RNase III-dependent hydrolysis of λ cII-*O* gene mRNA mediated by λ OOP antisense RNA

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The 77-nucleotide OOP antisense RNA of bacteriophage λ complements λ cII-*O* mRNA in a region that includes 55 nucleotides at the 3' end of the cII gene and 22 nucleotides in the intercistronic region between the cII and *O* genes. OOP RNA, produced from multicopy plasmids, inhibits λ cII gene expression by ~100-fold through an RNase III-dependent mechanism. Using primer extension analysis of cellular RNA isolated from an induced λ lysogen that contains an OOP DNA plasmid, we have identified a cleavage site in cII-*O* mRNA within the region of complementarity with OOP RNA, at 13 nucleotides from the 3' end of that region. Ribonuclease protection experiments demonstrate that almost all cII-*O* mRNA in this overlap region is cleaved when OOP RNA is overproduced in RNase III⁺ cells but not in RNase III⁻ cells. RNA fragments are detected that extend into the *O* gene from the cleavage sites, while the sister fragments that extend into the cII gene cannot be detected and must be eliminated by additional hydrolytic events. Differences in levels of uncleaved mRNA between RNase III⁺ and RNase III⁻ cells are much less at several hundred nucleotides to either side of the target region. An alternate OOP RNA-dependent hydrolytic process occurs in RNase III⁻ cells that results in cleavages in one of two regions, one close to the cleavage site observed in RNase III⁺ cells, and the second several nucleotides beyond the end of the complementary region between OOP RNA and cII-*O* mRNA. In this latter case, the fragments that extend into the cII gene are stable, while the sister *O* gene fragments are destroyed, in direct contrast to the RNase III-dependent process.

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Antisense RNAs are short, untranslated RNA molecules that form double-stranded complexes with complementary target RNAs. Antisense RNAs regulate transcription, translation, RNA degradation, or the initiation of DNA replication in various prokaryotic systems (for reviews, see Green et al. 1986; Inouye 1988; Inouye and Delihis 1988; Simons 1988; Simons and Kleckner 1988; Walder 1988). The OOP antisense RNA of bacteriophage λ is a short 77-nucleotide RNA that is transcribed from λ DNA both in vivo and in vitro (Blattner and Dahlberg 1972; Dahlberg and Blattner 1973; Hayes and Szybalski 1973; Schwarz et al. 1978). In all probability OOP RNA does not code for a polypeptide since it lacks a Shine/Dalgarno sequence (Shine and Dalgarno 1974) and since the only AUG codon in OOP RNA may be deleted without affecting biological activity (Krinke and Wulff 1987). Synthesis of the OOP antisense RNA begins in the intercistronic region between the cII and *O* genes of λ and proceeds into the cII-coding region, in the opposite direction from cII mRNA transcription. The final 55 nucleotides of the 77-nucleotide OOP antisense RNA transcript are complementary to the 3' end of the cII

gene. Although the promoter recognition region for the p_o promoter of OOP RNA overlaps the Shine/Dalgarno region and 5' end of the λ O gene, OOP RNA itself neither overlaps the *O* gene nor its ribosome recognition region.

OOP RNA, produced from multicopy plasmids, inhibits λ cII gene expression by 100-fold or more (Krinke and Wulff 1987). OOP RNA acts as an antisense RNA by forming a double-stranded complex with cII-*O* mRNA, as indicated by the observation that nonfunctional cII mRNA, produced from a second multicopy plasmid, competes with cII mRNA for OOP RNA and reduces the OOP effect. The inhibition of cII gene expression by OOP RNA is observed only in host strains that produce the endonuclease RNase III, an enzyme that is specific for double-stranded RNA (Robertson et al. 1968; Robertson 1982). This suggests that a double-stranded cII mRNA-OOP RNA complex is hydrolyzed by RNase III to yield a truncated cII mRNA fragment that lacks the 3' end of the cII transcript. This initial nucleolytic event may or may not be followed by additional nucleolytic events mediated by other cellular RNases.

This antisense RNA system is different from other systems that regulate gene expression in that the OOP antisense RNA is complementary to the 3' end of the cII

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target gene. Other antisense RNAs bind to the 5' ends of the mRNAs of the genes they regulate and may directly interfere with the initiation of translation of these genes (Simons and Kleckner 1988). A direct interference by OOP RNA with the initiation of translation of *cII* mRNA is highly unlikely, and the sole regulating action of OOP RNA on *cII* mRNA seems to be through the control of mRNA stability.

In this study we identify the precise location of an RNase III cleavage site at the 3' end of *cII* mRNA. In host cells that overproduce OOP RNA, we show that cleavage of *cII*-O mRNA by RNase III results in extensive mRNA degradation in the region of complementarity with OOP RNA. In the absence of RNase III, *cII*-O mRNA is degraded by an alternate pathway that is dependent on OOP RNA.

Results

Overproduction of OOP RNA from multicopy plasmids

We have shown previously that plasmids that overproduce OOP RNA inhibit *cII* gene expression from a derepressed λ prophage by 100-fold or more in RNase III⁺ but not RNase III⁻ hosts (Krinke and Wulff 1987). That these plasmids overproduce OOP RNA is shown here by a ribonuclease protection assay utilizing an RNA probe complementary to the final 55 nucleotides (3' end) of OOP RNA (Fig. 1). OOP RNA measurements were made 24 min after heat induction of isogenic RNase III⁺ and RNase III⁻ bacterial strains that contain, in addition to the multicopy OOP DNA plasmids, a heat-inducible λ prophage in single copy in the chromosome. The conditions were identical to those in which the effect of OOP RNA on *cII* gene expression had been measured previously (Krinke and Wulff 1987). In both RNase III⁺ and RNase III⁻ strains, substantial OOP RNA was detected with the plasmids pLK39 (p_o containing a wild-type OOP DNA fragment) (Fig. 2, lanes 2 and 6) and pLK40 (Δ , containing an OOP DNA fragment with a 13-bp internal deletion near the OOP promoter) (Fig. 2, lanes 3 and 7); and even larger levels of OOP RNA were determined for the plasmid pLK67 (p_L , in which the strong repressible λp_L promoter replaces the wild-type p_o OOP promoter region) (Fig. 2, lanes 4 and 8). However, no OOP RNA was detected in the RNase III⁺ strain with the control plasmid pLK38 (-; containing an OOP DNA fragment but with a deleted p_o OOP promoter) (Fig. 2, lane 1), and little OOP RNA was detected with pLK38 in the RNase

Figure 2. OOP RNA levels in isogenic RNase III⁺ and RNase III⁻ lysogenic strains that carry various OOP DNA plasmids. The OOP RNA probe (Fig. 1), which is complementary to the last 55 nucleotides of OOP RNA, was hybridized to total RNA from induced lysogens carrying various OOP DNA plasmids. RNA was digested with RNase A and RNase T1 for 60 min and separated by electrophoresis on a polyacrylamide sequencing gel in 8 M urea. The strains UC6171 (lanes 1–4) and UC6171 *rnc-105 glyA::Tn5* (lanes 5–8) carry a λ int-6 *clts857 cro27* *Pam3* prophage and plasmids pLK38 (deleted OOP promoter, lanes 1 and 5), pLK39 (wild-type OOP DNA, lanes 2 and 6), pLK40 (OOP DNA with a 13-bp internal deletion, lanes 3 and 7), or pLK67 (OOP DNA with the λp_L promoter, lanes 4 and 8). No OOP RNA is produced by pLK38, and the strain carrying this plasmid allows an estimate of the contribution of the prophage to OOP RNA levels. The internal deletion in pLK40 is wholly outside the region complementary to the probe, and as a result the pLK40 OOP RNA band is identical in length with the others. Similar results are seen with plasmids pLK38, pLK39, and pLK67 using an OOP RNA probe that is complementary to the entire OOP RNA molecule.

Figure 1. Genes and promoters in the λ *cro*-*cII*-O region. Also shown are RNA probes prepared by in vitro transcription with T7 or SP6 RNA polymerase. The 5' *cII* probe contains λ bp 38,359–38,453 (numbered according to Daniels et al. 1983); the OOP probe, λ bp 38,586–38,653; probe I, λ bp 38,586–38,735; probe II, λ bp 38,651–39,735; and the O probe, λ bp 39,064–39,173. Wavy lines represent RNA transcripts in λ infections. [For a review and complete discussion of transcriptional controls in this region, see Herskowitz and Hagen (1980); Wulff and Rosenberg (1983); Echols (1986).]

III⁻ strain (Fig. 2, lane 5). The low level of OOP RNA in this latter case is almost certainly transcribed from the prophage, which contains a wild-type OOP DNA region, and not from the plasmid lacking an OOP promoter. (We suspect that OOP RNA is also transcribed from the prophage in the RNase III⁺ strain but is effectively removed by an RNase III-dependent mechanism.) In summary, multicopy OOP DNA plasmids produce easily detectable quantities of OOP RNA in RNase III⁺ bacterial cells, where *cII* gene expression is inhibited, and also in RNase III⁻ bacterial cells, where *cII* expression is unaffected.

Primer extension analysis of OOP RNA from the *p_L*-OOP fusion plasmid (pLK67) reveals a primary OOP RNA transcript that is two nucleotides longer at its 5' end than wild-type OOP RNA (data not shown). These additional two nucleotides are complementary to *cII-O* mRNA (Krinke and Wulff 1987), so that the 79-nucleotide OOP RNA from *p_L* is capable of full base-pairing with *cII-O* mRNA.

Mapping of the RNase III cleavage site

Primer extension analysis was performed to determine whether RNase III cleaves *cII* mRNA when OOP RNA is overproduced. A specific 5' end was detected after prophage induction of an RNase III⁺ strain that carries pLK39 (*p_o*), the plasmid that overproduces full-length OOP RNA (Fig. 3, band AA, lane 7). This 5' end was not found in an RNase III⁻ strain with the plasmid pLK39 (*p_o*, containing a wild-type OOP DNA fragment) (Fig. 3, lane 8), and a much lower level was found in an RNase III⁺ strain with the control plasmid pLK38 (-; deleted for the *p_o* OOP promoter), in which the only potential source of OOP RNA is from the prophage present in single copy on the bacterial chromosome (Fig. 3, lane 5). This potential RNase III cleavage site in *cII-O* mRNA is located in the intercistronic region between the *cII* and *O* genes, 9 nucleotides downstream from the *cII* translation termination codon. It is within the region of complementarity between *cII-O* mRNA and OOP RNA and lies 13 nucleotides from the end of this region on the 3' side of *cII-O* mRNA (Fig. 4). The RNA molecule detected by primer extension contains the coding region and ribosome recognition region for the *O* gene, but no *cII* sequences.

If this represents a true RNase III-dependent 5' end, it should not be found when OOP RNA is provided by the plasmid pLK40 (Δ), which produces an OOP RNA with a 13-nucleotide internal deletion opposite this observed 5' end (Fig. 4). With pLK40 the 5' end is indeed not found at the usual site; instead, a new RNase III-dependent 5' end is observed in *cII-O* mRNA 22 nucleotides upstream (i.e., to the 5' side) of the site found with full-length OOP RNA (Fig. 3, band BB, lane 9). This new cleavage site lies within a region of continuous complementarity between *cII-O* mRNA and the (Δ) pLK40 OOP RNA. It is located 11–13 nucleotides from the end of this region (depending on where one places the endpoints of the deletion in pLK40) on the 3' side of *cII-O* mRNA (Fig. 4).

Figure 3. Primer extension analysis to determine RNase III cleavage sites on *cII-O* mRNA. Total RNA from induced lyogens was hybridized to a DNA primer complementary to nucleotides 48–64 of the *O* gene (58–74 nucleotides from the start site of OOP RNA), extended with reverse transcriptase, and run on a sequencing gel. The same gel contained DNA sequencing reactions (lanes 1–4; G, A, T, and C, respectively), using the same primer with single-stranded *cII-O* DNA cloned into M13. The strains UC6171 (lanes 5, 7, and 9) and UC6171 *rnc-105 glyA :: Tn5* (lanes 6, 8, and 10) carry a λ int-6 *clts857 cro27* *Pam3* prophage and plasmids pLK38 (deleted OOP promoter, lanes 5 and 6), pLK39 (wild-type OOP DNA, lanes 7 and 8), or pLK40 (OOP DNA with a 13-bp internal deletion, lanes 9 and 10). Note that the sequence on the gel is the complement to *cII-O* mRNA. (The faint bands in lane 6 are an artifact resulting from “spillover” from lane 7; see Fig. 5, lane 2.)

To show that the detected 5' end is the site at which RNase III cleaves, and is not created by other nucleases that trim the RNA molecule after initial RNase III cleavage elsewhere, we treated uncut cellular RNA, isolated after prophage induction of an RNase III⁻ strain that overproduces OOP RNA, with purified RNase III in vitro. Primer extension analysis revealed an RNA molecule with the same 5' end as that detected in vivo (Fig. 5), indicating that the 5' end found in vivo is the direct result of RNase III cleavage. [A number of additional bands are observed in the primer extension experiments in both Figures 3 and 5. In contrast to bands AA and BB, most of these bands cannot be correlated with fragments detected in experiments with radioactive probes (see below) and must be artifacts of the primer extension procedure.]

Figure 4. RNase III cleavage sites on *cII-O* mRNA, as determined by primer extension analysis. The *top* strand shows *cII-O* mRNA sequences in the region of complementarity to the 5' end of OOP RNA. The *bottom* strand shows the complementary OOP RNA sequence. The OOP RNA of pLK40 has an internal deletion of 13 nucleotides as indicated. (Note that the deletion ends may be moved two nucleotides toward the left without alteration of sequence.) The RNase III cleavage sites are indicated for wild-type OOP RNA and OOP RNA from pLK40.

cII-O mRNA levels

The primer extension experiment suggests that in the presence of OOP RNA, RNase III cleaves the *cII-O* transcript into two half molecules. One of these molecules has a 5' end at the cleavage site and extends into the *O* gene, whereas the second has a 3' end at the cleavage site and extends the opposite direction into the *cII* gene. We looked for these molecules using radioactive RNA probes in a ribonuclease protection experiment. Two probes complementary to overlapping *cII-O* mRNA sequences were used (Fig. 1). Probe I is complementary to 150 nucleotides of *cII-O* mRNA, including the final 68 nucleotides at the 3' end of the *cII* gene, the 32-nucleotide intercistronic region between the *cII* and *O* genes, and the first 50 nucleotides at the 5' end of the *O* gene. Probe II is a truncated version of probe I that lacks most of the region complementary to *cII* mRNA.

With the control plasmid in an RNase III⁺ strain, the predominant *cII-O* fragment detected by probe I extends the full length of the probe (Fig. 6, lane 9). With OOP overproduction provided by either pLK39 (*p_o*; 77-nucleotide OOP RNA) or pLK67 (*p_L*; 79-nucleotide OOP RNA transcribed from the strong λ *p_L* promoter), the 150-nucleotide fragment corresponding to uncut *cII-O* mRNA almost disappears, and a new band appears at ~73 nucleotides (Fig. 6, band A, lanes 10 and 12). With pLK40 (Δ ; OOP RNA with an internal deletion) a cluster of bands of ~95 nucleotides appears (Fig. 6, band B, lane 11) instead of the 73-nucleotide band. These are the expected lengths for fragments extending into *O* gene sequences that begin at the cleavage sites determined by the primer extension experiment (Fig. 3; e.g., band A of Fig. 6 corresponds to band AA of Figs. 3 and 5, and band B corresponds to band BB, respectively). [The cluster of bands observed with pLK40 (Δ) is not inconsistent with the single band observed in the primer extension experiment if the probe is trimmed to varying degrees by the ribonuclease mixture following hybridization to cellular mRNA. Band A also consists of a cluster of bands, including a major band at ~73 nucleotides and satellite bands ~71, 72, and 77 nucleotides. These satellite bands could represent a true heterogeneity in the mRNA endpoints, or they could be artifacts resulting from trimming of the radioactive probe to varying degrees by the

ribonuclease mixture following hybridization to cellular RNA. RNase III-dependent, OOP RNA-dependent satellite bands are detected in the primer extension experiments that correspond to the 72- and possibly 71-nucleotide bands, but not to the 77-nucleotide band.]

This identification of bands is confirmed by hybridization experiments to probe II. With the control plasmid in an RNase III⁺ strain, the predominant *cII-O* fragment extends the full length of the probe (Fig. 6, band A, lane 1). With pLK39 (*p_o*) or pLK67 (*p_L*), the 85-nucleotide fragment corresponding to uncut *cII-O* mRNA is greatly reduced, and a greatly enhanced band appears at precisely the same position as with probe I (Fig. 6, band A, lanes 2 and 4). A band that appears at an identical position with both probes I and II is indicative of an mRNA fragment extending into the *O* gene that has a 5' end in the region of complementarity to the two probes (Fig. 7). With pLK40 (Δ ; OOP RNA with an internal deletion), the predominant fragment extends the full length of probe II (Fig. 6, lane 3), as would be predicted, as in the case of pLK40 the presumptive RNase III cleavage site on *cII-O* mRNA lies outside the region of complementarity to this second probe (Fig. 9).

Figure 5. Cleavage of *cII-O* mRNA with RNase III in vitro. Cellular RNA, isolated from the RNase III⁻ strain UC6171 *mc-105 glyA::Tn5* carrying a λ *int-6 cIts857 cro27* Pam3 prophage and the OOP DNA plasmid pLK39, was treated with purified RNase III in vitro and analyzed with a primer extension experiment as described in Fig. 3. (Lane 1) Treated RNA; (lane 2) the same RNA without enzyme treatment; (lane 3) RNA isolated from the isogenic RNase III⁺ strain; (lanes 4–7) G, A, T, and C sequencing reactions, respectively. Band AA is at the same position as band AA in Fig. 3.

Figure 6. Ribonuclease protection analysis of *cII*-O mRNA levels in isogenic RNase III⁺ and RNase III⁻ lysogenic strains that carry various OOP DNA plasmids. Total RNA from induced lysogens, carrying the OOP DNA plasmids described in Fig. 3, was hybridized to probe I (lanes 9–16) or probe II (lanes 1–8) (Fig. 1). These probes are complementary to 150 and 86 nucleotides of *cII*-O mRNA, respectively (Fig. 1). RNA was digested with RNase A and RNase T1 for 60 min and separated by electrophoresis on a polyacrylamide sequencing gel in 8 M urea. The strains UC6171 (lanes 1–4 and 9–12) and UC6171 *rnc*-105 *glyA* :: Tn5 (lanes 5–8 and 13–16) carry a *λ*int-6 *cl*ts857 *cro*27 *P*am3 prophage and plasmids pLK38 (deleted OOP promoter; lanes 1, 5, 9, and 13), pLK39 (wild-type OOP DNA; lanes 2, 6, 10, and 14), pLK40 (OOP DNA with a 13-bp internal deletion; lanes 3, 7, 11, and 15), or pLK67 (OOP DNA with the *λ* *p*_L promoter; lanes 4, 8, 12, and 16). Approximate fragment lengths indicated at left and right were determined from migration of fragments of known length in several different experiments and by measuring distances between adjacent bands differing by a single nucleotide.

The primary cleavage event in the region of overlap with OOP RNA is at the same site in *cII*-O mRNA for both the 77-nucleotide *p*₀ OOP RNA and the 79-nucleotide *p*_L OOP RNA (Fig. 6). The 13-nucleotide distance from the end of the overlap region that was determined with *p*₀ OOP RNA in the primer extension experiments (Figs. 3 and 5) is therefore a 15-nucleotide distance with *p*_L OOP RNA. In addition to the fragments that extend from the cleavage site into O gene sequences that were

detected by both the primer extension experiment and the RNA probe experiment, sister fragments should also be generated that extend in the opposite direction into *cII* gene sequences. Using the 150-nucleotide probe I, these sister fragments should be 77 nucleotides in length in experiments with pLK39 (*p*₀) and pLK67 (*p*_L) and 55 nucleotides in length in the experiment with pLK40 (*Δ*), and they should not be detectable with probe II because they lie mostly or entirely outside the region complementary to probe II. No such fragments can be detected (Fig. 6, lanes 10–12). (The artifact band of 77 nucleotides is seen with both probes I and II; see above.) This indicates that the sister fragments are rapidly degraded, either by additional RNase III cuts or through other nucleolytic processes.

In summary, RNA probe experiments in RNase III⁺ strains indicate that OOP RNA leads to cleavage of *cII*-O mRNA at ~13 nucleotides from the 3' end of the region of continuous complementarity. The resultant fragments with 5' ends that extend into the O gene are relatively stable, while the fragments with 3' ends that extend into the *cII* gene are subject to additional nucleolytic events and cannot be detected.

Hydrolysis of cII-O mRNA in RNase III-deficient strains

In RNase III⁻ strains, the most intense bands correspond to the full length of the probe with both probes I and II, which demonstrates the RNase III dependence of the major hydrolytic events. An intriguing set of additional bands appears with probe I in the presence of OOP RNA in RNase III⁻ strains that are not seen in RNase III⁺ strains. These bands are seen most easily in Figure 8, where lanes 1, 3, 5, and 7 are identical to lanes 13, 14, 15, and 16, respectively, of Figure 6. (Fig. 8, lanes 2, 4, 6, and 8, shows that RNA transcription in the reverse direction from the multicopy OOP DNA plasmids accounts for only a small fraction of the RNA that hybridizes to probe I.) Each of the three different OOP DNA plasmids gives rise to two clusters of bands. These include band clusters of ~94–102 nucleotides (most intense at 96 nucleotides), and 72–78 nucleotides (most intense at 75–77 nucleotides) for pLK39 (*p*₀; wild-type OOP RNA) (Fig. 8, bands D and F, lane 3), 71–72 and 55–57 nucleotides for pLK40 (*Δ*; OOP RNA with an internal deletion) (Fig. 8, bands G and H, lane 5), and 96–104 nucleotides (most intense at 99 nucleotides) and 76–81 (most intense at 80 nucleotides) for pLK67 (*p*_L; OOP RNA with the strong *p*_L promoter) (Fig. 8, bands C and E, lane 7). (The clusters of bands could be real representations of fragments from *cII*-O mRNA or an artifact due to variable trimming of the radioactive probe RNA by the nuclease mixture after hybridization to cellular RNA.) These bands are not observed with probe II, suggesting that they contain extensive *cII* gene sequences. If the bands are generated by hybridization to mRNA fragments with 3' ends in the *cII*-O region that extend into the *cII* gene (Fig. 9), then hybridization to probe II should yield mostly bands that are too short for detection. How-

Figure 7. Origins of bands from hybridization of RNA from RNase III⁺ strains to probes I and II. (A and B) Radioactive RNA bands from Fig. 6. The length of radioactive probe participating in a double-stranded complex is shown for each band.

ever, two clusters of bands, one of ~33–35 nucleotides with probe II and pLK67 (p_L) (Fig. 6, band J, lane 8), and the second of ~30–32 nucleotides with probe II and pLK39 (p_O) (Fig. 6, band K, lane 6), are equivalent to the two longest bands, C and D, respectively, observed with probe I (Fig. 9) and support this interpretation of the banding pattern. The shorter cluster of bands in each pair arises from cleavage at a site near the RNase III-dependent site, whereas the longer cluster of bands results from cleavage several nucleotides beyond the region of complementarity between *cII-O* mRNA and the OOP RNA derivative. Sister bands to this set of bands, namely those with 5' ends in the region hybridizing to the probe and extending into *O* gene sequences, cannot be detected, indicating that they are degraded by additional hydrolytic events. [The sister bands to the most intense band in each cluster should be 44 nucleotides (band C), 73–75 nucleotides (band E), 41 nucleotides (band D), 70 nucleotides (band F), 78–79 nucleotides (band G), and 93–95 nucleotides (band H). Except for the last band, the sister bands should appear with both probes I and II (see Fig. 9).]

In summary, in the absence of RNase III, an alternate OOP RNA-dependent cleavage process results in cleavage in either of two regions, one located near the RNase III-dependent cleavage site and the second located in a single-stranded region beyond the region of complementarity between *cII-O* mRNA and OOP RNA. The *cII* gene fragments arising in this alternate event are stable, whereas the *O* gene fragments are degraded.

mRNA levels upstream and downstream from the region of complementarity to OOP RNA

It is shown in the preceding section that primary nucleolytic events in the region of complementarity of *cII-O* mRNA to OOP RNA are followed in some cases by subsequent nucleolytic events leading to degradation of longer segments of *cII-O* mRNA. We used two probes to investigate whether these subsequent nucleolytic events proceed hundreds of nucleotides on either side of the primary nucleolytic event. One probe (5' *cII* probe; Fig. 1) detects the first 96 nucleotides of *cII* mRNA, 145–240 nucleotides to the 5' side of the region of complementarity to OOP RNA. The second probe (*O* probe; Fig. 1) detects nucleotides 379–488 of the *O* gene, 389–498 nucleotides to the 3' side of the region of complementarity to OOP RNA. The results (Fig. 10) reveal much smaller RNase III-dependent effects on mRNA levels at these distances from the region of complementarity to OOP RNA.

Reproducible differences in mRNA levels in these regions are, however, observed. Band intensities are always greatest when OOP RNA is not overproduced from a plasmid [e.g., with pLK38 (–)], which may reflect OOP RNA-dependent cleavages that occur in both RNase III⁺ and RNase III[–] strains or, alternatively, a competition between plasmid and phage promoters for RNA polymerase. In the presence of a multicopy plasmid, bands are usually more intense in RNase III[–] than in RNase III⁺ strains, although differences are not

Figure 8. Ribonuclease protection analysis of *cII*-*O* mRNA in RNase III⁻ strains with various OOP DNA plasmids in the presence or absence of a prophage. Total RNA was hybridized to probe I, digested with RNase A and RNase T1 for 60 min, and separated by electrophoresis on a polyacrylamide sequencing gel in 8 M urea. The strains UC6171 *mc105 glyA* :: Tn5 with a λ int-6 *cts857 cro27* Pam3 prophage (lanes 1, 3, 5, and 7) and UC6689 [an RNase III⁻ derivative of UC6444, which lacks a prophage but has a λ N *cts857* DNA fragment inserted into the *galK* gene; Krinke and Wulff 1990] (lanes 2, 4, 6, and 8) carry plasmids pLK38 (deleted OOP promoter; lanes 1 and 2), pLK39 (wild-type OOP DNA; lanes 3 and 4), pLK40 (OOP DNA with a 13-bp internal deletion; lanes 5 and 6), and pLK67 (OOP DNA with the λ *p_L* promoter; lanes 7 and 8). Approximate fragment lengths indicated at right were determined as in Fig. 6.

large. This could result from a limited RNase III-dependent degradation in these regions, possibly due to initial RNase III cleavages in the OOP region.

The 5'-*cII* mRNA level is about two to three times higher in an RNase III⁻ than in an RNase III⁺ cell when OOP RNA is not overproduced from a plasmid (Fig. 10a, lanes 1 and 2). Under the same conditions, *O* mRNA levels show little variation (Fig. 10b, lanes 1 and 2). Possible explanations include internal folding of *cII* mRNA to form regions sensitive to RNase III in the area detected by the probes or subsequent degradation of this region following initial cleavage of double-stranded regions elsewhere in the mRNA. These double-stranded regions could result from pairing to the endogenous OOP antisense RNA produced by the prophage or pairing to the *p_{RE}* transcript.

Discussion

These experiments support a simple model for RNase III-dependent degradation of *cII*-*O* mRNA. Pairing of OOP RNA with *cII*-*O* mRNA is followed by RNase III cleavage of *cII*-*O* mRNA at a site on the double-stranded complex that is 13 nucleotides from the 3' end of *cII*-*O* mRNA. This yields two product molecules: one that contains the entire *cII* gene and ends with a 3' end at the cleavage site, and the second that begins with a 5' end at the cleavage site and contains the entire *O* gene and its ribosome recognition region. OOP RNA may be cleaved as well, but this has not been investigated. The *O* gene fragment is relatively stable, but the *cII* gene fragment is subject to additional nucleolytic events, including possible additional RNase III cleavages at other sites. The degradation of *cII* gene mRNA is incomplete, and sequences at the 5' end of the gene are affected less. There are several examples in *Escherichia coli* and its phages where cleavage by RNase III or other endonucleases initiate mRNA degradation (Schmeissner et al. 1984; Cannistraro et al. 1986; Plunkett and Echols 1987; Portier et al. 1987; for review, see Belasco and Higgins 1988; Bardwell et al. 1989). Endonucleolytic events are thought to be the rate-limiting factor in mRNA degradation. These endonucleolytic events are followed by exonucleolytic degradation in the 3' → 5' direction, probably by polynucleotide phosphorylase and RNase II (Donovan and Kushner 1986). No exonuclease with 5' → 3' directionality has yet been found in *E. coli*. These events can lead to differential stability of the two mRNA fragments after initial cleavage (Panayotatos and Truong 1985; Newbury et al. 1987), usually with the fragment on the 5' side of the cleavage site being considerably less stable.

The experiments reported here provide no direct information on the initial pairing between OOP RNA and *cII*-*O* mRNA but strongly support the other features of the model. The finding of a cleavage site 13 nucleotides from an end of a complementary region (15 nucleotides for the 79-nucleotide *p_L* OOP RNA) is in agreement with reported cleavage sites with *sar* antisense RNA (Liao and McClure 1988) and *copA* antisense RNA (Blomberg et al. 1990). The creation of a relatively stable fragment containing the complete ribosome recognition region and coding region of the *O* gene could be important physiologically if this truncated *O* gene fragment is translated at a greatly different rate from full-length *cII*-*O* mRNA. The effect of OOP RNA on *O* gene expression has not been investigated.

The OOP RNA-dependent, RNase III-dependent degradation of λ *cII* mRNA parallels the *sib*-dependent, RNase III-dependent degradation of λ int gene mRNA (Schmeissner et al. 1984; Plunkett and Echols 1989). In this latter case, transcription through the *sib* region, which is distal to the *int* gene, leads to hydrolysis by RNase III in the *sib* region. Secondary degradative events generate a shortened RNA ending within the *int*-coding sequence.

RNase III-dependent hydrolysis of *cII*-*O* mRNA is observed when the sole source of OOP RNA is from the

Figure 9. Origins of bands from hybridization of RNA from RNase III⁻ strains to probes I and II. (C–J) Radioactive RNA bands from Figs. 6 and 8. The length of radioactive probe participating in a double-stranded complex is shown for each band.

induced prophage (Fig. 3, lane 5), suggesting that OOP RNA-dependent degradation of *cII* mRNA may occur during normal λ growth. In infections by an OOP⁻ λ mutant, which has a strong down mutation in the OOP promoter, the steady-state level of mRNA at the 3' end of the *cII* gene is severalfold higher than in λ^+ infections (L. Krinke, M. Mahoney, L. Yelich, and D. Wulff, unpubl.), confirming that OOP RNA has an appreciable influence on mRNA levels during normal λ growth.

In the absence of RNase III, *cII*-O mRNA is subject to an alternate cleavage process that is also dependent on OOP RNA. The alternate cleavage process differs from the RNase III-dependent process in several important respects. (1) With each of the three OOP RNAs, the alternate process results in cleavage at two sites (or two clusters of sites). One site is near but not necessarily identical to the corresponding site of RNase III cleavage, and the second site lies several nucleotides beyond the end of the complementary region, a region that is presumably single stranded where RNase III never cleaves. (2) The 77-nucleotide ρ_0 OOP RNA and the 79-nucleotide ρ_L OOP RNA result in banding patterns that are slightly shifted with respect to each other, suggesting that the alternate cleavage process is keyed to the precise end of complementarity between OOP RNA and *cII*-O mRNA. In contrast, the RNase III-dependent cleavage site is identical for both ρ_0 and ρ_L OOP RNAs. (3) In the alternate pathway, cleavage products with 3'

ends that extend into the *cII* gene are relatively stable, whereas the products with 5' ends that extend into the O gene cannot be detected—exactly the opposite to what is observed in the RNase III-dependent process. These observations raise intriguing questions. What enzyme mediates the secondary nucleolytic process, and what determines its specificity? Why do the products of RNase III⁺ digestion yield relatively stable O mRNA fragments and unstable *cII* mRNA fragments while the products of the minor nucleolytic process have the reverse order of stability? Because no exonucleases with a 5' $\rightarrow$ 3' specificity are known, the undetected fragments with 5' ends that extend into the O gene are presumably subject to additional endonucleolytic attack. Where is this presumed additional cleavage site, what is the responsible enzyme, and what determines its cleavage specificity?

Materials and methods

Bacteria and plasmids

E. coli bacterial strains are UC6171 (F⁻ *galK*⁻ *hfl*-1 *str*⁻ *sup*^o) (Fien et al. 1984) and its isogenic RNase III⁻ derivative UC5993 (F⁻ *galK*⁻ *hfl*-1 *enc105* *glyA* :: Tn5 *str*⁻ *sup*^o), both of which are lysogenic for *lnt6* *clts857* *cro27* Pam3 (Krinke and Wulff 1987). The OOP DNA plasmids pLK38, pLK39, pLK40 (listed as Δ 11), and pLK67 are described by Krinke and Wulff (1987). The radio-

Figure 10. Ribonuclease protection analysis of *cII* (a) and *O* (b) mRNA levels in isogenic RNase III⁺ and RNase III⁻ lysogenic strains that carry various OOP DNA plasmids. Total RNA from induced lysogens, carrying the OOP DNA plasmids described in Fig. 3, was hybridized to the 5' *cII* probe or the *O* probe (Fig. 1). These probes are complementary to the first 96 nucleotides of *cII* mRNA and nucleotides 376–498 of *O* mRNA, respectively (Fig. 1). RNA was digested with RNase A and RNase T1 for 60 min and separated by electrophoresis on a polyacrylamide sequencing gel in 8 M urea. The strains UC6171 (odd-numbered lanes) and UC6171 *rnc*-105 *glyA* :: Tn5 (even-numbered lanes) carry a λ int-6 *cl*ts857 *cro*27 Pam3 prophage and plasmids pLK38 (deleted OOP promoter; lanes 1 and 2), pLK39 (wild-type OOP DNA; lanes 3 and 4), pLK40 (OOP DNA with a 13-bp internal deletion; lanes 5 and 6), or pLK67 (OOP DNA with the λ ρ_L promoter; lanes 7 and 8).

active RNA probes were made using plasmids that contain fragments of λ DNA inserted into the polylinker region of the vector plasmid pGEM-1 (Promega Biochemicals).

Isolation of cellular RNA

Strains carrying an OOP DNA plasmid and a temperature-sensitive prophage were grown to 1.6×10^8 cells/ml at 32°C and shifted to 42°C. At 24 min chloramphenicol was added to 100 μ g/ml, and at 25 min, the cultures were chilled in ice water. The cells were collected by centrifugation, washed twice with 10 mM Tris-Cl (pH 7.3) + 100 μ g/ml chloramphenicol, and frozen at -80°C. RNA was extracted as described by McKenney et al. (1981): Pellets were resuspended in lysis buffer [10 mM Tris-Cl (pH 7.3), 1 mM EDTA, 10 mM DTT, 200 U/ml RNase inhibitor, and 270 mg/ml lysozyme], frozen at -80°C,

and thawed at room temperature three times. The extracts were digested with DNase I (ribonuclease free) (final concentration, 250 U/ml) in 25 mM magnesium acetate for 45 min at 0°C. Acetic acid was added to 10 mM and SDS to 0.5%. After 5 min at room temperature, RNA was purified by two phenol extractions, chloroform extraction, two ethanol precipitations at -20°C, and a wash with 70% ethanol. After desiccation the RNA was resuspended in water at ~5 μ g/ μ l. RNA concentrations were measured by determining absorbance at 260 nm.

Ribonuclease protection experiments with radioactive probes

Ribonuclease protection experiments were done essentially as described by Gilman (1989). To prepare ³²P-labeled RNA probes, plasmid DNAs were first linearized with a restriction enzyme. RNA was synthesized in 50 mM Tris-Cl (pH 7.3); 8 mM MgCl₂; 2 mM spermidine; 50 mM NaCl (omitted with SP6 RNA polymerase); 0.4 mM ATP, UTP, and GTP; 1.2 U/ μ l RNase inhibitor; 100 μ ci of [α -³²P]CTP (600–800 Ci/mM); 1 μ g linearized template DNA; and 10 units T7 or SP6 RNA polymerase in a total volume of 25 μ l for 1 hr at 37°C (T7 RNA polymerase) or 42°C (SP6 RNA polymerase). DNA templates were removed by DNase I treatment (10 units) for 15 min at 37°C. RNA was phenol-extracted, ethanol-precipitated, and purified on a denaturing gel. Following brief exposure to X-ray film, the radioactive RNA band was visualized, cut, diced, and eluted overnight on a rotary wheel into 2 ml gel elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS) with 20 μ g tRNA as a carrier. The acrylamide was removed by centrifugation, and the RNA was purified by phenol extraction and two ethanol precipitations, followed by washing with 75% ethanol/25% of 0.1 M sodium acetate, desiccation, and resuspension in hybridization buffer [80% formamide, 20% of 40 mM PIPES (pH 6.4), 1 mM EDTA].

A mixture of 30 μ l of purified RNA probe (5×10^5 cpm) in hybridization buffer and 1 μ l of RNA template (1 μ g/ μ l) in TE buffer was incubated at 85°C for 5 min and then overnight at 45°C. To each sample was added RNase A (40 μ g/ml) and RNase T1 (12 U/ml) in 350 μ l RNase digestion buffer [10 mM Tris-Cl (pH 7.3), 300 mM NaCl, 5 mM EDTA]. Following incubation for 1 hr at 30°C (probes I and II and 5' *cII* probe) or 37°C (OOP probe and *O* probe), SDS (final concentration, 0.5%) and proteinase K (50 μ g) were added. After 15 min at 37°C, the samples were extracted with phenol, precipitated with ethanol (using 20 μ g glycogen as carrier), desiccated, and resuspended in 5 μ l of RNA loading buffer (80% formamide/20% of 1 mM EDTA + 0.1% xylene cyanol). RNA bands were separated on denaturing polyacrylamide gels [10% acrylamide, 50 mM Tris-Cl, 50 mM boric acid, 1 mM EDTA, 8 M urea (pH 8.3)] and visualized by autoradiography. Under these conditions the intensity of the bands was proportional to the quantity of cellular RNA added to the hybridization mixture. A control substituting tRNA for cellular RNA was run with each probe.

Primer extension analysis

Primer extension analyses were performed according to Kingston (1987). A DNA oligonucleotide with the sequence 5'-GCCACATTACGCTCCTG-3', which is complementary to nucleotides 48–64 of the *O* gene, was labeled by incubation with [λ -³²P]ATP (3000 Ci/mM) and polynucleotide kinase. [A DNA oligonucleotide complementary to OOP RNA was used to determine the 5' ends of OOP RNA and ρ_L OOP RNA.] The labeled primer was precipitated three times with ethanol, desiccated, and dissolved in 100 μ l 0.3 M sodium acetate. For hybridizations, 5×10^4 cpm of labeled oligonucleotide and 30 μ g

purified cellular RNA were ethanol-precipitated, air-dried, resuspended in 30 μ l aqueous hybridization solution [3 M NaCl, 0.5 M HEPES (pH 7.5), 1 mM EDTA], and incubated overnight at 30°C. The next day 170 μ l of 0.3 M sodium acetate was added to each sample, followed by ethanol precipitation and air-drying. Samples were resuspended in RT mix [50 mM Tris-Cl (pH 8.0), 5 mM MgCl₂, 5 mM DTT, 50 mM KCl, 50 μ g/ml BSA, 4 mM each of dATP, dCTP, dGTP, and TTP, 30 units RNase inhibitor] and incubated for 90 min at 42°C with reverse transcriptase. After addition of EDTA to 20 mM and 1 μ g RNase A, each sample was incubated for 30 min at 37°C, followed by the addition of 100 μ l 2.5 M ammonium acetate and ethanol precipitation. Each sample was washed with 70% ethanol, desiccated, and resuspended in 3 μ l TE buffer and 4 μ l Sequenase loading buffer. Samples were run on a 10% polyacrylamide denaturing gel in 8 M urea alongside a DNA sequencing ladder generated by the dideoxy method with Sequenase using the same ³²P-labeled primer and single-stranded cII-O mRNA.

RNase III enzyme reactions

Purified cellular RNA (30 μ g) was incubated with 3 μ l purified RNase III (a gift of Professor H. Robertson) in 100 μ l RNase III buffer [100 mM NH₄Cl, 10 mM magnesium acetate, 20 mM Tris-Cl (pH 7.5)] for 1 hr at 37°C [Robertson et al. 1968]. The RNA was phenol-extracted, ethanol-precipitated, washed with 70% ethanol, air-dried, and subjected to primer extension analysis.

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