (102; cf. reference 160). Finally, mRNA processing of a kind must occur in bifunctional transcripts containing structural RNA cistrons as well as mRNA cistrons (3, 74, 77). These have not been characterized in detail, but the processing activity or activities which act on the structural RNA should serve to separate the mRNA cistron from the structural RNA. Whether additional mRNA processing occurs in these situations is unknown.

mRNA turnover in *E. coli*. The factors which control mRNA turnover in *E. coli* are not well understood. A useful distinction which has been made is that between functional inactivation and chemical decay. Functional inactivation is an alteration of an mRNA species which renders it unsuitable for further translation (in the absence of bulk mRNA degradation). Chemical decay, on the other hand, refers to the degradation of an mRNA species to oligo- or mononucleotides. These two processes are theoretically and experimentally dissociable, in at least some cases. For instance, a temperature-sensitive mutant exists in which the chemical half-life of bulk mRNA is increased at the nonpermissive temperature but the functional half-life of specific messages (in regard to translation) is not altered (25, 138).

Functional inactivation is generally thought to involve an endonucleolytic cleavage (or other type of reaction) near the 5′ end of an mRNA molecule, resulting in the destruction (or blockage) of the ribosome-binding site or proximal coding sequences. Since translation of an mRNA by ribosomes is thought to protect the mRNA from nucleolytic attack, functional inactivation would be expected to promote chemical decay by blocking the protective effect of ribosomes. (Chemical decay is thought to result largely from the action of 3′→5′ exonucleases, starting from the 3′ end of the message or from internal, endonuclease-induced breaks or from both [103].) Specific endonucleolytic cleavages have been implicated in the functional decay of *lac* mRNA. Discrete cleavage products are produced when *lac* mRNA is incubated with RNase III in vitro (164), and functional inactivation of *lac* mRNA in vitro is largely associated with such RNase III action (163). One of these cleavages occurs near the 5′ terminus of the mRNA, at a site consistent with a *lac* mRNA 5′ terminus found in vivo (22). The other cleavages observed occurred in regions of potential secondary structure 200 to 250 nucleotides from the 5′ end. It is not certain which if any of these in vitro cleavages is important for *lac* mRNA functional inactivation in vivo. Discrete endonucleolytic cleavages near the 5′ end of *trp* mRNA have been observed as well (86). These results taken together suggest a role for RNase III in message metabolism.

It is extremely important to note that mechanisms of functional inactivation other than endonucleolytic cleavage are possible. For example, the binding of a protein or RNA to the ribosome-binding site of an mRNA could block further ribosome addition, thus exposing the message to decay. This possibility must be taken more seriously with the recent work of Nomura and his collaborators, who have demonstrated that certain ribosomal proteins bind to initiation sites, shutting down further translation of the mRNAs for those r-proteins (135). This form of autoregulation controls the translation of 16% of total cellular protein and so is not an insignificant mechanism.

Other examples of non-nucleolytic functional inactivation have been well characterized. In bacteriophage T4, *regA* protein is a translational repressor of its own message and that of several other early genes (121). The *regA*-binding site on mRNA species has been shown to overlap the ribosome-binding site of the message (87). The functional and chemical half-life of mRNA species controlled by *regA* is increased in *regA*-deficient mutants, although the stability of other T4-encoded mRNA species (not controlled by *regA*) is unaffected (88). Another type of functional inactivation is mediated by an antisense RNA. A small RNA (MIC RNA; see "Small RNAs" and reference 124) has been shown to inhibit translation by hybridizing to the ribosome-binding site of a particular mRNA species. This form of regulation by translational inhibitors may apply to other groups of mRNAs.

Further support for the notion that the binding of proteins at or near the initiation site for protein synthesis can determine the fate of an mRNA comes from the T4 phage gene 32 mRNA (57). In this case, the gene product shows autoregulation, providing another example of "non-nucleolytic functional inactivation." In addition, a phage-encoded factor markedly stabilizes this mRNA. Stabilization is dependent on a specific 5′-leader sequence in the mRNA and transfer of this 5′-leader sequence to an otherwise unmodified *lac* operon mRNA confers 10-fold stabilization on the hybrid RNA! Although the factor which mediates stabilization is phage encoded, the result is no less compelling and is highly suggestive as a possible means of control in other organisms.

All of these results are most consistent with models in which the rate-determining event in mRNA decay is functional inactivation, with a critical competition and interplay between proteins that determine the loading rate of ribosomes at initiation codons. The direct action of a nuclease (or the preliminary [intrinsically reversible] binding of an inactivating [or stabilizing] factor followed by nuclease action) would terminate ribosome addition and could be followed by chemical destruction of the mRNA. Thus, for example, RNase III cleavage might follow a functional inactivation event mediated by the binding of another protein.

A crucial observation is that chemical decay of many mRNA cistrons occurs in a net 5'→3' direction, even though no 5'→3' exonuclease activity has ever been purified from *E. coli* (83, 103, 125). This net directionality implies the cooperation of an endonuclease with a 3'→5' exonuclease (probably RNase II and polynucleotide phosphorylase; see below) in message metabolism. Multiple internal endonucleolytic cleavages beginning near the 5' end and progressing 3' coupled with 3'→5' exonucleolytic degradation from exposed 3' ends could account for the observed pattern. If the presence of ribosomes on mRNA serves as protection from endonucleolytic attack, as has been suggested, then a 5' cleavage (resulting in functional inactivation) would block ribosome initiation and progressively expose the mRNA to nucleases in a 5'→3' direction. In *trp* mRNA, as might be expected, nonsense mutations leading to premature ribosome release destabilize mRNA sequences distal to the mutation (128).

These results must be reconciled with the observation that mRNA decay is paradoxically slowed when protein synthesis is blocked by chloramphenicol (31, 76). The slowing or blockage of ribosomes on mRNA by chloramphenicol could result in the same form of protection as active translation of the message if, for instance, only the density or spacing of ribosomes on an mRNA, or the fraction of time that an initiation site was occupied by ribosomes, was important for inactivation. Detailed interpretation of results again depends on understanding the nature of the initial inactivation events. The endonucleolytic cleavages occurring in a cistron might be specific or nonspecific. As noted above for *lac*, there is a suggestion of specific cleavage sites, but the cleavages may not be the initial events in decay.

The above model depends heavily on the protection of mRNA by ribosomes. Consistent with that notion, mRNA is labilized when ribosomes are released by puromycin action (32 and references therein). This suggests that messages with more efficient ribosome-binding sites might be more stable. This strong inference has not been tested as yet. If the efficiency of ribosome initiation does affect mRNA half-life, then the peptide yield from a given mRNA should be extremely sensitive to changes in the affinity of the ribosome-binding site, as an increase in affinity would increase both the rate of initiation of protein synthesis and the half-life of the mRNA species. Model calculations suggest a roughly exponential dependence of mRNA functional half-life on the rate of ribosome initiation (data not shown). If this is an important means of control, then differing codon usage might drastically affect the protein yield from some mRNAs. (Ribosomes tend to pause at rare codons in some mRNAs transcribed from multicopy plasmids [185].)

In one case mRNA decay has been shown to occur in a net 3'→5' rather than 5'→3' direction. OmpA mRNA has a very long half-life (15 min) compared with most mRNAs (0.5 to 2 min) and decays in a 3'→5' direction. (In this mRNA, coding sequences are as stable as noncoding sequences! This mRNA may thus be a model case, or it may, given its very long half-life, represent an unusual case [186].)

Metabolism of the individual cistrons in multicistronic messages appears largely independent. In some operons the most 5' cistron has the longer half-life, whereas in other operons the more 3' cistrons are more stable (10, 17, 48, 91, 144). Therefore, there is no obligate net 5'→3' or 3'→5' direction to the decay of multicistronic mRNAs. This result strengthens the idea that endonucleolytic cleavages or other individual functional inactivation events within coding sequences or at ribosome-binding sites are important for decay. If a simple 3'→5' exonuclease activity were responsible for all decay, then the more 3' messages would always have a shorter half-life than 5' messages in a multicistronic operon. Furthermore, in the two cases in *E. coli* where processing of an multicistronic operon is known to occur (*rplJL-rpoBC* and *rpsO-pnp*), RNase III-mediated separation of the cistrons has not been shown to affect the half-life of the 5' messages (9, 179) (if a 3'→5' exonuclease were the primary activity involved, RNase III cleavage should decrease the half-life of the 5' cistrons).

Regardless of the initial events in decay, if endonucleolytic cleavage within coding sequences often occurs during mRNA degradation, a significant number of ribosomes translating a message should be blocked on mRNA fragments with no termination codon. This would result in the release of peptidyl tRNA species with partially completed proteins attached. These incomplete protein species could be released from tRNA by peptidyl tRNA hydrolase (115) and subsequently degraded. Estimation of the maximum number of peptidyl tRNAs formed as a fraction of the total number of peptides made can be inferred from the rates of accumulation of peptidyl tRNAs in a mutant deficient in peptidyl tRNA hydrolase (116, 117), from the fraction of incomplete β-galactosidase chains formed in vivo or in vitro (112), or from the fraction of newly formed protein which turns over very rapidly after its formation (194). All three methods suggest that up to 10 to 20% of translational products end prematurely. The fraction of these incomplete proteins that result from translation of partially degraded mRNA species is unknown. However, these estimates place an upper limit on the percentage of ribosomes which become blocked on cleaved mRNA molecules.

The translational yield of mRNAs in *E. coli* is 20 to 30 peptides per mRNA, on average (90, 108). This implies that up to six ribosomes would be caught on an average truncated mRNA if the 20% estimate (see above) were correct. If all mRNAs were initially inactivated at their 3' terminus (by a 3'→5' exonuclease), then all of the ribosomes loaded on each mRNA at the time of inactivation would release incomplete peptides. Most mRNAs fully loaded with ribosomes and especially longer mRNAs are expected to have more than six ribosomes bound at a given time (90). Therefore, it seems unlikely that initial inactivation occurs only at the 3' terminus. The argument becomes stronger if a large number of abortive translations are due to misincorporation of amino acids or attenuation rather than to mRNA fragmentation. If this were the case, initial mRNA inactivation would necessarily occur near the 5' terminus.

A tacit assumption of the overall 5'→3' decay mechanisms is that 3'→5' exonucleolytic decay beginning at the 3' terminus is slow relative to endonuclease cleavages. Secondary structural features at 3' termini could serve as a barrier to an exonuclease. For example, the major site for transcription termination in the trp operon is at *trp* t', but most of the

transcripts have 3′ termini at a more proximal site (*trp* t) at a hairpin structure. This major 3′ terminus is thought to be generated as a point at which 3′ exonuclease cleavage which starts at *trp* t′ is arrested. This limited degradation from *trp* t′ to *trp* t can be produced in vitro by RNase II action.

An interesting possibility is that REP sequences could serve as barriers to 3′ decay. These highly conserved sequence elements are found at the ends of operons and in intercistronic regions in many operons. They often are present as inverted repeats capable of forming long double-stranded stems (70). They have been shown not to be sites for RNase III cleavage, so that it is unlikely they have a function similar to the *sib* site in λ retroregulation (176). They also do not appear to act as terminators, as the expression of distal genes is little affected by the insertion of a REP sequence proximal to them. Recent work suggests that deletion of REP sequences decreases the half-life of mRNA cistrons located 5′ to the sequence (C. F. Higgins, personal communication). This result is consistent with REP sequences serving as barriers to 3′→5′ exonuclease degradation (as appears to occur in the *trp* operon). These highly conserved sequences may serve other roles as well.

There is some evidence that double-stranded RNase III recognition sites may protect mRNA from 3′→5′ exonucleolytic attack. As mentioned above, when the R1 RNase III site from bacteriophage T7 is cloned distal to a eucaryotic gene expressed in *E. coli*, the functional and chemical half-lives of the mRNA are increased (142). This result indicates that the placement of this RNase III site distal to the structural gene in an mRNA increases its half-life. The question of whether RNase III cleavage at this site affects the half-life of the mRNA has not been addressed, however, since this cleavage occurred very efficiently in vivo. (Even in an RNase III-deficient strain, processing occurred to a considerable extent.) The portion of the transcript 3′ to the RNase III cleavage site was found to decay more rapidly than the portion 5′ to it, but this result does not bear on the question of whether RNase III cleavage affects the stability of proximal sequences.

Failure of processing at this cleavage site during T7 infection causes decreased expression of the gene immediately distal (see above). The mechanism by which this "transplanted" RNase III site functions to stabilize sequences proximal to it is probably unrelated to its function during T7 infection (since it then controls a gene distal to it instead of one proximal to it). A direct test of the effect of RNase III cleavage on the stability of proximal sequences requires the construction of a mutant RNase III site which retains the capacity to form a hairpin but blocks RNase III processing.

Another example of sequences located 3′ to a structural gene controlling its stability is found in φX174. In bacteriophage φX174, mutations in a region just proximal to the terminator TJ cause destabilization of the polycistronic mRNA terminated at this point (66). The half-life of this mRNA is reduced from 10 (in wild-type phage) to 1 to 2 min by insertion or deletion mutations in this region. Both wild-type and mutant forms of this sequence element have been cloned into the 3′ region of φX174 gene B or D in a pBR322 vector. When these plasmids were expressed in *E. coli*, the wild-type sequence element yielded a stable mRNA, while the mutant sequence elements led to a relatively unstable mRNA species, implying that the effect of this element on mRNA half-life is independent of other φX174-encoded activities. The mechanism by which this sequence element stabilizes cistrons located 5′ to it is unknown. A potential 6-base-pair helix is present in the wild-type sequence element, but disruption of this helix by mutation does not always abolish its stabilizing effect.

The functional as well as the chemical half-life of these mRNA species is increased by this φX174 sequence element; in other words, the protein yield of each mRNA is increased. The functional half-life of the stabilized mRNA species is still less (5 to 6 min) than their chemical half-life (8 to 10 min). It is not known if other φX174 transcripts contain similar 3′-sequence elements.

Clearly then, there is precedent for sequences located either proximal or distal to a cistron which modulate its half-life. These need not be mutually exclusive possibilities, and conceivably both could operate upon a single cistron.

The identity of endonucleases involved in mRNA functional inactivation or turnover is uncertain. RNase III is implicated in several systems and its role in mRNA metabolism may be more general than is currently appreciated. RNase III mutants (which retain some RNase III activity) show altered expression of many different proteins (55). This could result from altered mRNA metabolism or from the abnormal 50S ribosomal subunits present in these strains (see above). No other endonucleases are clearly involved in message metabolism, although a number of partially purified activities could have a role in functional or chemical decay or both (Table 1). In particular, RNase N has been speculated to play a role in mRNA metabolism (122).

Experimental evidence implicates RNase II and polynucleotide phosphorylase as the major exonucleolytic activities involved in mRNA chemical decay. Temperature-sensitive mutations in both of these activities slow chemical decay of mRNA species at the nonpermissive temperature (43, 94). In one study functional decay was slowed as well (94), but in another the cells showed little effect until long after temperature shift, suggesting that functional decay was not greatly affected (although it was not specifically assessed [43]). Other exonucleases may participate in mRNA decay in *E. coli*, but their roles would appear minor compared with RNase II and polynucleotide phosphorylase. Mutants deficient in one or the other of these enzymes do not show any obvious changes in mRNA metabolism, suggesting that the two are largely complementary (43).

Small RNA Processing and Turnover

In general, little is known about the processing or turnover of small RNAs in *E. coli*. A number of them (MIC RNA and spot 42 RNA) require no processing, as the primary transcript is identical to the mature species. 6S RNA requires post-transcriptional processing, but the activities involved are not known and the rates of reaction have not been measured. M1 RNA processing is blocked in an RNase E temperature-sensitive mutant, but purified RNase E does not cleave the M1 RNA precursor (64), leaving its mechanism of processing in doubt as well. A 4.5S RNA precursor accumulates in an RNase P-deficient mutant and p4.5S RNA is processed by RNase P at the base of its long double-stranded stem (18). *divE* and *dnaY* are presumably processed, as are other tRNA species.

RI RNA can apparently be processed by RNase E both in vivo and in vitro (181). This cleavage removes the first five nucleotides from the 5′ end of the RNA. Removal of these same nucleotides by RNase A has been shown to decrease binding of RI RNA to primer RNA, thus diminishing the inhibitory effect of RI RNA on ColE1 DNA replication (182). This nuclease may then play a role in the maintenance of

plasmid copy number in ColE1 and related plasmids. ColE1 DNA replication is defective in an RNase III-deficient host, and it has been suggested that RNase III may play a role in RI RNA processing as well (28).

As far as turnover is concerned, M1 RNA, 6S RNA, and 4.5S RNA are stable (though M1 RNA with a particular point mutation has a much decreased half-life [158]). *divE* and *dnaY* are probably stable (as they are tRNAs), but no experimental information is available. RI RNA is unstable but accumulates in an RNase E-deficient mutant, suggesting that RNase E action is involved in its degradation (80). MIC RNA is presumably unstable, as its role is clearly regulatory, but no information is available concerning its fate. Spot 42 RNA has a half-life of about 20 min at 37°C. The pathway and enzymes responsible for its degradation are unknown.

The mechanism by which RNA primers for DNA replication are removed in *E. coli* is uncertain. RNase H could remove all but the last nucleotide of each primer but cannot cleave the bond between RNA and DNA (34). DNA polymerase I is capable of degrading RNA as well as DNA, making it a candidate for this role. The activity or activities responsible for removing these ribonucleotides have not been defined, however (98). More information is available about the excision of primer RNAs in *B. subtilis*. These primers appear to be sequentially (exonucleolytically) removed, since the 5′ residue changes from adenosine to guanosine (see above) as Okazaki fragments increase in length (8). Removal of the RNA primers is quite rapid in *B. subtilis* (80% of Okazaki fragments 40 nucleotides in length are ribosubstituted, but essentially no fragments 200 nucleotides in length are ribosubstituted) (8), but the enzymatic activities involved have not been identified.

RNA fragments derived from the processing of rRNA and tRNA are rapidly degraded, most likely by RNase II and polynucleotide phosphorylase. The fragments with extensive secondary structure (e.g., the RNase III stems flanking 16S and 23S rRNA) may be longer-lived, requiring other enzymes for their degradation. RNase III is capable of hydrolyzing double-stranded RNA to form oligonucleotides under some conditions (33, 101) and may contribute to the degradation of the double-stranded stems removed from precursor RNAs (150).

TABLE 2. Some kinetic constants of nucleases

Enzyme	Substrate	K_m (μM)	kcat[a] (per min)	Reference
RNase A[b]	CpA		3,000	
	CpG	5×10^{-6}	500	
	UpA		1,200	
	UpC		40	
RNase P M1 RNA	pTyr tRNA	5×10^{-7}	2	62
		5×10^{-7}	1	
RNase M5 α protein	p5S	0.3×10^{-7}	3.4	140
		1.5×10^{-7}	0.4	
L-19 IVS RNA[c]	pC_5	42×10^{-6}	2	198

[a] Absolute catalytic rate constant (turnover number).
[b] Data for RNase A are for step 1 of its action (cleavage and cyclization), as summarized by F. M. Richards, and H. W. Wyckoff (in *The Enzymes*, vol. 4, p. 647–806, 1971).
[c] Self-splicing rRNA intron from *Tetrahymena* spp. (24).

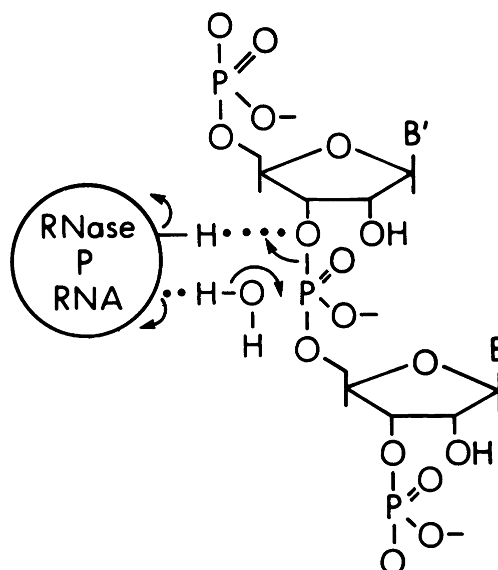

FIG. 5. Phosphodiester bond to be cleaved by RNase P RNA, indicating the electron shifts that must be promoted by the RNA catalyst (114).

Efficiency of RNA Processing Enzymes Made of Protein and RNA

No processing enzyme has been studied with the decades of biochemical mastery lavished on pancreatic RNase. Nevertheless, it is possible to compare some features of the mechanism and efficacy of sample processing enzymes with the RNase A model.

The initial cleavage by RNase A forms a 5′ OH and a cyclic 2′,3′-phosphate (Table 2). Presumably, in processing reactions as well as in hydrolysis by RNase A or base, the attacking nucleophilic group enters a face of the tetrahedral phosphate moiety at an apex, forming a pentocovalent intermediate. A leaving group is subsequently expelled from an apical position. Although the precise geometry of any processing event is simply unknown at present, consideration of the different mechanisms by which the intramolecular bonds are labilized is fruitful.

One formulation of events during RNase A action includes removal of a 2′ OH proton by a basic imidazole residue, protonation of 5′ O of the leaving nucleoside (perhaps by an acidic function on another amino acid), and attack by the 2′-alkoxide to yield the cyclic phosphate. Similar considerations apply for processing cleavages. For example, RNase M5 or RNase P must labilize bonds as indicated in Fig. 5. The major difference is of course that in the one case the catalytic labilization of bonds can be ascribed to protein histidines (analogous to RNase A), but with RNase P, labilization must be managed by RNA.

Even in the case of RNA-catalyzed cleavages of RNA, more than one mechanism is now known. Zaug and Cech have shown that, during the action of the L-19 intervening sequence (IVS) RNA from *Tetrahymena* spp., shorter and longer oligocytidylate chains can be formed starting from pentacytidylate, in a series of transesterification reactions (198). The reactions are initiated by an attack of the 3′-hydroxyl of the 3′-terminal guanosine on a phosphodiester bond within an oligocytidylate base paired to an internal

sequence in the catalytic RNA. In this case, the bond between the particular active G residue and an adjacent nucleotide can be 10^{10} more sensitive to base hydrolysis than any other internucleotide bond in the catalytic RNA, a lability that must be conferred by as yet undefined interactions.

In the case of 5′ maturation of pre-tRNA by the M1 RNA of RNase P, the mechanism is quite different, because periodate-oxidized M1 RNA (which lacks a terminal hydroxyl group) is still catalytically active (114). The pH dependence of this reaction shows an optimum at 9 to 8.5. Since there are no reported pK values for nucleic acid groups in this range, the actions (Fig. 5) analogous to those carried out by histidines in proteins or a 3′ OH group in the L-19 IVS RNA must be performed by nucleoside base tautomers, by nonterminal 2′ OH groups, or by an internucleotide bond with aberrant pK values (possibly abetted in some way by base pairing or Mg^{2+} chelation complexes).

Whatever the sources of catalytic action prove to be in various enzymes, Table 2 indicates that both RNA and protein processing enzymes work about equally well. One can compare the action of RNase A; RNase P and its M1 RNA catalytic component; RNase M5 and its alpha protein catalytic component; and L-19 IVS RNA with the indicated substrates. As tabulated, K_m values are comparable for all of these types of enzyme. RNase A shows very much greater turnover numbers; however, this may be due to its less stringent specificity. As one might anticipate, more site-specific nucleases tend to act more slowly. (Consistent with this notion, the DNA restriction enzyme *Eco*RI shows a turnover number in the range of RNA processing enzymes [see note 26 in reference 198].)

FUNCTION OF RNA PROCESSING

The need for processing is clear from some RNA species, since many precursor species are not functional. But in many cases, the need for a precursor RNA, and a fortiori the function of processing, is less obvious. Similarly, the function of turnover is relatively clear for mRNA but could subserve multiple functions or be an undesirable side effect for other RNA species.

tRNA

The function of tRNA processing is clear, as unprocessed tRNA precursors cannot function in protein synthesis. It seems likely that the existence of extra sequences on nascent tRNAs in part reflects a limitation of the transcriptional apparatus, as in no case does a tRNA primary transcript begin or end at the tRNA's mature termini. Since mature tRNAs have only a single known secondary structure which is easily recovered after thermal denaturation, it is unlikely that precursor sequences have any role in determining this structure. The functional significance of cotranscribed mRNA genes found in three tRNA operons is also uncertain, though it provides a way to control the relative abundance of the cotranscribed species. Whether tRNA processing serves other functions is less clear (see below).

rRNA

As with tRNAs, transcription of a mature rRNA species as a primary transcript may not be possible with the *E. coli* transcriptional apparatus. However, not all and possibly very few of the rRNA processing reactions observed are required for the resultant rRNA species to function. Other functions of processing for rRNA and possibly for other stable RNAs must be postulated.

Usually, the existence of signals for cleavages, methylation, etc., in extra sequences has been invoked as a rationalization for processing, and it has even been suggested, for example, that ribose methylation of pre-rRNA may function by providing signals for processing cleavages! Rather than arguing in a circle that extra sequences are formed so that they can catalyze their own removal, we have argued instead that extra transcribed sequences are in themselves important and that processing removes the extra sequences after a mature conformation is adopted (92).

At least eight kinds of functions for transcribed spacers and processing can then be envisaged.

(i) *Equimolar synthesis of rRNA species.* Extra sequences join rRNA species, permitting their cotranscription. The organization of *rrn* gene transcripts ensures that equal quantities of the 5S, 16S, and 23S rRNA species found in 70S ribosomes are formed. This argument is suggestive but not compelling, since many proteins are formed in equimolar amounts from different promoters, many tRNAs are formed in monocistronic or polycistronic transcripts without any obvious relationship to cell physiology (49), and polarity effects might still act to produce unequal amounts of 16S and 23S rRNAs. Nevertheless, the argument has a force similar to that for the existence of polycistronic mRNAs for many biosynthetic operons, and the cotranscription of certain structural RNAs with specific mRNA species suggests the possibility of a functional relationship between them (see below).

(ii) *Signals for regulatory events.* The extra sequences in RNAs may have special metabolic roles. It has been suggested that the various 23S rRNA termini found in *E. coli* are modulated under different growth conditions and may affect ribosome function (165).

(iii) *Promotion of secondary structure formation.* Any biological role of transcribed spacer sequences must occur during ribosome formation, since they are eliminated as ribosomes are formed. In fact, pre-rRNA seems to form ribosomal particles in vitro more easily than does mature rRNA (133, 191). Spacer sequences could facilitate ribosome formation by promoting alternate conformational features in rRNA. In particular, the structure of 16S rRNA in the 30S pre-rRNA contains several very large loops that may preclude shorter-range base-paired stems known to exist in the secondary structure of mature rRNA (97). Formation of 30S ribosomes from mature 16S rRNA in vitro has been found to require a greater activation energy than is apparent in intact cells (184), and alternate conformation features in pre-rRNA may allow a more favorable kinetic route. Long-range interactions might be favored by the base-paired stems, which act as a kind of clamp on conformation, increasing the proximity of otherwise distant sequences. Whether this speculation is valid or not, the importance of the double-stranded stem at the base of p16S rRNA has been strongly supported by recent findings that tracts of nucleotides with a different sequence but comparable complementarity are found in pre-rRNA sequences of *B. subtilis* and an archaebacterium (Fig. 3). Evolutionary conservation of the capacity to form such a stem implies that it has a function in the pre-rRNA.

Conversely, a short range interactions involving precursor sequences may sometimes preclude undesirable long-range interactions, as in *B. subtilis* 5S rRNA (172). If a p5S rRNA

species is prepared lacking precursor residues −20 to −12, then the 5S rRNA species adopts an abnormal conformation due to base pairing between nucleotides −12 to −6 and 95 to 101 (solid boxes, Fig. 4) (172). In the full-length precursor species, base pairing to nucleotides −21 to −16 apparently competes for the binding of the −11 to −6 region and prevents the abnormal association between mature and precursor sequences. The prevention of potentially undesirable long-range interactions by short-range interactions could be a general feature of RNA structure and might help to explain the existence of some precursor structures.

(iv) *Verification of functionality: quality control.* The signals for processing steps can be very complex; especially for the final steps, the demands on secondary and tertiary structure of rRNA and tRNA can be very exacting (see above). Thus, processing both verifies that a newly forming ribosome is developing normally and ensures that the final structure will be rigorously standardized. For example, single point mutations in 16S rRNA can prevent the correct processing of the RNA to its fully functional form (175). If either conformation or sequence is aberrant, processing tends to fail and prevents a distorted precursor from becoming an aberrant mature RNA chain.

(v) *Time of production: quantity control.* Theoretically, the slowing of processing steps could lead to the accumulation of precursors of an RNA species, reducing the mature fraction of RNA for that species. In this way, processing could regulate the rate of production of individual types of RNA. Although the rates of different processing reactions are not precisely coordinated in cells growing at different rates (92), the fraction of any RNA species in the form of precursors is usually relatively small (<10%). This observation suggests that the rate of processing could subserve a regulatory function.

(vi) *Integration of cellular processes.* Ordinarily, every kinetic process in a cell is arranged so that particular steps are required before others can occur, but in a number of cases, the blockage of later steps leads to a feedback regulation of earlier steps. An example is the inhibition of rRNA and tRNA syntheses when protein synthesis is blocked. The final processing of rRNA may be analogous in that it occurs in polysomes (65, 109, 196) and may require active protein synthesis (23, 166). At present it is not clear how extensive the coupling of protein synthesis and rRNA processing may be, but it is conceivable that the rate of one can directly influence the rate of the other.

(vii) *Instability and turnover.* Because *E. coli* contains enzymes capable of catalyzing the turnover of nearly any cellular RNA in vitro (50), it is clear that special measures are required to render some species of RNA metabolically stable. Mutations in tRNA structure which labilize the molecule in vivo (169) demonstrate that the intact normal secondary structure is required for stability. It seems likely that, during transcription and further biosynthetic reactions, extra transcribed sequences might help to prevent degradation of unfinished RNA species until their final resistant form is attained.

(viii) *Promote dissociation of mature and precursor sequences after processing.* In *B. subtilis*, pre-5S rRNA precursor sequences −21 to −11 (dashed box, Fig. 4) form part of the RNase III stem at the base of 23S rRNA, in the complete pre-rRNA. The base pairing of these sequences with precursor sequences −11 to −6 has been suggested to promote the dissociation of precursor sequences from mature sequences after processing reactions have been completed (171).

mRNA

Although processing of mRNA, including splicing, clearly has a regulatory function in mammalian cells (where it determines the rate of production of a species and can even produce more than one mRNA from a single transcript by alternative pathways), similar events in bacteria are rare. Again, cotranscription of related cistrons as polycistronic mRNAs requires the existence of spacer sequences. Signals for attenuation and leader peptide production are other possible functions of spacer sequences (see above). However, the metabolism of mRNA is dominated not by processing reactions which lead to its maturation, but by processing reactions which lead to its degradation.

The chief reasons for mRNA instability are, first, the rapid control of the protein synthetic repertoire of cells, so that shifts in response to changing metabolic needs can be rapid and decisive; and second, the differential translation yield from different mRNA species, so that the balance of the rates of ribosome initiation and translation with the rates of mRNA inactivation and degradation determine just how much of a given protein is made from a certain amount of the transcribed mRNA (134).

In the metabolism of unstable mRNA, extra transcribed sequences can be involved in several ways which have already been mentioned: the provision of structural blocks to nuclease action or sites of nuclease attack. How important such sites are in translated compared to untranslated sequences has not been determined, but it seems likely that, as in the lambda *sib* and phage T4 gene 32 cases, sites in untranslated sequences will be quite important.

Many alterations within the coding or precursor sequences of an mRNA (at both its 5′ and 3′ ends [10]) have been demonstrated to affect greatly the stability of that species, and the strong inference from these state-of-the-art studies is that one or more such sequences are critical in determining the stability of the wild-type mRNA molecule. However, systematic deletions or insertions in hybrid mRNAs which modify their half-life drastically may not indicate what is most critical for the stability of the native species in vivo, precisely because they have changed the structure of the mRNA. An alteration may expose the hybrid species to a type of decay or inactivation event which is relatively unimportant for the wild-type species in vivo, or it may protect it from a usually critical event. This approach will therefore become even more valuable when it is combined with the use of mutants deficient in particular degradative enzymes (e.g., RNase II and polynucleotide phosphorylase [43]). That should allow dissection of the effects of a particular sequence alteration. For instance, does a particular deletion destabilize an mRNA species because of an effect on susceptibility (i) to a 3′ to 5′ exonuclease; (ii) to a specific endonuclease; (iii) to a specific binding protein; or (iv) to ribosome binding or translation. Furthermore, is that type of inactivation event important for the wild-type species in vivo?

Small RNAs

As the number of small RNAs with defined function increases, their stability properties and any functions of processing should become clearer. For example, it is obvious that the MIC RNA must be metabolically unstable; but for M1 RNA, only speculative possibilities (similar to some of those dicussed above for tRNA) are available to guess at possible functions for extra transcribed sequences or proc-

essing steps. The role of cotranscribed open reading frames found in a number of these RNA species is also speculative.

CONCLUSION

It now seems increasingly likely that evolution initially developed extra transcribed sequences as important functional features. Such sequences can promote particular conformations or help to protect an incomplete RNA chain until it is in its final form. According to this view, processing reactions were developed later, to remove extra sequences and generate an RNA molecule in its final mature form. The processing cleavages and trimming might then be analogous to modifications such as methylation: fine-tuning reactions to improve the performance of an already partially functional RNA (as in the case of the 23S pre-rRNA). In turn, the processing steps become a series of checkpoints to verify that the assembly of an RNA or RNA-protein complex has proceeded correctly before the final production of the maximally active cellular species. It is important to distinguish function from stability: some structural RNAs can be unstable, and some mRNAs can be long-lived. But in each case, the structure of an RNA transcript serves as a blueprint for its own maturation and, where appropriate, for its own turnover.

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