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3′-End Formation at the Phage λ tR1 ρ-Dependent Transcription Termination Site†

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ABSTRACT: The ρ-dependent transcription terminator tR1 of bacteriophage λ stops RNA synthesis downstream of the major rightward promoter, P_R, shortly after the *cro* gene. Terminated transcripts produced in a purified in vitro transcription system display a heterodisperse set of 3′ termini, occurring in clusters located at +290–300, 308–312, 340–345, 385–390, and 440–450 nucleotides from the transcription start site [Morgan, W. D., Bear, D. G., & von Hippel, P. H. (1983) *J. Biol. Chem. 258*, 9553–9564]. However, transcripts from the same promoter in vivo have been reported to end primarily at +310–312 [Court, D., Brady, C., Rosenberg, M., Wulff, D. L., Behr, M., Mahoney, M., & Izumi, S. (1980) *J. Mol. Biol. 138*, 231–254]. In order to understand the nature of this discrepancy, we have carried out a comparative analysis of λ P_R transcription products producd in translationally active S30 cell extracts, in a purified in vitro system and in vivo. RNAs from the cell extracts coupled to translation show primarily three P_R-derived transcripts beginning at one predominant 5′ end and terminating at +263, 308, and 318. Sites +263 and +308 appear to be RNA processing sites. S1 nuclease mapping studies of RNAs produced in vivo show one 5′ end and two 3′ termini ending at +263 and 311; the +263 site is the predominant 3′ end. When transcripts produced in a purified in vitro transcription system are incubated in the S30 cell extract under various conditions, the RNAs are degraded to two primary products with lengths of 263 and 308–311 nt. Processing of Cro transcripts to the 263- and 308-nt species is not observed in S30 extracts containing a defective RNase II enzyme and lacking polynucleotide phosphorylase activity. These studies indicate that the 3′ termini of λ P_R transcripts are formed by a combination of at least two processes: ρ-dependent transcription termination and RNA processing by 3′-exonucleolytic digestion.

It has become increasingly apparent that prokaryotic RNA transcripts generated in purified in vitro transcription reactions often differ from those found in vivo. The nature of these differences can be due to a variety of factors including in vitro transcription reaction conditions [nature and concentration of particular ionic species, nucleoside triphosphate (NTP)[1] concentrations, solution polarity], the presence of specific proteins such as the ρ, NusA, or τ proteins, and the coupling of transcription to translation (von Hippel et al., 1984; Chamberlain et al., 1985; Platt, 1986; Yager & von Hippel, 1987; Friedman et al., 1987; Bear & Peabody, 1988). In addition, numerous examples of prokaryotic posttranscriptional RNA processing reactions have been identified, including endo- and exonucleolytic cleavages (Mott et al., 1985; Deutscher, 1985; Platt, 1986; Belasco & Higgins, 1988), polyadenylation (Taljanidisz et al., 1987), and even splicing in selected phage transcripts (Chu et al., 1984).

Transcription from the phage λ P_R promoter directs the synthesis of the Cro protein, and terminates at a ρ-dependent site referred to as tR1 (Roberts, 1969), the most widely studied of all ρ-dependent terminators. Termination at tR1 in a purified in vitro transcription system yields transcripts mapping to at least five clusters of sites dispersed over a 150-nt region (Lau et al., 1982, 1983; Morgan et al., 1983a,b, 1984), which have been previously mapped to positions at approximately +290, 310, 340, 385, and 440 (Morgan et al., 1983a,b). However, only one major transcript has been previously found in vivo, with its terminus located at +310–312 (Court et al., 1980). It is known that NusA protein can eliminate termination at +290 in the purified in vitro transcription system (Lau et al., 1982). In addition, it is thought that termination at +340, 385, and 440 may be inhibited in vivo by concomitant translation of the cII protein whose coding region spans these termination sites (Morgan et al., 1983a).

In an attempt to understand the nature of the differences in Cro mRNA transcripts generated in the purified in vitro system and those in vivo, we have characterized the transcripts produced in translationally active and inactive cell extracts, and compared them to those made in vivo and in a purified system. Our rationale was to test whether translation, nucleolytic processing, or previously unidentified termination-inhibiting protein factors might be present in the cell extracts that could alter the transcript pattern from that observed in the purified reaction. From our studies, we find that transcripts produced in the extracts closely resemble those made in vivo. Translation coupled to transcription does not appear to significantly alter the pattern of 3′-end formation in the extracts. Although other unknown protein factors could play a role in 3′-end formation, we find that RNases process transcripts synthesized in the extracts to the species found in vivo. When the transcripts made in a purified in vitro transcription system are incubated in the extract, the in vivo sized

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[1] Abbreviations: bp, base pair; DTT, dithiothreitol; nt, nucleotide; NTP, nucleoside triphosphate; PNPase, polynucleotide phosphorylase; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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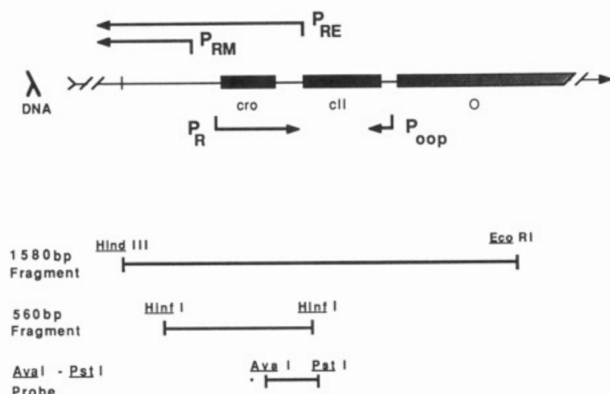

FIGURE 1: Restriction fragments containing P_R and tR1 of λ. The 1580 bp template, 560 bp fragment, and 191 bp S1 mapping probe used in this study are shown in the context of a partial genetic map of λ (Wulff & Rosenberg, 1983).

transcripts are generated. Evidence is presented that suggests that the 3′-exonucleases RNase II and/or polynucleotide phosphorylase (PNPase) are responsible for processing of the Cro transcripts. Our results are discussed in the context of other examples of processed ρ-dependent transcripts.

Materials and Methods

Enzymes, DNA Templates, and Strains. Escherichia coli ρ and RNA polymerase were isolated as described previously (Bear et al., 1985; Lowe et al., 1979).

The 1580 and 560 bp DNA restriction fragment templates (see Figure 1) were isolated from phage λ as described previously (Lau et al., 1982; Morgan et al., 1983a). To facilitate the procurement of large yields of the fragments, the templates were cloned into high copy number plasmids using standard techniques (Maniatis et al., 1982). The 1580 bp fragment was cloned into pUC18 (Yanisch-Perron et al., 1985), and the resulting plasmid was designated pNB. The 560 bp *Hin*fI fragment was filled in at the ends with the Klenow fragment of DNA polymerase I and ligated into pUC18 that had been cut with *Hin*cII. This plasmid was designated pHINF. The inserts were excised from the plasmids by *Eco*RI and *Hin*dIII digestion followed by preparative electrophoresis (Maniatis et al., 1982).

Cell extracts for coupled transcription/translation reactions were prepared from *E. coli* strain MRE600 (obtained from Grain Processing, Muscatine, IA), which lacks RNase I (Cammack & Wade, 1965). Isogenic strains MG1693 (*thyA715*) and SK5726 [*thyA715*, *pnp-7*, *rnb-500*^{ts}/pDK39 (*rnb-500*^{ts}), Cm^R] were obtained from Sidney Kushner (Arraiano et al., 1988). SK5726 lacks PNPase activity and contains a temperature-sensitive RNase II enzyme. The presence of a plasmid containing the *rnb-500* allele is necessary for the survival of the double mutant (Donovan & Kushner, 1986). Strain N3431 [*lacZ43*, *rne-3071*(ts), *relA1*, *spoT1*, *thi-1*] is temperature-sensitive for RNase E (Goldblum & Apirion, 1981) and was obtained from the *E. coli* Genetic Stock Center at Yale University.

In Vitro Transcription Reactions. Transcription reactions were carried out in S30 cell extracts prepared as previously described (Pratt et al., 1984). Extracts were prepared from MRE600 or MG1693 cells grown at 37 °C, or from SK5726 cells grown at 30 °C. The extract reactions contained 0.25 pmol of template, 3.5 μL of 0.1 M $Mg(OAc)_2$, 7.5 μL of low molecular weight components (LM), 5 μCi of [α-^{32}P]CTP, 5 μL of S30 extract, and 10 mM Tris–acetate, pH 7.0, in a total volume of 30 μL. The final concentrations of the LM components in the reaction mixture were the following: Tris–acetate, pH 8.2, 56 mM; DTT, 2 mM; ATP, 1.2 mM; GTP and UTP, 0.85 mM; CTP, 0.085 mM; phosphoenolpyruvate, pH 7.0, 27 mM; each of the amino acids, 0.35 mM; PEG, 1.9%; folinic acid, 35 μg/mL; cAMP, pH 7, 0.64 mM; tRNA, 0.17 mg/mL; NH_4OAc, 36 mM; KOAc, 72 mM; $Ca(OAc)_2$, 9.7 mM. The final Mg^{2+} concentration was 14 mM. Reactions were stopped by the addition of 1:1 phenol/$CHCl_3$, extracted with $CHCl_3$, and precipitated with 2 volumes of cold 100% ethanol. The precipitates were resuspended in a formamide sample buffer, electrophoresed on an 8% sequencing gel, and subjected to autoradiography using Kodak XAR-5 film and an intensifying screen. For preparation of RNAs to be S1-mapped or analyzed on Northern blots, radiolabeled CTP was omitted, CTP concentration was increased to 0.85 mM, and the RNAs were purified by phenol/$CHCl_3$ extraction and ethanol precipitation.

For analysis of translational products, [^{35}S]methionine was substituted for unlabeled methionine in the S30 cell extracts, and cell lysates were loaded onto an SDS–polyacrylamide gel (20% acrylamide). Ovalbumin and lysozyme were used as molecular weight standards. After electrophoresis, the gel was autoradiographed and stained with Coomassie brilliant blue to reveal the markers.

The purified transcription reactions were carried out in a 100-μL volume, containing a buffer composed of 20 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM EDTA, and 100 mM KCl. The 1580 bp DNA template (0.25 pmol) was preincubated with 2 pmol of *E. coli* RNA polymerase for 5 min at 37 °C. The ratio of RNA polymerase to template within the range of 10:1 to 1:100 did not affect the lengths of the transcripts ending in the tR1 region nor their relative distribution (data not shown). In reactions where ρ was present, 2.5 pmol of protein was included in the preincubation mixture. An NTP mix was added to a final concentration of 200 μM ATP, GTP, CTP, and UTP and 10 μg/mL rifampicin, and the reactions were incubated for an appropriate amount of time (2–10 min) at 30 or 37 °C. RNAs were then purified by phenol/$CHCl_3$ extraction and ethanol precipitation. For preparation of transcripts to be used as molecular weight markers or in processing reactions, 5 μCi of [α-^{32}P]CTP was included, and the final CTP concentration in the reactions was 20 μM.

S1 Mapping of 3′ Termini. The *Ava*I–*Pst*I fragment of pHINF (Figure 1) was hybridized to RNA derived from purified in vitro reactions, from extract reactions, or from cells. Cellular RNA was isolated as described (Aiba et al., 1981) from *E. coli* strain JM109 (Yanisch-Perron et al., 1985) harboring the pHINF plasmid. RNA was harvested from cells grown to an optical density of 0.35–0.45 absorbance unit at 600 nm. RNA from the in vitro reactions was purified by phenol/$CHCl_3$ extraction and ethanol precipitation prior to hybridization. Hybridizations were carried out in 30 μL at 35 °C in a NaTCA solution for 4 h and digested with S1 nuclease for 1 h as previously described (Murray, 1986). Protected fragments were electrophoresed on an 8% denaturing polyacrylamide gel next to sequencing ladders and were subjected to autoradiography. The ladders were generated by either dideoxy or chemical sequencing reactions (Sanger et al., 1977; Maxam & Gilbert, 1980) carried out on pHINF or the *Ava*I–*Pst*I restriction fragment.

Northern Analysis of Cro mRNAs. RNA transcripts were generated in purified in vitro reactions and S30 extracts by using the 1580 bp fragment as a template, and were purified by phenol/$CHCl_3$ extraction and ethanol precipitation. RNA was isolated from XL1-Blue cells (Stratagene Cloning Sys-

tems) harboring the pNB plasmid as described above. The RNAs were separated on an 8% denaturing polyacrylamide gel, blotted onto nylon (Zeta-Probe from Bio-Rad), and hybridized to the nick-translated 560 bp fragment. The blot was then autoradiographed.

Incubation of Transcripts in S30 Extracts. Internally radiolabeled transcripts were synthesized in vitro from the 1580 bp template and were separated on an 8% sequencing gel. Transcripts were eluted from the gel as described for DNA fragments (Dybczynski & Plucienniczak, 1988), except that elutions were carried out at room temperature for 16 h on a rotation platform. Carrier tRNA was added to the eluted RNAs. The RNAs were purified by phenol/$CHCl_3$ extraction and ethanol precipitation. The purified transcripts were incubated in a 30-μL volume containing 5 μL of S30 extract, the LM components as described above, and 3.5 μL of 0.1 M magnesium acetate.

Secondary Structure Prediction. The secondary structure of Cro mRNA was analyzed by using PCFOLD 4.0 (Zucker & Sankoff, 1984), employing the free energy parameters described by Freier et al. (1986).

Results

Transcription in S30 Extracts Yields a Set of Transcripts That Are Similar to Those Found in Vivo. The primary aim of our initial studies was to determine if ρ-dependent termination at the λ tR1 terminator in S30 cellular extracts yields transcription products that are more similar to those found in vivo. The extracts were prepared from *E. coli* strain MRE600 as described under Materials and Methods. MRE600 is commonly used for coupled transcription/translation systems because it lacks RNase I (Cammack & Wade, 1965).

Two restriction fragments carrying the λ P_R transcription unit and the tR1 ρ-dependent terminator were used as templates for transcription or transcription/translation in our studies, and as probes for 3′- and 5-end mapping; these are shown in Figure 1. The 1580 bp template contains three other promoters in addition to P_R; these are P_{RE}, P_{RM}, and P_{oop}. In purified transcription reactions, however, these other promoters do not interfere with the study of the tR1 terminator. The P_{RE} and P_{RM} promoters are both quite weak compared to P_R (Morgan et al., 1983a). P_{RE} requires the protein cII for activity, while P_{RM} requires the cI protein for activity (Ptashne, 1986); neither of these are included in purified transcription reactions. The P_{oop} promoter is active, but is located near the end of the 1580 bp template, and codes for a very small transcript (the 4S RNA) that does not interfere with P_R-initiated transcription (Morgan et al., 1983a). In extracts, the cII protein (transcribed from P_R) is expected to be produced when amino acids are included in transcription reactions, so that activation of P_{RE} could theoretically occur. The 560 bp template carries only the P_R promoter and encodes the Cro protein, but not cII.

To verify that only the Cro and cII protein coding sequences on our templates were functional, and to demonstrate the transcription/translation activity of our S30 extract, the 1580 and the 560 bp restriction fragments were each tested for template-directed translation activity. Figure 2 shows the autoradiographs of SDS–polyacrylamide gels of [^{35}S]-methionine-labeled translation products synthesized in the MRE600 S30 cell extract. When the 1580 bp fragment was used as a template in the S30 extracts, two primary products (Figure 2, lane 2) are found with electrophoretic mobilities that correspond to those expected for λ Cro and cII proteins (data not shown). A truncated transcription unit encoding

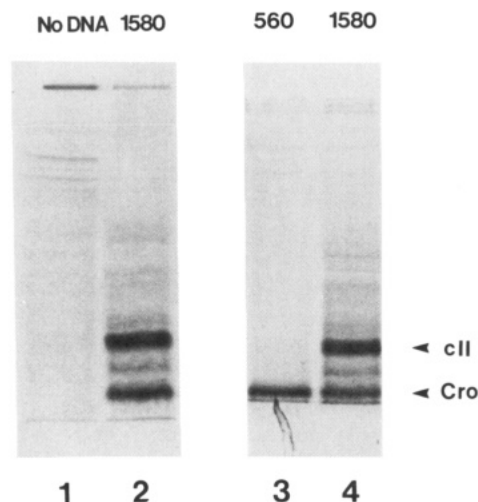

FIGURE 2: Coupled transcription/translation of restriction fragment templates. Restriction fragment DNA templates (see Figure 1) were used to direct transcription/translation in S30 extracts from *E. coli* strain MRE600. ^{35}S-labeled translation products were electrophoresed on SDS–polyacrylamide gels and subjected to autoradiography. Lanes 1 and 2 and lanes 3 and 4 are two separate experiments. Lanes 1 and 2, reactions were performed in the absence and presence of the 1580 bp template, respectively. Products were loaded alongside the molecular weight standards ovalbumin (43 000) and lysozyme (14 300). The electrophoretic mobilities of the two predominant products of the transcription/translation of the 1580 bp fragment (lane 2) are consistent with the proteins cII (11 000) and Cro (7400) that are expected to be produced from the 1580 bp template (see Figure 1). Lanes 3 and 4 show comparison of translation products derived from the transcription/translation of the 560 and 1580 bp fragments, respectively. As expected, transcription/translation of the 580 bp template does not produce the cII protein.

the cI protein is also located on the 1580 bp fragment; however, no significant quantities of this peptide were detected. When the shorter 560 bp template was transcribed, only a band corresponding to the Cro protein was observed (Figure 2, lane 3).

An autoradiogram of ^{32}P-labeled transcripts generated by the 1580 bp template in both the purified and MRE600 S30 extracts is shown in Figure 3. The transcripts produced in the purified system have been previously classified into five major groups (Morgan et al., 1983a,b, 1984), and are shown in Figure 3, lane 1. The lengths of the transcripts in the five classes are as follows: class I, 290–300 nt; class II, 308–312 nt; class III, 340–345 nt; class IV, 385–390 nt; class V, 440–445 nt (Morgan et al., 1983a). However, a different set of transcripts was found in reactions carried out in S30 extracts. After 2 min of transcription, three major RNAs accumulated in the extract reactions. Two of these (labeled "b" in Figure 3, lanes 3 and 5) were similar in size to the class II transcripts, and the other (labeled "a" in Figure 3, lanes 3 and 5) was shorter than the class I transcripts. After 5 min of transcription, several larger transcripts were produced (e.g., those labeled "d" in Figure 3, lanes 4 and 6); these were longer than class V transcripts, but were shorter than the expected read-through product. Uncoupling of translation in the extracts (no amino acids) resulted in no major qualitative differences in termination in the region where in vitro class III–V transcripts are expected to be found; however, there was a slightly enhanced amount of an RNA species approximately 370 nt in length (labeled "c" in Figure 3, lanes 5 and 6). Our length estimates indicated that this transcript apparently terminates at a site distal to the initiation codon of cII protein (Rosenberg et al., 1978). Most importantly, the transcript classes III–V that are synthesized in the purified in vitro transcription system were not detected among the products

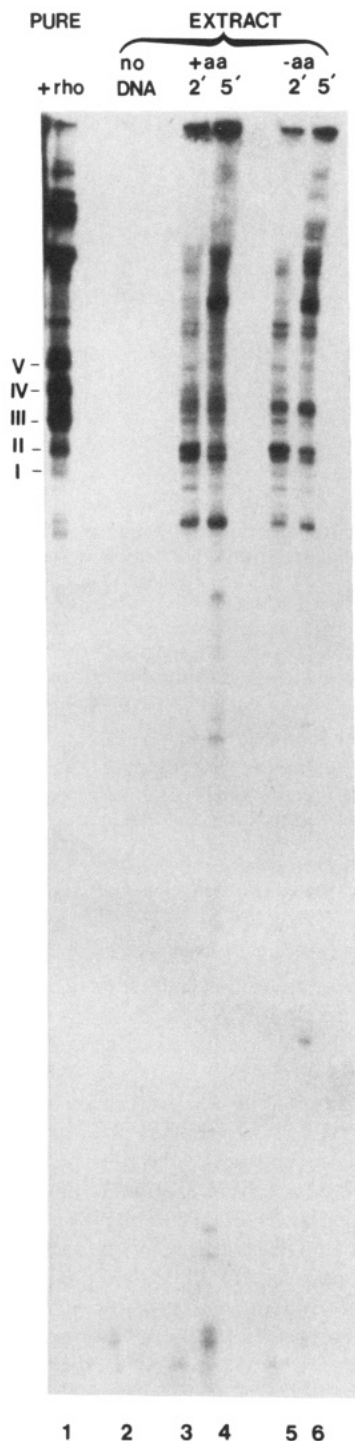

FIGURE 3: Transcription of Cro mRNA in extracts and in vitro. Restriction fragments used in this study contain the P_R promoter and tR1 terminator. Fragments were transcribed in vitro in a coupled transcription/translation S30 extract or in a purified system containing only RNA polymerase, NTPs, rifampicin, and ρ (see Materials and Methods for details). Lane 1, RNAs synthesized for 2 min in the purified transcription reaction in the presence of ρ. Lane 2, RNAs synthesized in extracts with no exogenous DNA added. Lanes 3 and 4, RNAs synthesized in extracts in the presence of amino acids for 2 and 5 min, respectively. Lanes 5 and 6, RNAs synthesized in extracts in the absence of amino acids for 2 and 5 min, respectively.

made in extracts, regardless of whether translation was occurring.

The levels of the class "b" transcripts appeared to decrease as the reaction time in the extracts was increased from 2 to 5 min, while the levels of class "a" transcripts increased. This observation is consistent with a precursor–product relationship

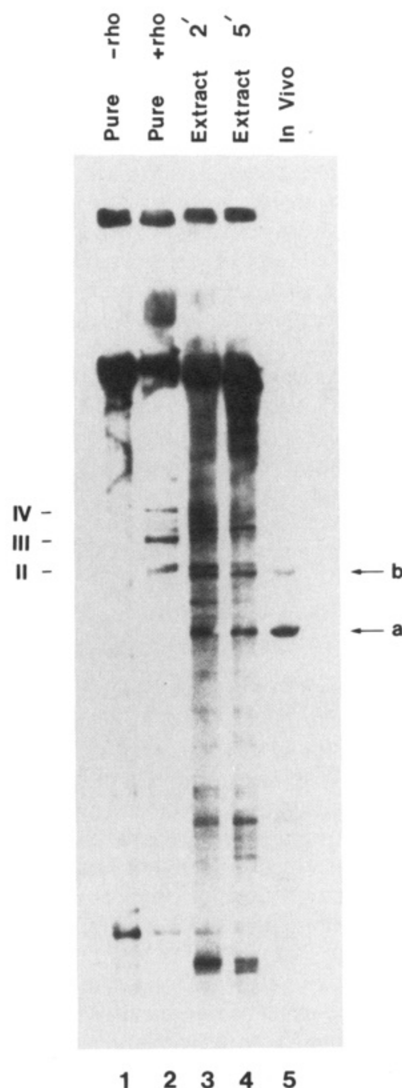

FIGURE 4: Northern blots of Cro mRNA produced in vitro and in vivo. RNA was transcribed from the 1580 bp template in purified transcription reactions and in extracts. In vivo RNA was isolated from *E. coli* harboring a pUC18 plasmid containing the 1580 bp template. RNAs were separated on 8% denaturing gels, blotted onto nylon, and probed with a nick-translated 560 bp fragment (see Figure 1); the blot was then subjected to autoradiography. Lanes 1 and 2, transcripts produced from the 1580 bp template in the absence and presence of ρ, respectively, in the purified system. Lanes 3 and 4, transcripts produced from 1580 bp templates for 2 and 5 min, respectively, in extracts. Lane 5, in vivo RNA.

between the "b" and "a" classes.

S1 mapping and primer extension analyses indicated that the 5′ ends of the transcripts produced in the purified system and the extracts are identical (data not shown) and that these 5′ ends coincide with the transcription start site for P_R-derived transcripts previously reported (Blattner & Dahlberg, 1972). Thus, the differences between the purified transcription products and those of extract reactions are not due to differences in initiation start sites.

Northern analysis of transcripts derived from cells containing the 1580 bp template cloned on pNB (see Materials and Methods) demonstrated that two major Cro transcripts are produced (Figure 4). These correspond in size to transcript classes "a" and "b" produced in the extract transcription reactions in which the 1580 bp fragment (Figure 1) was used as template (Figure 4, compare lanes 3–5). In the steady state, in vivo transcript class "a" was more prevalent than class "b". The differences in length between the two in vivo transcripts are most likely due to different 3′ ends, as only one 5′ end

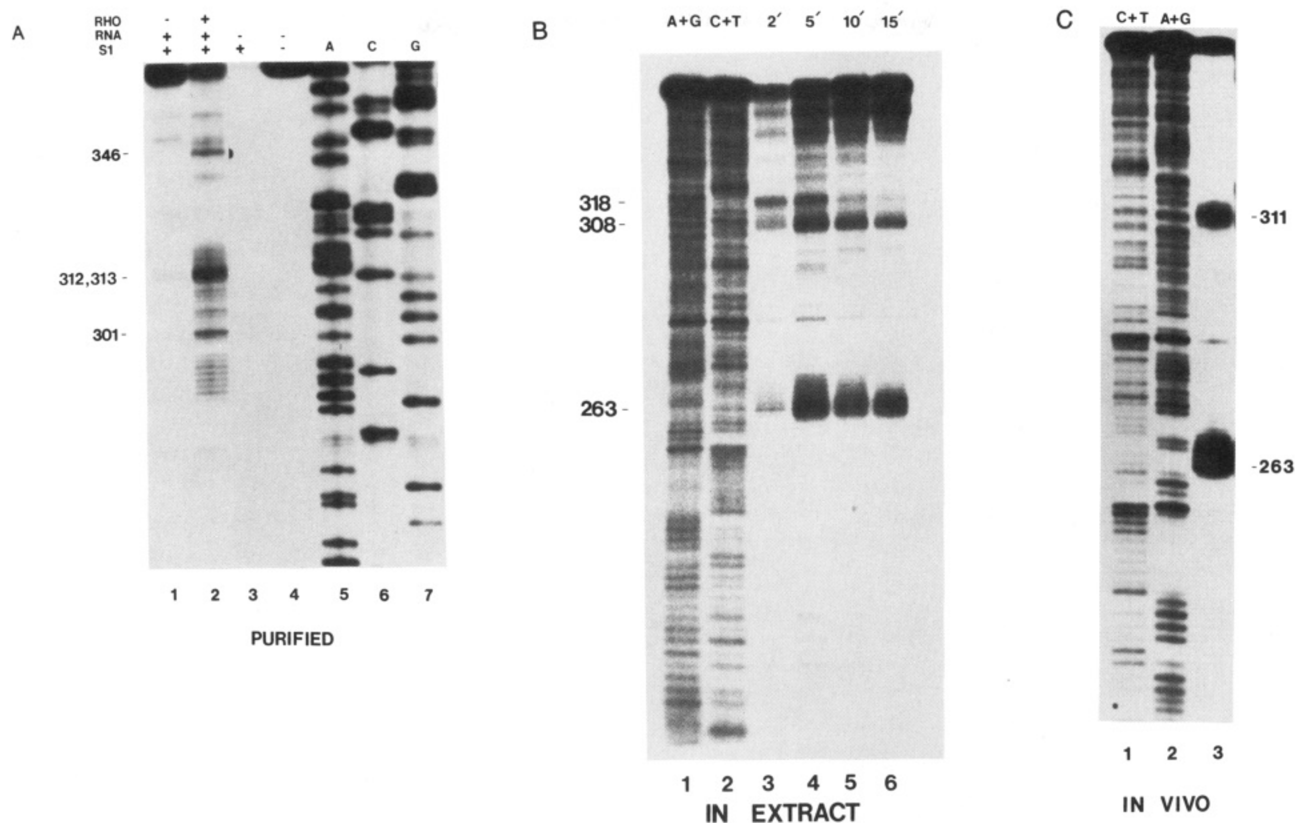

FIGURE 5: S1 mapping of Cro mRNAs synthesized in vitro, in vivo, and in extracts. An *Ava*I–*Pst*I restriction fragment probe derived from pHINF (see Materials and Methods) was labeled at the *Ava*I end and was hybridized to the RNAs described below. Hybrids were treated with S1 nuclease and were loaded onto an 8% sequencing gel next to a sequencing ladder. (A) DNAs protected by RNAs synthesized from the 1580 bp template in the purified systems. Lanes 1 and 2, synthesis in the absence and presence of ρ, respectively. Lanes 3 and 4, the *Ava*I–*Pst*I probe in the presence and absence of S1 nuclease, respectively (no RNA). Lanes 5–7, molecular weight markers derived from dideoxy sequencing reactions. (B) DNAs protected by RNAs synthesized in extracts from the 1580 bp template. Lanes 1 and 2, Maxam–Gilbert sequencing reactions of the probe. Lanes 3–6, synthesis carried out in a translationally coupled system for 2, 5, 10, and 15 min, respectively. (C) DNAs protected by RNAs synthesized in vivo from 560 bp template contained on the pHINF plasmid. Lanes 1 and 2, Maxam–Gilbert sequencing reactions of the probe. Lane 3, in vivo transcripts.

species was detected by 5′ S1 mapping (data not shown). This 5′ end was the same as that determined for the in vitro derived transcripts.

The 1580 bp template migrates in the same region of the gel as the read-through transcripts, and gives rise to the dense hybridized bands on the autoradiogram near the top of lanes 1–4 of Figure 4. Thus, we were unable to estimate the amount of terminator read-through in vivo transcription from these experiments. However, using a luciferase expression vector, we have previously estimated read-through of the tR1 termination region to be approximately 20% in vivo (Peabody et al., 1989).

S1 Mapping Indicates That +263 and 308–311 Are the Primary 3′ Ends Found in Vivo and in Extract Transcription Reactions. We used a standard S1 nuclease mapping technique (see Materials and Methods) to precisely determine the 3′ termini of the transcripts produced in the purified system, in extracts and in vivo (Figure 5). The 1580 bp fragment was used as a template for transcription reactions. Northern analysis showed that all of the major in vivo Cro transcripts were shorter than 340 nt. Therefore, to simplify the 3′-end analysis, a probe derived from pHINF (Figure 1) was employed in the subsequent S1 mapping experiments. This probe is an *Ava*I–*Pst*I restriction fragment that spans sites I, II, and III of the tR1 termination cluster (see Figure 1), and includes eight bases derived from the pUC18 polylinker at the *Pst*I end. In the purified transcription system, we found major protected DNA fragments corresponding to RNA lengths of 301, 312–313, and 346 nt (see Figure 5A), previously identified as the transcript classes I, II, and III (Lau et al., 1982; Morgan et al., 1983a). S1 nuclease digestion usually resulted in the generation of clusters of protected fragments; thus, the most predominant transcript(s) in each cluster is (are) reported. Extract-generated transcripts appeared to terminate at 263, 308, and 318 nt (Figure 5B). Comparison of the 3′ termini of transcripts produced in extracts after 2 min to those produced after 5, 10, or 15 min suggests a precursor–product relationship between the 318- and 308-nt transcripts, and between the 318- and 263-nt transcripts. The 308-nt transcript may be an intermediate in the processing of the 318-nt species to the 263-nt species. In vivo, we found protected fragments with 3′ termini at sites +263 and +311 (Figure 5C), which correspond to in vitro extract transcript classes "a" and "b". The latter size is the same as that reported by Court et al. (1980) on the basis of RNase fingerprinting studies of isolated in vivo transcripts, and also corresponds to transcripts terminating at the "tR1" site originally characterized by Rosenberg et al. (1978). However, the 263-nt Cro transcript has not been reported previously.

Transcripts Isolated from Purified Transcription Reactions Are Degraded or Processed in Extracts to Sizes Found in Vivo. In order to determine the reason for the differences in transcripts synthesized in the purified transcription reaction and those synthesized in the extracts and in vivo, we wanted to know if the longer in vitro synthesized transcripts could be processed to the shorter species by incubation in the extracts. ρ-Dependent transcripts produced in the purified in vitro system were separated on sequencing gels, and RNAs from

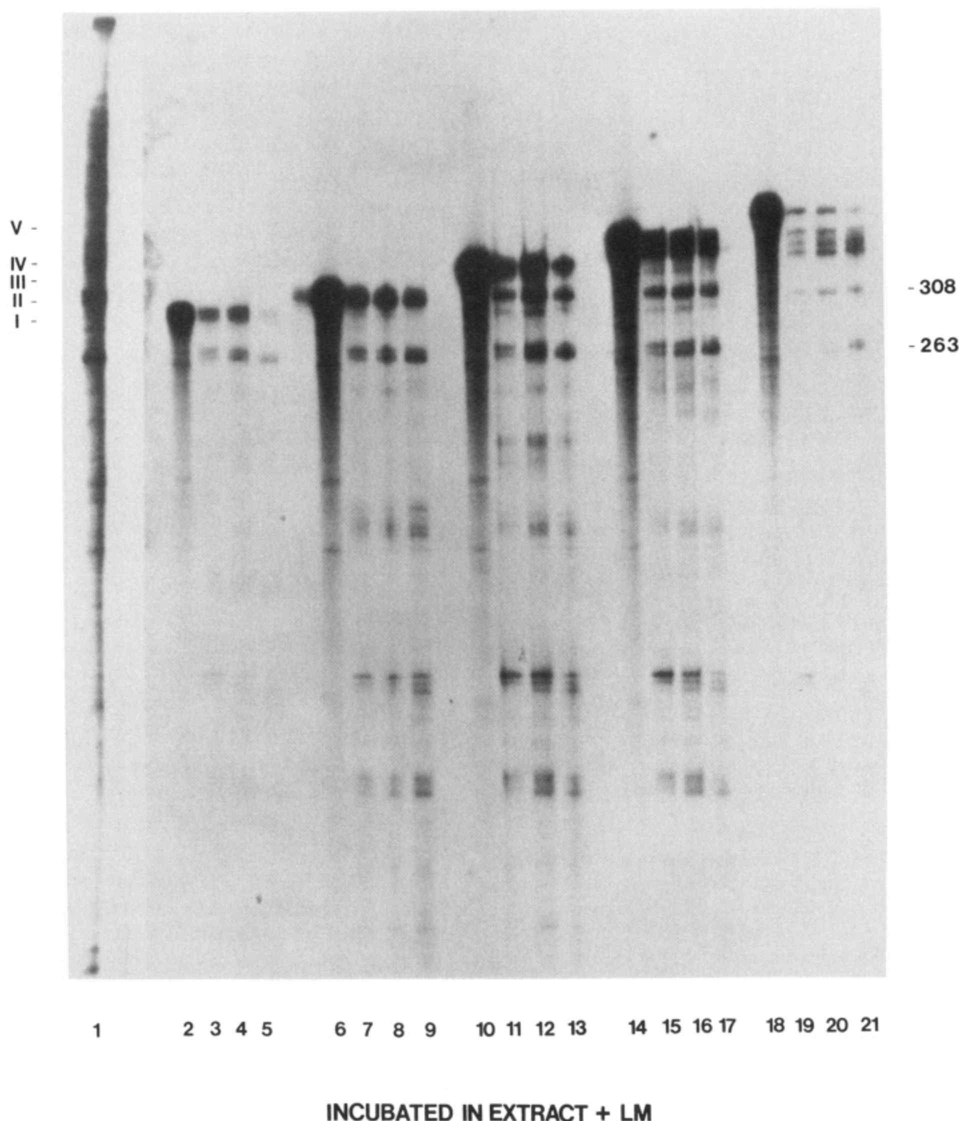

FIGURE 6: Incubation of transcripts derived from the purified system in the S30 extract. RNA transcripts were synthesized on the 1580 bp template in a purified system in the presence of ρ, and were separated on an 8% sequencing gel. The five classes of transcripts were eluted separately from the gel and were incubated in the S30 extract: I, 290–300 nt; II, 300–320 nt; III, 320–360 nt; IV, 360–410 nt; V, 430–470 nt. Lane 1, RNAs synthesized in a complete extract after 2 min. Lanes 2–5, class I treated with the S30 extract for 0, 0.5, 1, and 2 min, respectively. Lanes 6–9, class II treated for 0, 0.5, 1, and 2 min, respectively. Lanes 10–13, class III treated for 0, 0.5, 1, and 2 min, respectively. Lanes 14–17, class IV treated for 0, 0.5, 1, and 2 min, respectively. Lanes 18–21, class V treated for 0, 0.5, 1, and 2 min, respectively.

the individual bands corresponding to classes I–V were purified from the gel (see Materials and Methods for details). The products of the time-dependent processing of each of the isolated classes of transcript in the MRE600 S30 extract are shown in Figure 6. All of the exogenously synthesized transcripts were rapidly processed in the extracts to smaller species. The longest transcript degradation intermediate corresponds in length to the 308-nt transcript observed in the in vitro extract transcription reactions. In addition, transcript classes I–IV are processed to a species corresponding to the 263-nt transcript. Class V transcripts are processed to species corresponding in length to class IV, as well as to the 263- and 308-nt transcripts. Processing of the longer transcripts to the 308- and 263-nt transcripts most likely occurs at the 3′ end, as the 5′ ends of transcripts derived from purified reactions were not changed after 2 min of incubation in extracts (data not shown).

Extracts Deficient in both PNPase and RNase II Are Defective in Processing of Cro Transcripts to +308 and +263. The 3′ ends of *E. coli* mRNAs are susceptible to processing by the 3′–5′-exonucleases RNase II and PNPase (Belasco & Higgins, 1988). The endonuclease RNase E has also been reported to process mRNA in *E. coli* (Mudd et al., 1988). Therefore, we analyzed the Cro transcripts produced in vitro by S30 extracts made from an *E. coli* strain lacking PNPase and having a defective temperature-sensitive RNase II enzyme. We also examined RNA species made in extracts of a strain containing a defective temperature-sensitive RNase E enzyme.

S30 extracts were made from isogenic strains MG1693 (*pnp*$^+$, *rnb*$^+$) and SK5726 (*pnp-7*, *rnb-500*^{ts}). A plasmid carrying the 1580 bp fragment was transcribed in extracts preheated at 30 and 45 °C for 20 min. Transcription reactions were then carried out for 10 min at 30 °C. Use of plasmid rather than linear template had no effect on the RNAs synthesized in the extracts except for the production of an RNA of about 100 nt in length that is encoded by the parent plasmid pUC18 (data not shown).

Preheating of the extract made from MG1693 at either 30 or 45 °C for 20 min had no effect on the processing of Cro transcripts; both the +308 and +263-nt transcripts were observed (Figure 7, lanes 2 and 3) and appear to be identical with those produced in the MRE600 extract used in all of our

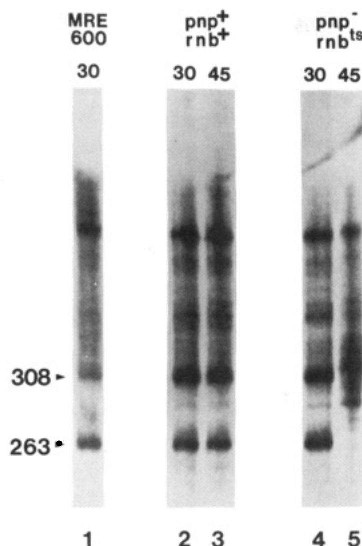

FIGURE 7: Transcription of Cro mRNA in a PNPase$^-$ and RNase II^{ts} S30 extract. The plasmid pNB was transcribed in extracts made from strain MRE 600 and two isogenic strains containing wild-type (MG1693) and mutant (SK5691) 3′-exonucleases (*pnp-7*, *rnb500*^{ts}). RNA transcripts were internally labeled with [α-^{32}P]CTP. Prior to transcription, the extracts were heated at 30 or 45 °C for 20 min. Transcription reactions were carried out at 30 °C. Lane 1, transcripts made in extracts from strain MRE600, preheated at 30 °C. Lanes 2 and 3, transcripts made in extracts from strain MG1693, preheated at 30 and 45 °C, respectively. Lanes 4 and 5, transcripts made in extracts from strain SK5691 (*pnp*$^-$, *rnb*^{ts}), preheated at 30 and 45 °C.

previous studies (Figure 7, lane 1). Preheating of the extract made from SK5726 at 45 °C resulted in the complete inhibition of the formation of the 263- and 308-nt processed species (Figure 7, lane 5). A major band corresponding to the 318-nt species was generated. Several RNAs accumulated that were longer than the 318-nt transcript, but were not identical with the longer transcript classes III–IV synthesized in purified transcription reactions. Preheating of the extract made from SK5726 at 30 °C for 20 min prior to transcription did not result in the disappearance of the processing products (Figure 7, lane 4), indicating that inactivation is temperature-sensitive. Preheating of extracts made from strain N3431 (containing a temperature-sensitive RNase E) at 45 °C prior to the transcription reaction had no effect on the processing reactions (data not shown).

Discussion

3′ Ends of Phage λ Cro mRNA Generated in Extracts Are Formed by a Combination of Transcription Termination and RNA Processing. Our observations are summarized as follows (see Figure 8A): (i) Transcription in bacterial cell extracts produces RNAs with 3′ ends that are comparable to those found in vivo; longer ρ-dependent transcripts that are prevalent in the purified transcription reaction are not found in the extracts. (ii) The most prevalent transcripts found both in extract transcriptions after 5 min and in vivo are two classes that map to +263 and +308–311 from the start of transcription; the 263-nt transcript is not detected in the purified in vitro transcription system. The 263-nt transcript lies within the ρ binding site on the transcript (Chen et al., 1986, 1987; Faus & Richardson, 1989), immediately adjacent to the *boxB* sequence of the *nutR* site (see Figure 8B), which is required for N-mediated antitermination (Drahos & Szybalski, 1982). Therefore, the 263-nt transcript is unlikely to be a direct product of transcription termination. (iv) Longer transcript classes generated in the purified in vitro transcription reaction can be processed to the 263- and 308-nt transcripts when incubated in the S30 extract. (v) An S30 extract made from a strain defective in RNase II and lacking PNPase activity, and preheated before transcription, does not produce the 308- or 293-nt transcripts.

From these observations, we conclude that +263 site is an in vivo RNA processing site and that the +308–311 site functions as both a termination site and an RNA processing site. Since the translational stop codon for the *cro* gene is located at +217, the processed 263-nt transcript still retains the entire coding region for the Cro protein, and should be translationally active.

Cro Transcripts Are Processed by RNase II and/or PNPase. The number of RNases that have been identified in *E. coli* is quite large (Deutcher, 1985), but only a small number have been unambiguously shown to participate in mRNA processing and degradation. Two 3′→5′ single-stranded exonucleases, PNPase (the product of the *pnp* gene) and RNase II (the product of the *rnb* gene), have been previously implicated in *E. coli* mRNA processing [see Belasco and Higgins (1988) for a recent review]. These enzymes appear to be functionally redundant, and can substitute for each other in mutants that fail to produce one or the other (Donovan & Kushner, 1983, 1986). Two endonucleases, RNase III (the product of the *rnc* gene) and RNase E (the product of the *rne* gene), have also been shown to be important in phage and *E. coli* mRNA processing (Belasco & Higgins, 1988). Computer analysis of the secondary structure of the entire tR1 region contained on our templates failed to identify any stem–loop structures that correspond to previously identified substrates for the RNase III site (Robertson, 1982). Thus, we restricted our initial search for possible processing enzymes to RNase II, PNPase, and RNase E.

In our studies, we found that the preheating of extracts made from a strain lacking PNPase and containing a temperature-sensitive RNase II mutation inhibits the formation of the 263- and 308-nt processing intermediates. In a strain where only the PNPase is defective, no change in the processing is observed (data not shown); however, because of the functional redundancy, we are presently unable to determine whether one or both of the exonucleases are responsible for the processing.

We had initially expected that the 263-nt transcript might be due to RNase E cleavage. RNase E cuts at single-stranded regions in a sequence lying upstream from a stem–loop structure. The sequence where the cut takes place is only loosely conserved among the phage T4 gene 32 transcript (Mudd et al., 1988) and other known RNase E substrates such as the *E. coli* 9S RNA (the precursor to 5S rRNA) (Ghora & Apirion, 1978), *E. coli* DicF-RNA (Faubladier et al., 1990), and colE1 RNA1 (Tomcsanyi & Aprion, 1985). One of the RNase E cleavage sites of *E. coli* 9S RNA, 9Sb (AUCAA/AUAAA), is almost identical with that at site +263 (AUCAA/AUUAA). The sequences at the 3′ termini of the 263- and 308–312-nt transcripts both end with the sequence AUCAA which could suggest that the same nuclease is involved in processing at both sites. We were unable to detect any inhibition of processing in preheated extracts made from a temperature-sensitive RNase E mutant.

Our results indicate that the 3′-exonucleases, RNase II and/or PNPase, are at least in part responsible for the generation of the processing intermediate. The location of 3′-exonucleolytic processing sites at +263 and +308–312 is consistent with the predicted structure of the 3′-untranslated region of the Cro mRNA. Each 3′ terminus is located a few nucleotides 3′ proximal to a predicted stable stem–loop

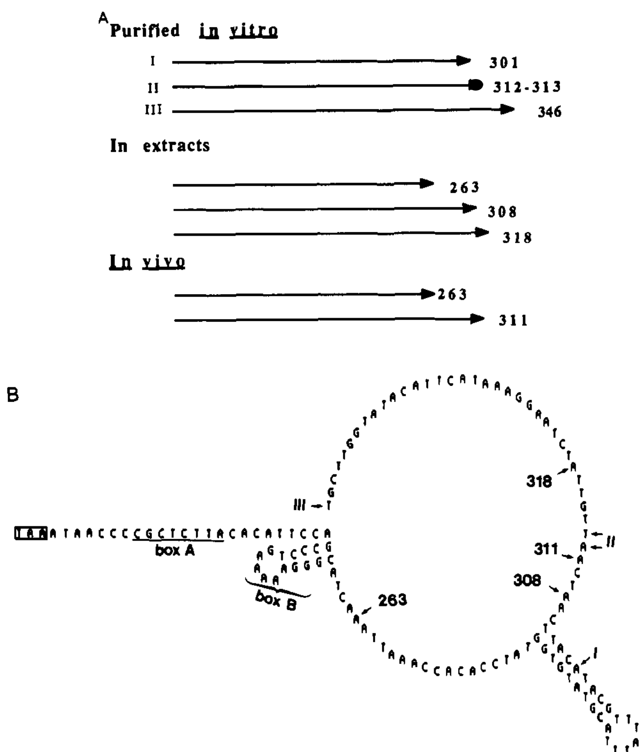

FIGURE 8: (A) Summary of S1 mapping data. Numbers indicate lengths of major transcripts generated under conditions shown, as determined from S1 mapping. (B) The predicted RNA secondary structure for the phage λ P_R transcript. The 3′ sequences of Cro mRNA, from the Cro stop codon through site III (346 nt), were analyzed by using PCFOLD (Zucker & Sankoff, 1984). The 3′ sequences of extract and in vivo derived transcripts mapped in this study are indicated by numbers in the center. The 3′ termini of class I–III transcripts derived from purified transcription reactions are also indicated. The stop codon for Cro protein is boxed, and the positions of *boxA* and *boxB* are noted. In this figure, T's are substituted for U's.

structure (see Figure 8B). Secondary structures are known to impede degradation of RNA by 3′-exonucleases (Mott et al., 1985; Plamann & Stauffer, 1990; Belasco & Higgins, 1988). The physical interaction between the exonuclease and the stem–loop may limit the digestion, generating the small single-stranded segment after the stem–loop structure.

Utilization of Termination Sites. An open question is whether the downstream termination sites observed in the purified in vitro transcription system are utilized in vivo and are substrates for processing. Transcription termination at tR1 has been estimated to function at efficiencies ranging from 50 to 85%, depending upon whether the terminator is in the context of the λ genome or on a plasmid expression vector (Court et al., 1980; Luk & Szybalski, 1982; Peabody et al., 1989). Using Northern analysis, we are unable to detect transcripts in vivo that correspond to transcript classes III–V generated in the purified transcription reaction. An explanation often invoked to explain the absence of sites III–V in vivo is translational interference (Morgan et al., 1983b). Translation is thought to normally inhibit the termination activity of ρ, presumably by blocking ρ binding to the transcript (Adhya et al., 1976). Since translation of cII protein starts at nucleotide +338 of the P_R transcript (Figure 8B), in vitro termination sites III (+346), IV (+391), and V (+450) could be nonfunctional in vivo due to translational interference. We find that the omission of amino acids from the S30 extracts does not restore the function of sites III–V (see Figure 3); however, an increased accumulation of a class "c" species with a size intermediate to in vitro classes III and IV (approximately 370 nt) is detected.

The failure to detect transcripts in the extracts at sites III, IV, and V could be explained by rapid processing of these particular forms to the 263- and 308-nt species; however, if these transcripts were processed by RNase II, one might have expected them to accumulate in the extracts where processing activity had been heat-inactivated. Although transcripts longer than 312 nt do in fact accumulate in the S30 extracts, their lengths do not correlate with the longer species found in the purified in vitro reaction. Thus, the failure to find these longer transcripts in vivo or in the S30 extracts suggests that other unknown factors and processes may be involved in the suppression of termination at in vitro termination sites III–V and that the use of these termination sites is truly an artifact of the purified in vitro transcription termination reaction.

The failure to detect in vitro class I transcripts (290–300 nt) either in the extracts or in vivo may be due to processing and/or the action of the NusA protein. Termination at this site cluster can be completely eliminated in the in vitro purified transcription system by the addition of NusA protein (Lau et al., 1982; C. L. Andrews and D. G. Bear, unpublished results), and the transcripts are not detected in the extracts where NusA is expected to be present. Thus, it is unlikely that this transcript is produced in significant quantities in vivo; if the transcript is produced, our studies suggest that it may be rapidly processed to the 263-nt transcript as well as to smaller intermediates.

Is 3′-Terminal Processing a General Feature of ρ-Dependent Transcripts? Several transcription units have been found to produce ρ-dependent transcripts that are processed to more stable intermediates. These include the *trp* operon (Mott et al., 1985), the *leu* operon (Rosenthal & Calvo, 1987), the *ilv* GMEDA operon (Wek et al., 1987), the tyr-tRNA^{suIII} gene cluster, the phage T4 *gene41* (Hinton, 1989), and perhaps the *speED* operon (Xie et al., 1989). It is not surprising that the 3′ termini of ρ-dependent transcripts would be susceptible to 3′-exonuclease and single-stranded endonuclease digestion. One of the important general features of a ρ-dependent termination site is a long single-stranded region on the transcript where ρ binds and which activates its ATPase activity (Morgan et al., 1985); the ATPase is required for the helicase-like transcript release reaction (Galluppi et al., 1976; Howard & de Crombrugghe, 1976; Brennan et al., 1987). Such regions may be highly susceptible to exonucleases specific for single-stranded RNAs. Mott et al. (1985) have shown that RNase II treatment of transcripts generated in vitro by ρ-dependent termination at the *trp t′* terminator yields in vivo sized transcripts. The 3′ terminus of the processed *trp* transcript is the highly stable *trp t* ρ-independent terminator. On the basis of our results together with studies described above on systems that are yet to be completely characterized, we would like to suggest that coupled ρ-dependent termination/3′-exonucleolytic processing may be a general phenomenon. Thus, the generation of ρ-dependent transcript 3′ termini may resemble eukaryotes—a heterogeneous population of transcripts is generated by a downstream termination process, and subsequently processed to a much smaller population of stable functional intermediates (Proudfoot, 1989).

In conclusion, our studies strongly suggest that the comprehensive in vitro analysis of prokaryotic RNA 3′-end formation must include studies in both purified and extract in vitro transcription systems if a valid comparison with in vivo systems is to be made.

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