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Nucleotide sequence surrounding a ribonuclease III processing site in bacteriophage T7 RNA

(intercistronic region/polycistronic mRNA precursor/hairpin structure/endoribonuclease III)

MARTIN ROSENBERG* AND RICHARD A. KRAMER† ‡

* Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014; and † Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

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ABSTRACT **We have determined a nucleotide sequence of 87 residues surrounding a ribonuclease III (endoribonuclease III; EC 3.1.4.24) processing site in the bacteriophage T7 intercistronic region between early genes *0.3* and *0.7*. The structural requirements necessary for proper recognition and cleavage by RNase III are discussed. In addition, other structural features characteristic of this intercistronic boundary are described.**

When bacteriophage T7 infects *Escherichia coli*, the host RNA polymerase (RNA nucleotidyltransferase, EC 2.7.7.6) transcribes only the early region of the phage genome (i.e., leftmost 20%, Fig. 1) (2–4). Transcription initiates at three closely spaced sites near the left end of the DNA and terminates predominantly at a single site at the distal end of the early region (4–6). The resulting transcript is a high-molecular-weight (2.2×10^6), polycistronic mRNA precursor which is subsequently cleaved at specific sites into individual monocistronic mRNAs by ribonuclease III (RNase III; endoribonuclease III; EC 3.1.4.24) a host-specified endonuclease (7, 8). RNase III is known to function in *E. coli* as a "processing" enzyme involved in the normal maturation of the primary ribosomal RNA transcription products (8, 9).

The individual T7 early RNAs can be either isolated directly from T7-infected cells or synthesized *in vitro* by transcription of T7 DNA with *E. coli* RNA polymerase and digestion of the RNA products with purified RNase III (3–5, 10). We have previously examined sequences at the 5′ and 3′ termini of the early mRNA species obtained in both ways (11–13). Our results indicated that the *in vivo* mRNAs are the direct products of RNase III cleavage. In addition, we found that the processed RNAs have identical 3′-terminal sequences (C-C-U-U-U-A-U_{OH}) as well as identical 5′-terminal sequences (pG-A-U). This conserved structure at the various T7 cleavage sites suggested that sequence specificity might be important to the processing event. Moreover, the fact that RNase III is known to exhibit a general specificity for double-stranded RNA (although it degrades this RNA rather non-specifically) (14–16) suggests that single-stranded RNAs that serve as natural substrates for RNase III might contain specific double-stranded regions that are recognized by the enzyme.

To elucidate further the action of RNase III in processing natural substrates, we have isolated and characterized several overlapping RNA fragments that span the intercistronic region between the T7 early genes *0.3* and *0.7* (1). Preliminary analysis of these fragments indicated that: (*i*) RNase III produces a single endonucleolytic break between the 3′ end of the gene *0.3* and the 5′ end of the gene *0.7* mRNAs, (*ii*) some fragments that contain the RNase III cleavage site are not recognized and cleaved by the enzyme; and (*iii*) the 3′-terminal oligoadenylate sequences found on the ends of the *in vivo* T7 early mRNAs (11) are not encoded by the genome, but presumably represent a nontemplate-dependent post-transcriptional modification. Here, we report the complete nucleotide sequence of the gene *0.3–0.7* intercistronic region of T7 and propose a specific role for RNA secondary structure in substrate recognition and action of RNase III.

Abbreviations: RNase, ribonuclease; *0.3* mRNA, *0.7* mRNA, *1.0* mRNA, and *1.1* mRNA, the messenger RNAs of bacteriophage T7 for the protein products of genes *0.3*, *0.7*, *1.0*, and *1.1*, respectively; ∇, deletion mutant.

‡ Present address: Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Md. 20014.

MATERIALS AND METHODS

Preparation of Polycistronic T7 Early RNA. RNA was synthesized *in vitro* from T7 DNA as previously described (12). Fragments of this transcript that spanned the gene *0.3–0.7* intercistronic boundary were isolated by a two-step hybridization procedure (outlined in Fig. 1) using DNA obtained from appropriate deletion mutants of T7. The procedure and preliminary characterization of the RNA fragments obtained have been described in detail elsewhere (1). Deletion phage strains C116, C114, C74, and H3 (17) were provided by F. W. Studier.

Fingerprint Analysis. Digests of the RNA fragments were prepared with T_1 and pancreatic ribonucleases and analyzed by standard RNA sequencing techniques (18, 19). The procedures and the results of these analyses have been previously published (1).

Partial Enzymatic Digestion of the RNA Fragments: Separation and Characterization of Large Oligonucleotide Products. Limited enzymatic digestion of the RNA fragments with either T_1 or pancreatic RNase was carried out in 0.01 M Tris·HCl (pH 7.5), 0.02 M $MgCl_2$, at 4° for 20–40 min; enzyme-to-substrate ratios varied from 1:250 to 1:1000. The products were then fractionated by standard two-dimensional techniques; electrophoresis on Cellogel (Kalex) in 8.0 M urea at pH 3.5 in the first dimension and homochromatography on thin-layer plates of DEAE-cellulose (Analtech; 9:1, cellulose: DEAE-cellulose; 40 × 20 cm) in the second. Homochromatography solutions (18, 19) were used which effectively separated oligomers up to 45 residues in chain length. Alternatively, partial digestion products were separated by electrophoresis on 15% polyacrylamide slab gels containing 8.0 M urea using Tris–borate, pH 8.3, as running buffer (20); bands were located autoradiographically and the samples were eluted as previously described (11).

All partial products were further characterized by complete digestion with the appropriate enzymes and fractionation in either one or two dimensions by standard electrophoretic and/or chromatographic techniques (18, 19, 21).

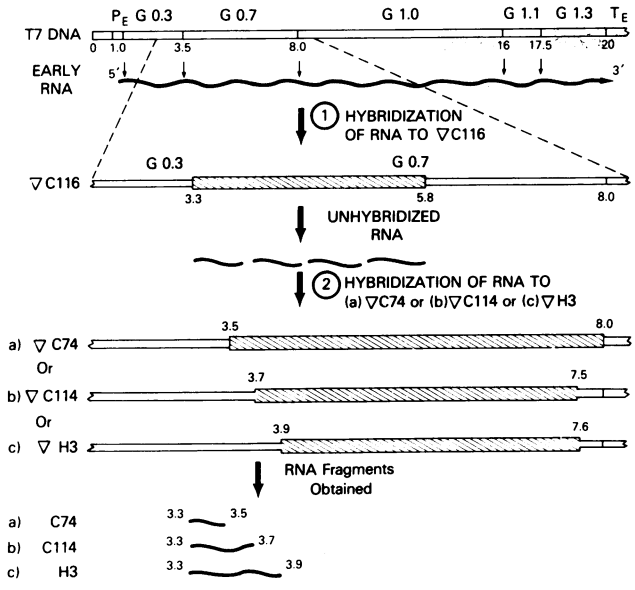

FIG. 1. Diagrammatic outline of the procedure used to isolate RNA fragments containing an RNase III cleavage site from the gene *0.3–0.7* intercistronic boundary of the T7 early RNA precursor. RNA is synthesized *in vitro* from wild-type T7 DNA and hybridized on filters to the DNA from T7 deletion mutant C116. Under appropriate hybridization conditions (1) the region of the transcript that corresponds to the deleted segment of DNA does not hybridize to the filter and remains in the supernatant portion of the hybridization mixture (step 1: unhybridized RNA). This RNA is then hybridized to DNA from one of the T7 deletion mutants C74, C114, or H3 (step 2). Only RNA derived from the leftmost segment of the C116 deletion will hybridize to these deletions. The filter-bound hybrids are trimmed with ribonuclease (to remove unhybridized "tails") and subsequently eluted from the DNA. The RNA fragments obtained span the gene *0.3–0.7* boundary and contain a single RNase III cleavage site. All distances given are in T7 map units: 1 map unit = 1% of T7 genome = 380 base pairs. P_E, region of initiation for T7 early transcription; T_E, site of termination of T7 early transcription; ↓, an RNase III cleavage site; ∇, deletion mutant of T7.

RESULTS

Deletion Mapping of Oligonucleotides from the Gene *0.3–0.7* Intercistronic Region. We previously described the isolation and characterization of RNA fragments derived from the gene *0.3–0.7* boundary of the T7 early RNA precursor (1). These RNAs were obtained by a two-step hybridization procedure in which DNAs from appropriate T7 deletion mutants were used to define the approximate end-points of the fragments (see Fig. 1). Thus, three distinct, successively larger, overlapping regions of RNA between T7 map positions 3.3 and 3.5, 3.3 and 3.7, and 3.3 and 3.9 were isolated. The RNAs obtained were digested with T_1 and pancreatic RNase and the oligonucleotide products were characterized by standard sequencing techniques (18, 19, 21). Comparison of the two-dimensional fingerprints of the three sets of RNA fragments indicated that groups of oligonucleotides could be assigned specific relative positions within the nucleotide sequence of this region. Oligonucleotides common to all three sets of fragments must be located in the 3′-terminal region of the gene *0.3* mRNA, between T7 map positions 3.3 and 3.5. Oligonucleotides unique to the larger fragments must represent sequences to the right (i.e., 3′ side) of the common sequences, sequences positioned in the 5′-terminal region of gene *0.7* mRNA between T7 map positions 3.5 and 3.7 and 3.7 and 3.9, respectively. Although certain small products that recur in the sequence cannot be accurately located, the majority of oligonucleotides were positioned in this manner. The data are summarized in Table 1 and Fig. 2.

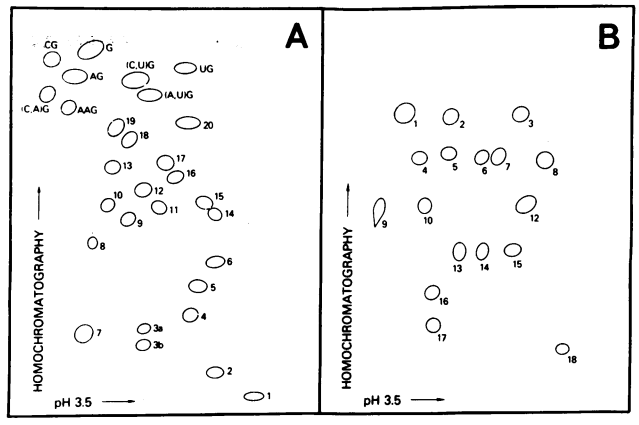

FIG. 2. Composite sketches of the two-dimensional fingerprints obtained from T_1 (*A*) and pancreatic (*B*) RNase digestion of the isolated fragments. The assignment of particular oligonucleotides to specific regions of the template is indicated in Table 1. For comparison with the autoradiographs of these fingerprints, see Kramer and Rosenberg (1).

Determination of the Complete Sequence; Oligonucleotide Overlaps. Partial T_1 and pancreatic RNase digestions were carried out on the three sets of RNA fragments and the products were fractionated and analyzed as described in *Materials and Methods*. The results (Fig. 3) are consistent with the deletion mapping data presented above and allow deduction of an unambiguous sequence of 87 residues. Partial digests were also performed on each of the three sets of RNA fragments after initial treatment with purified RNase III (12). Analyses of the products confirmed the sequence shown in Fig. 3 and support our earlier conclusions that (*i*) RNase III produces a single endonucleolytic break in this region of the T7 mRNA and (*ii*) although all of the RNA fragments contain the site of RNase III cleavage, the smallest fragments (defined between T7 map positions 3.3 and 3.5) are not cleaved by the enzyme.

DISCUSSION

The primary structure of the bacteriophage T7 intercistronic region surrounding an RNase III processing site between genes *0.3* and *0.7* has been determined. Overlapping fragments of RNA spanning this region were isolated by hybridizing the high molecular weight early RNA transcript prepared *in vitro* to DNAs obtained from certain deletion mutants of T7. A unique sequence of 87 residues was deduced by analysis of partial T_1 and pancreatic RNase digestion products and by grouping oligonucleotides derived from the overlapping fragments into regions defined by the end-points of the T7 deletion mutants used in their isolation.

Secondary Structure. A possible secondary structure for the gene *0.3–0.7* intercistronic region is shown in Fig. 4. This structure maximizes the number of Watson–Crick base pairs and is consistent with the observed relative susceptibility of various residues within the sequence to endonucleases. Our results indicate that RNase III produces a single endonucleolytic cleavage within this structure (between positions −1 and +1, Fig. 4). Cleavage occurs on one side of an internal loop that separates two relatively stable base-paired regions of RNA. As observed both *in vivo* and *in vitro* (11, 12), the processing event gives rise directly to the 3′ end of the gene *0.3* mRNA (. . . C-C-U-U-U-A-U_{OH}, at position −1) and the 5′ end of gene *0.7* mRNA (pG-A-U . . . , at position +1).

Table 1. Deletion mapping of oligonucleotides from the genes *0.3–0.7* intercistronic region, and oligonucleotide sequences

Deletion group I[a] (between T7 map positions 3.3 and 3.5) oligonucleotides				Deletion group II[a] (between T7 map positions 3.5 and 3.7) oligonucleotides				Deletion group III[a] (between T7 map positions 3.7 and 3.9) oligonucleotides	
T_1		Pancreatic		T_1		Pancreatic		T_1	
No.[b]	Sequence[c]	No.[b]	Sequence[c]	No.[b]	Sequence[c]	No.[b]	Sequence[c]	No.[b]	Sequence[d]
6	CCUUUAUG[A]	1	GC[A,U]	1	AUUAUCACU-	5	AAU[G]	2	UUCCUAC-
7	A_4CAACAAG[C]	2	AU[G]		UUACUUAUG[A]	18	GAGGGAGU[A][e]		UUUACCG[C]
8	ACUCAAG[G]	4	AGC[G,U]	16	UAAUG[U]	2	AU[G,C]	3a	UCUACUCACCG[C]
9	CUCCUAG[C]	5	AAU[A]		AG[G]		AC[U]	3b	n.d.
10	CAAUAG[C]	6	GGC[C]		G[G]			4	CUUACUAUCG
11	AAUCUG[C]	7	GAU[A,U]					5	CAUCCUUG
13a	AACUG[U]	8	GGU[C]					12	CUCUG
13b	CAAUG[G]	9	AAC[A]					14	UAUAUG
15	CUUUAG[A]	10	AGAC[U]					17	UCUAG
18	AUAG[A]	12	GAGU[G]					20	CUUG
19	UACG[A]	13	AAGGC[A]						(C,U)G
	AUG[G]	14	AGAAU[C]						AG
	(C,U)G[A,C]	15	AAGGU[C]						G
	AG[U]	16	GA_4C[A]						
	UG[A,C]	17	A_3GAAC[U]						
	G[A,C,U]								

n.d., not determined.

[a] Groups of oligonucleotides were assigned to specific regions of the template defined by the left-hand end-points of certain T7 deletion mutants (see Fig. 1 and text for details). Group I, II, and III: Oligonucleotides positioned between the left-hand end-points of ▽C116 and ▽C74, ▽C74 and ▽C114, ▽C114 and ▽H3, respectively.

[b] No. refers to numerical designation used in Fig. 2.

[c] Sequences given are those deduced by Kramer and Rosenberg (1).

[d] Sequences given were deduced identically as those in c but from unpublished data. These oligonucleotides were not incorporated into the final sequence shown in Figs. 3 and 4.

[e] The sequence previously reported for pancreatic product 18 was ambiguous (i.e., G(A,G)G-G-A-G-U[A]). The correct sequence was deduced from the analysis of certain partial nuclease digestion products (see Fig. 3). Pancreatic product 18 follows and partially overlaps T_1 product 1. Since this T_1 product has nearest neighbor A, pancreatic product 18 must have the sequence *G-A*-G-G-G-A-G-U[A].

The proposed structure predicts that subsequent to RNase III cleavage the two mRNA species could remain non-covalently associated by means of the base-pairing interactions within the lower stem region (i.e., residues −33 to −41 of the *0.3* mRNA complementary to residues +3 to +11 of the *0.7* mRNA). Indeed, under the conditions employed for partial nuclease digestion (4°, 0.02 M Mg^{2+}) residues in the lower stem were resistant to endonuclease both prior and subsequent to cleavage of the RNA with RNase III. On the other hand, calculation of the thermal stability of this helical region (according to Gralla and Crothers, ref. 22) suggests that it should denature under physiological conditions, thereby giving rise to the individual *0.3* and *0.7* mRNA species observed in T7-infected cells.

Structure Required for RNase III Recognition and Cleavage. Limited information on the primary structure surrounding several different RNase III cleavage sites is now available (10, 24–27). Sites of one class (i.e., primary sites) occur in natural substrates of the enzyme and are readily cut under conditions identical to those that generate the T7 early mRNAs from the T7 early RNA precursor. Comparison of terminal nucleotide sequences from these primary sites [including the T7 early mRNAs (refs 12, and 27; this paper) and several RNA species derived from the ribosomal RNA precursor of *E. coli* (14)] indicates that variations in nucleotide sequence can occur around the site of cleavage.

Other RNase III cleavage sites that are poorly recognized by the enzyme under standard conditions have also been examined (25–28). Efficient cleavage at these "secondary" sites requires extremely low monovalent salt concentrations and/or excess enzyme. These conditions produce a variety of aberrant breaks in the T7 early mRNA precursor (28). Although some sequence

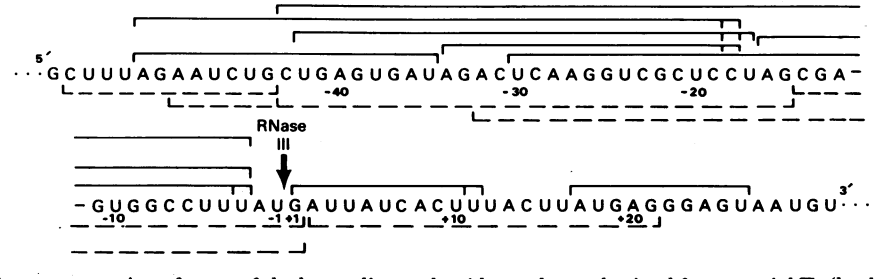

FIG. 3. Diagrammatic representation of some of the large oligonucleotide products obtained from partial T_1 (broken lines) and pancreatic (solid lines) ribonuclease digestion of the RNA fragments. The products indicated were present in various yields depending on digestion conditions (see *Materials and Methods*) and the relative susceptibility of sites to nucleases. The sequence shown was deduced from these data and the data presented in Table 1.

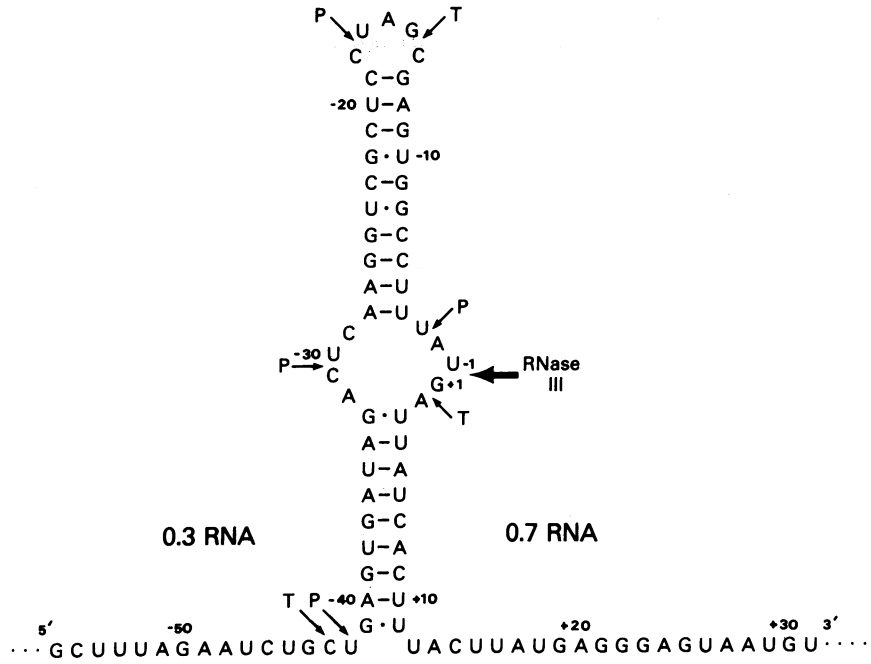

FIG. 4. The primary and possible secondary structure for the gene *0.3–0.7* intercistronic region of the T7 early RNA precursor. Residues are numbered from the site of RNase III cleavage: +1 to +31, 31 residues at the 5′ end of the *0.7* mRNA; −1 to −56, 56 residues of the 3′-terminal region of the *0.3* mRNA. Sites found to be quantitatively most susceptible to nuclease (T, T_1 RNase; P, pancreatic RNase) are indicated. The secondary structure suggested is based on both thermodynamic considerations (22, 23) and relative susceptibility of the structure to partial enzymatic cleavage.

similarities between primary and secondary sites can be noted, it remains unclear whether there are specific sequence elements that are absolutely required or of selective advantage to recognition and action by RNase III.

Robertson *et al.* (27) have determined the nucleotide sequence of a 29-residue RNA fragment (designated F5) that is released from the region between T7 genes *1.1* and *1.3* following *in vitro* RNase III cleavage of the T7 early RNA precursor (29). *In vivo*, as well as *in vitro* under standard cleavage conditions, a single scission predominates in this region (27, 29). The small fragment, however, can then be released from the 3′ end of the gene *1.1* mRNA species by cleavage at what is apparently a "secondary" site 29 residues 5′ to the "primary" cleavage site. Comparison of the sequence of F5 fragment with the 3′-terminal 29 nucleotides of the *0.3* mRNA (positions −1 to −29, Fig. 4) indicates a remarkable homology of structure. Twenty-five of the 29 residues are identical and a similar hairpin loop structure can be drawn. In both cases there are five unpaired residues in the hairpin loop (positions −14 to −18, Fig. 4) and cleavage occurs after a uridylate residue, 13 nucleotides 3′ to the top base pair in the stem structure. Although sequence homology between these two sites may simply reflect a common evolutionary origin (i.e., genetic duplication), the fact that the primary structures of these regions have been so strongly conserved indicates that the overall structure specified by this sequence is highly important to enzyme recognition and cleavage.

Although the hairpin structure discussed above is a common feature of the two T7 processing sites, it is probably not sufficient for RNase III action. The polycistronic early mRNA precursor from T7 deletion mutant ∇C74 (see Fig. 1) is not cleaved by RNase III at the 3′-end of gene *0.3 in vivo* (17). Instead, the 0.3 mRNA is found linked to the gene *1.0* mRNA species. This observation suggests that the deletion removes certain structural information within the *0.3–0.7* junction that is required for RNase III cleavage. (Alternatively, but less likely, that portion of the gene *1.0* mRNA which is brought into the proximity of this cleavage site could directly interfere with cleavage.) Our sequencing data indicate that the left-hand end point of the C74 deletion does not lie within gene *0.3;* RNA fragments isolated using this deletion contain the entire 3′-terminal region of the *0.3* mRNA and extend for several residues into the 5′ end of the *0.7* mRNA (Table 1). Thus, these fragments (and likewise the T7 ∇C74 early RNA precursor) should contain the hairpin structure defined above, as well as the actual site of RNase III cleavage. Nonetheless, neither these fragments nor the early RNA precursor of the deletion mutant is cleaved by RNase III.

A second T7 deletion, ∇C114, has its left-hand end point approximately thirty residues further from the *0.3* to *0.7* junction than ∇C74. The RNase III cleavage site in this mutant early RNA precursor is efficiently processed (17). Likewise, RNA fragments that were obtained using the C114 deletion, and thereby contain more of the 5′-terminal region of the *0.7* mRNA, were readily cleaved by RNase III (1). Thus, we conclude that sequence information in the 5′-terminal region of the *0.7* mRNA (between residues +3 and +31, Fig. 4) is necessary for proper enzyme recognition and cleavage. As shown in Fig. 4, part of this structure (residues +3 to +11) is complementary to sequences in the *0.3* mRNA (residues −33 to −41) and hence specifies a second stem structure located "below" the cutting site. A sequence of five nucleotides, G-A-G-U-G, occurs both in this stem (residues −41 to −37) and in an identical position and orientation in the upper stem structure (residues −13 to −9). The fact that RNase III is isolated as a dimeric protein consisting of identical subunits (28) suggests that the symmetric features of this duplex region of RNA may be used by the enzyme to achieve recognition and cleavage.

Potential Control Signals of Translation and Transcription. The region depicted in Fig. 4 may include the entire untranslated region between the gene *0.3* and *0.7* polypeptides. A number of translation termination triplets occur in the 3′-

terminal region of the *0.3* mRNA (at positions −52, −42, −38, and −35). Although there is no evidence to indicate which, if any, of these codons are functional, they do occur in all three reading frames. Thus, any ribosome translating the polycistronic early T7 mRNA must terminate protein synthesis prior to the site of RNase III cleavage and would not traverse the intercistronic boundary into gene *0.7*. Correspondingly, a potential ribosome binding site appears in the 5′-terminal region of the *0.7* mRNA. Recognition of mRNAs by *E. coli* ribosomes has been shown to involve certain purine-rich segments which are complementary (to various degrees) to the 3′ end of the 16S ribosomal RNA (30, 31). The stretch of purine residues between positions +19 and +25 in the *0.7* mRNA exhibits at least a four-base-pair complementarity and is followed at an appropriate distance by an AUG start codon (position +28). Again, there is as yet no direct evidence indicating that this site functions to initiate translation of a gene *0.7* polypeptide.

Several laboratories have demonstrated that transcriptional termination occurs to some extent in or near the various intercistronic boundaries of the T7 early region (32–35). Termination at these sites is thought to be mediated by the host protein factor rho, and results in a natural polarity of transcriptional expression of the T7 early genes. RNase III processing of resulting polycistronic transcripts would then give rise to the individual early mRNA species in their observed non-equimolar ratios (promoter-proximal mRNAs > promoter-distal mRNAs). More specifically, Hercules *et al.* (35) have suggested that the efficiency of termination between genes *0.3* and *0.7* is approximately 50% (i.e., one out of every two polymerases terminates at this boundary). However, it is not known exactly where in this region termination of transcription occurs, nor whether there is any correlation between the nucleic acid structures involved in cleavage by RNase III and those involved in the termination event. Recent studies on rho-dependent sites of transcriptional termination in bacteriophage λ indicate that certain stem and loop structures that appear at the 3′ end of the RNA transcript do play a role in the termination event (M. Rosenberg, unpublished data). Thus, the stem and loop structures in the T7 RNase III processing sites could also be implicated in specifying the weak transcriptional termination events, suggesting a structural relationship (perhaps evolutionary) between sites of transcriptional termination and those of post-transcriptional processing.

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