Transcriptional control via translational repression by *c4* antisense RNA of bacteriophages P1 and P7

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The *c4* repressors of bacteriophages P1 and P7 are antisense RNAs that inhibit antirepressor synthesis. This antisense inhibition is unusual in that the *c4* repressor and the repressed genes *orfx* and *ant* are cotranscribed in that order from the same promoter, and *c4* RNA is processed from a precursor RNA. Here, we show that *c4* RNA directly represses translation of *orfx*, a small open reading frame, to which *ant* is translationally coupled. This translational repression blocks *ant* transcription via a ρ -dependent terminator. Thus, *c4* RNA controls expression of the *ant* gene by a novel indirect mechanism combining translational repression, translational coupling, and ρ -dependent termination.

[Key Words: *ant* mRNA; *c4-orfx* regulatory genes; *immI* proteins; ρ termination; site-directed mutagenesis; translational coupling]

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During the last few years, antisense inhibition of gene expression has been demonstrated in a wide variety of systems, and several antisense RNAs in prokaryotes have been analyzed in detail. Antisense inhibition of gene expression is generally post-transcriptional; however, two cases of direct inhibition of target transcription by antisense RNA have been demonstrated. In *Escherichia coli*, *tic* RNA interferes with transcription of the cAMP receptor protein (Okamoto and Freundlich 1986); and in the plasmid pT181, RNA I inhibits synthesis of the plasmid-encoded *repC* mRNA (Novick et al. 1989). In a typical antisense system (except for the *micF* system in *E. coli*; Mizuno et al. 1984), antisense and target RNA are transcribed from two different promoters, which are in opposite orientations. The resulting divergent transcription of antisense and target RNA leads to complementarity along the entire overlapping region (for review see Simons and Kleckner 1988; Takayama and Inouye 1990; Eguchi et al. 1991).

We have recently described a novel antisense system in the immunity region *immI* of the temperate bacteriophages P1 and P7 (for all but selected references on P1 and P7, see Yarmolinsky and Sternberg 1988). Here, synthesis of an antirepressor, the product of the *ant* gene, is repressed by the product of the *c4* gene. The *c4* repressor is an antisense RNA. This system is novel, because antisense and target RNAs are transcribed from the same promoter. In addition, the *c4* RNA is generated by processing of a precursor RNA. Mature *c4* RNA of P1 has

been identified as a 77 ± 1 -base RNA (see Fig. 2, below), and P7 *c4* RNA has been found to have the same or a similar size as P1 *c4* RNA. Antisense RNA function is exerted by binding to sequences downstream in the target RNA (Citron and Schuster 1990, 1992).

The organization of the P1 *immI* region has been described previously (Baumstark and Scott 1987; Hansen 1989; Heisig et al. 1989). Two antirepressor proteins Ant1 and Ant2 are translated from a single open reading frame, with the smaller protein initiating at an in-frame start codon. An open reading frame, *orfx*, of unknown function overlaps the start of *ant1* and is preceded by a presumptive ribosome-binding site (Figs. 1 and 2). Intactness of *orfx* is a prerequisite for the expression of *ant1*. This is demonstrated by the existence of a P1 *orfx16* mutant that is phenotypically *ant*⁻ (Fig. 2; Heisig et al. 1989). The *c4* gene is located upstream of *orfx*; and a tandem promoter P51a/b and a *c1* repressor-controlled operator, Op51, overlapping the -35 region of P51a, are located in front of the *c4* gene (Baumstark and Scott 1987; Heisig et al. 1989). It is important to note that *c4*, *orfx* and *ant1/2* are cotranscribed in that order and that transcription starting from P51b is sufficient to express *c4* and *ant*, the latter only if *c4* cannot act (Heisig et al. 1989).

P1 and P7 are closely related, but heteroimmune and the immunity difference has been mapped to the *c4* gene region. It has therefore been postulated that the *c4* repressor and its target are phage specific (Chesney and Scott 1975; Scott et al. 1977; Wandersman and Yarmolinsky 1977). *c4* RNA contains two short sequence elements *a'* and *b'* that are complementary to the down-

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Figure 1. The *immI* operon of P1/P7. (Top) The *immI* region contains in that order the *cI*-controlled operator Op51 (○), the tandem promoter P51a/b (arrowheads), and the genes *c4*, *orfx*, and *ant1/ant2* (open regions with a vertical broken line marking the start of *ant2*). In a P1 or P7 lysogen, transcription of the *immI* operon starts from P51b (+1 position). Op53 controls the lytic replicon. Its first gene, *kilA*, has been characterized by Hansen (1989). Transcripts found in P1/P7 lysogens and/or infected bacteria are represented by wavy lines (with the broken line indicating a ρ -dependent transcript termination). The angular arrow indicates the site of *c4* RNA action. (Center) Plasmids used in this study. Horizontal thin, thick, and combined thick/thin lines represent P1, P7, and P7–P1 hybrid DNA, respectively, inserted into different vectors. Natural and/or artificial mutations are indicated above the lines. Plasmids in parentheses contain the mutation (in parentheses) additionally. (Bottom) P1 DNA restriction enzyme fragments used as probes for Northern blots are indicated by horizontal lines. Numbers above the lines indicate the nucleotide positions of the cleavage sites of the restriction enzymes shown at the top.

stream target RNA sequences a2 and b2 (Fig. 2). Complementarity of a'/a2 and b'/b2 is essential for *c4* action. The heteroimmunity of P1 and P7 is the result of just two substitutions in each of the complementary sequences (Fig. 2). We have suggested that b'/b2 base-pairing blocks the Shine–Dalgarno sequence of *orfx*, thereby also blocking *ant* translation. This would mean that *c4* RNA action is solely post-transcriptional. A switch to *ant* expression may involve a flip-flop from an a'/a2 and b'/b2 to an a'/a1 and b'/b1 base-pairing (Fig. 2), thereby unmasking the ribosome-binding site of *orfx* (Citron and Schuster 1990).

Here we analyze the transcriptional regulation of the *immI* region. We identify the *ant* mRNA of P1 and P7 as a 1.5-kb transcript starting at the promoter P51b and covering the entire *immI* region. We show that transcription of *ant* mRNA is regulated by *c4*, which is constitutively synthesized. *c4* RNA acts as a translational repressor of *orfx*. This inhibition of *orfx* translation blocks *ant* transcription via a ρ -dependent terminator. Thus, *c4* RNA indirectly represses transcription of *ant* by blocking translation of *orfx*.

Results

c4 RNA represses *orfx* translation

To provide direct evidence that *c4* blocks translation of *orfx* mRNA, we constructed an in-frame *lacZ* fusion

plasmid using the translational fusion vector pNM480 (Minton 1984). This vector contains a *lacZ* gene that lacks both an initiation codon and a Shine–Dalgarno sequence. We inserted into the *Sma*I site of this vector a *Pvu*II–*Nla*IV fragment from pAH1018, which contains the T7 ϕ 10 promoter but not the ribosome-binding site and P1 sequence from position 308 to 505 (Fig. 2). In the resulting plasmid pNMC1 a hybrid *orfx*–*lacZ* frame is transcribed from the T7 ϕ 10 promoter if T7 RNA polymerase is present. The transcript contains the *c4* RNA target sites a2 and b2 and is translated from the Shine–Dalgarno sequence of *orfx*. T7 RNA polymerase was supplied in *trans* by a pGP plasmid (Table 1). β -Galactosidase activity is detectable when T7 RNA polymerase is supplied by plasmid pGP1-2. If the T7 RNA polymerase plasmid also produces P1 *c4* RNA (pGP1), however, β -galactosidase activity is reduced to background level. In contrast, no reduction of β -galactosidase activity is observed if the T7 RNA polymerase plasmid produces P7 *c4* RNA (pGP7). This indicates that the effect is target specific and that *c4* RNA does not interfere with T7 RNA polymerase function. In a Northern blot, all pNMC1-containing cells used for β -galactosidase assays showed the same hybridization pattern with the *ant* probe (Fig. 1; data not shown) indicating that *c4* RNA does not affect synthesis or stability of the *orfx*–*lacZ* fusion RNA, which is transcribed by T7 RNA polymerase. Therefore, we conclude that *c4* RNA directly blocks translation of *orfx*.

for the binding of *E. coli* ribosomes (Shine-Dalgarno) is shown below the P1 sequence; the positions where the P1 sequence is identical to, or divergent from, the consensus sequence are indicated by uppercase and lowercase letters, respectively. The *orfx* gene and the beginning of the *ant1* gene are framed.

ant mRNA is indirectly controlled by *c4* RNA

The model of a post-transcriptional action of *c4* RNA on its target mRNA predicts in its simplest form that two kinds of *immI* transcripts should be detectable in P1-infected cells or P1 lysogens: (1) a small *c4* RNA encompassing the antisense sequences *a'* and *b'* (and possibly its nonprocessed precursor) and (2) a rather large mRNA ~1.5 kb in size, which comprises the complementary target sequences *a2* and *b2* and the entire *ant* gene (Figs. 1 and 2). To test this, *E. coli* C600 bacteria were infected with P1wt, P1vir^s, or P1vir^s*orfx16* and were subjected to a Northern blot analysis using the *c4* probe (Fig. 1). In bacteria infected with P1wt, however, no 1.5-kb transcript can be seen (Fig. 3A). Instead, there are two smaller hybridizing RNA species, ~200 and 80 bases in size. The smaller one is mature *c4* RNA, whereas the larger one

most probably is a mixture of *c4* precursor molecules (starting at the promoter P51a or P51b). Mature *c4* RNA was recently identified as a 77 ± 1 -base RNA (Citron and Schuster 1992). A large RNA of the expected size is only

Figure 3. (A) Northern blot analysis of P1wt-, P1vir^s-, and P1vir^s*orfx16*-infected C600. Bacteria were grown at 37°C to $A_{600} = 0.2$ and infected with phages at a m.o.i. of 2 for 40 min. Total RNA (20 μ g) was subjected to 1.0% agarose glyoxal gel electrophoresis and hybridized to the *c4* probe (Fig. 1). Approximate RNA sizes indicated at the left were determined with the 0.16- to 1.77-kb RNA ladder (Bethesda Research Laboratories) run in parallel. (B) Northern blot analysis of P1wt and P7c4.2ts lysogens of C600 and of plasmid carrying SCS1 bacteria. The latter carried the pUA plasmid as indicated and plasmid pAC8. Bacteria were grown at 30°C to logarithmic phase. An aliquot of each culture was heat induced for 20 min at 42°C. Total RNA from heat-induced (+, 42°C) and uninduced (–, 30°C) bacteria was subjected to 1.5% agarose glyoxal gel electrophoresis, and the RNA was hybridized to the *ant* probe (Fig. 1). The same markers as in A were used (not shown).

Table 1. β -Galactosidase assay of *orfx*–*lacZ* fusion constructs

Plasmids	<i>c4</i> RNA	β -Galactosidase activity (units)
pNM480 + pGP1-2	—	<1
pNMC1 + pGP1-2	—	24 ± 4
pNMC1 + pGP1	P1	<1
pNMC1 + pGP7	P7	56 ± 6

E. coli CB454 bacteria carrying the plasmids as indicated were assayed for β -galactosidase activity as described in Materials and methods. All pGP plasmids express T7 RNA polymerase and *c4* RNA of P1 or P7 as indicated. pNM480 + pGP1-2 was used as a negative control.

seen in P1vir^s-infected bacteria (Fig. 3A). Here, antirepressor (and therefore also *ant* mRNA) is synthesized constitutively (Heilmann et al. 1980), because functionally active *c4* RNA cannot interact properly with its target because of the *vir^s* mutation (Fig. 2; Citron and Schuster 1990). The size of *ant* mRNA corresponds with an expected start at P51b and termination behind the end of the *ant* gene. The *ant* mRNA start site of an induced lysogen (see below) could be mapped to the +1 position of P51b by primer extension (Fig. 2; data not shown). Thus, the *ant* transcript covers all *immI* genes. In contrast, in P1wt-infected bacteria *ant* mRNA is detected only in minor amounts early after infection (data not shown). It decreases further in the course of infection so that after 40 min, *ant* mRNA is no longer detectable. Presumably this reflects the establishment of lysogeny in the majority of infected cells is due to the proper interaction of *c4* and target RNA. No *ant* mRNA is detectable at all in P1vir^s*orfx16*-infected bacteria (Fig. 3A). Here, the frameshift mutation *orfx16* suppresses the effect of an improper *c4*-target interaction. P1 Ant-defective phages grow poorly in sensitive bacteria, unless they are also *c1* deficient (Yarmolinsky and Sternberg 1988). This may be the reason for the low amount of *c4* RNA molecules in P1vir^s*orfx16*-infected cells (Fig. 3A). We conclude from these results that (1) an active *c4* RNA interferes with synthesis or stability of *ant* mRNA, possibly as an indirect transcriptional repressor of *ant*, and (2) an interruption of *orfx* has the same effect.

For a more detailed analysis of this phenomenon, we constructed a series of pUA plasmids (Fig. 1; Materials and methods) carrying the thermosensitive P7c4.2ts mutation (Scott et al. 1977), so that *ant* transcription is inducible at will. Furthermore, to mimic the regulation in the prophage state, all experiments with pUA plasmids were done in the presence of pAC8. This second plasmid codes for the *c1* repressor and *bof* corepressor of P1. These regulatory proteins form a ternary complex with the operator Op51, thereby repressing promoter P51a but not P51b (Fig. 2; Velleman et al. 1992). Using this two-plasmid system, *ant* mRNA synthesis as observed in phage-infected bacteria (see above) can be mimicked (Fig. 3B): No *ant* mRNA is detectable by Northern blotting in the lysogens of P1wt and P7c4.2ts at 30°C. It is only observed in the P7c4.2ts lysogen at 42°C when *c4* becomes inactive. Similarly, a large amount of *ant* mRNA is found at 42°C with plasmid pUA1ts, which contains the whole *immI* region of P7c4.2ts (Fig. 1). Roughly the same amount of *ant* mRNA is obtained with pUA2ts. In this plasmid, the P7 *EcoRI* fragment (410 – 2292) containing part of *orfx* and the whole *ant1/ant2* region is replaced by the corresponding P1 *EcoRI* fragment (Fig. 1). This exchangeability of the *EcoRI* fragments, at least with regard to the transcriptional regulation, allows the effect of mutations in P7–P1 hybrid plasmids to be tested. Thus, plasmid pUA3ts, containing the P1*orfx16* mutation in addition to P7c4.2ts (Figs. 1 and 2) does not yield *ant* mRNA (Fig. 3B) as expected from the P1vir^s*orfx16* infection experiment (Fig. 3A). These results clearly demonstrate that a derepressed and intact

orfx is a prerequisite for *ant* mRNA synthesis or stability. Two functions of *orfx* can be envisioned: (1) a purely passive role, where only the translation process but not the Orfx protein itself is essential for *ant* transcription or *ant* mRNA stability, or (2) an active role, for example, as an antiterminator or transcript stabilizing protein.

immI proteins are dispensable for *ant* transcription

To decide whether *orfx* translation solely or Orfx protein itself is necessary for *ant* mRNA synthesis, Orfx protein was altered without impairing the translation pattern. Starting with the frameshift mutant *orfx16* (Fig. 2, position 439) we restored the original reading frame by deletion of 1 nucleotide downstream at position 471 (Fig. 2, *orfx1*). In the resulting plasmid pUA12ts (Fig. 1) 10 of 73 amino acids in about the middle of *orfx* are exchanged. We assume that Orfx protein is inactivated in that way for the following reason. Although there is no known function of Orfx protein, its expression from a cloned *orfx* gene causes cell filamentation (J. Heinrich, in prep.). Accordingly, bacteria containing pUA2ts form filaments at 42°C, whereas those containing pUA3ts do not; likewise, bacteria containing pUA12ts do not show filamentation either. We then wanted to confirm that in pUA12ts only the function of the Orfx protein but not the translation of the *immI* region is affected. Therefore, we inserted the *orfx16,1* double mutant into the protein expression vector pT7-6 and obtained plasmid pTA12 (Fig. 1). In the pT7-6 expression system the inserted genes are transcribed by T7 RNA polymerase, which overruns *E. coli* terminators (McAllister and Morris 1981). This allows the normal transcriptional regulation to be bypassed, whereas translational regulation is maintained. In bacteria carrying the wild-type plasmid pAH1018 (Fig. 1) three proteins are induced (Heisig et al. 1989): Ant1, Ant2, and the fusion protein Kila* (Fig. 4, lane 3). If *c4* is supplied in *trans* by plasmid pKM10 (Fig. 4, lane 4) or if translation of *orfx* is interrupted (Fig. 4, lane 5), synthesis of Ant1 protein is abolished, whereas that of the downstream Ant2 and Kila* proteins is not affected. In contrast, plasmid pTA12 again produces Ant1 (Fig. 4, lane 6) and therefore behaves like plasmid pAH1018. Thus, inactivation of Orfx protein does not affect translation of the *immI* region. This result also proves that *ant1* is translationally coupled to *orfx*, as has been suggested previously (Heisig et al. 1989).

Because translation of the *immI* region is not affected in pTA12, the double mutation *orfx16,1* could now be subjected to the transcription test (Fig. 5). As already shown above, *ant* mRNA is induced at 42°C with plasmid pUA2ts but not with pUA3ts. With plasmid pUA12ts, *ant* mRNA is again detectable in amounts comparable to that of pUA2ts carrying no *orfx* mutation. We conclude that the Orfx protein has no function in *ant* mRNA transcription or stabilization. Because of the translational coupling of *orfx* and *ant1*, the *orfx16* mutation also inhibits *ant1* translation. Therefore, the possibility that Ant1 protein promotes synthesis or stability of *ant* mRNA cannot be excluded. To test this, several

Figure 4. In vivo synthesis of P1 antirepressor. Bacteria carrying a plasmid with the *orfx/ant1/ant2* genes were grown at 37°C. Antirepressor synthesis was induced for 2 hr at 37°C as described in Materials and methods, except for the bacteria (–) in the lane next to the markers. After induction, bacteria were lysed, and crude extracts were subjected to 15% SDS-PAGE. Proteins were stained with Coomassie blue. Positions for the Ant1, Ant2, and the *kilA* fusion protein named KilA* (Heisig et al. 1989) are indicated. Markers are in descending order (with molecular sizes in kD): phosphorylase B, glutamate dehydrogenase, lactate dehydrogenase, and trypsin inhibitor.

frameshift mutations in the *ant1* gene were analyzed. In the plasmid pUA17ts, 2 nucleotides are deleted at positions 640/641 (Figs. 1 and 2; Δ PvuI). As a consequence of the resulting frameshift, which creates a stop codon at position 693, *ant* mRNA is not detectable. In contrast, filling in the *AccI* site at position 798, which also causes a frameshift and creates a premature translation termination signal at position 827, has no effect on the synthesis of *ant* mRNA (see Fig. 5). Similarly, frameshifts introduced downstream of the *AccI* site are without effect (data not shown). These results suggest that if the *ant1* product is required for *ant* transcription, only the amino-terminal 67 amino acids could be involved. This suggestion seems to be unlikely, however, for the following reason: Using the same strategy as shown above for *orfx*, we constructed plasmid pUA18ts from pUA17ts and restored the reading frame by the insertion of 2 bases after position 736 (Figs. 1 and 2; *ant* b); in addition, a stop codon resulting from the first frameshift had to be destroyed by a single substitution at position 693 (Fig. 2, *ant* a). As a result, 33 of 67 amino-terminal amino acids are exchanged in pUA18ts. Nevertheless, *ant* mRNA synthesis is not affected and does not differ quantitatively from that of pUA10ts (Fig. 5). This demonstrates that *ant* transcription also does not require an intact Ant1 protein. In summary, these results prove that *immI*-encoded proteins are dispensable for *ant* transcription.

A ρ -dependent terminator controls *ant* transcription

The results described above prove that *ant* transcription is translation dependent. Such a link between translation and transcription may be explained if a ρ -dependent terminator controls the elongation of *ant* mRNA. We tested this hypothesis using *E. coli* *pts* mutants (*pts15*, ρ -4, ρ -702) infected with P1 phages at 42°C. The corresponding ρ^+ strains were used as controls. With all three *pts* mutants, virtually identical results were obtained; one example is shown in Figure 6. In accordance with the data presented in Figure 3A, *ant* mRNA is only synthesized in P1^{vir}- but not in P1^{wt}- or P1^{vir}*orfx16*-infected ρ^+ bacteria. In ρ^- bacteria, however, *ant* mRNA as well as a larger *ant*-specific RNA are found, irrespective of whether *orfx* translation is prevented by *c4* (P1^{wt}) or whether translation is interrupted (P1^{vir}*ant16*). In P1^{vir}-infected ρ^+ bacteria, *orfx* translation is derepressed but can be blocked by the addition of chloramphenicol (Fig. 6). Therefore, a chloramphenicol-dependent transcription termination is to be expected, which should be suppressible again by ρ mutations (Adhya and Gottesman 1978). In P1^{vir}-infected ρ^- bacteria *ant* mRNA is found in the presence of chloramphenicol (Fig. 6). In summary, these results show that blocking translation by *c4* RNA, an *orfx* frameshift mutation, or chloramphenicol results in premature transcription termination. The transcription block can be lifted in the absence of ρ . Therefore, we conclude that *ant* transcription is regulated via ρ -dependent termination. A site of ρ action must be located in the transcript between the premature translation termination signals at positions 693 and 827 created by the frameshifts at the *PvuI* and *AccI* sites, respectively (see above).

Figure 5. Northern blot analysis of bacteria carrying plasmids. With the exception of pUA2ts, all plasmids contained one or two frameshift mutations in *orfx* or *ant1*. SCS1 bacteria carried the pUA plasmid, as indicated, and plasmid pAC8. Bacteria were grown at 30°C to logarithmic phase and heat induced for 20 min at 42°C. Total RNA was subjected to 1.5% agarose glyoxal gel electrophoresis, and the RNA was hybridized to the *ant* probe (Fig. 1). The same markers as in Fig. 3A were used (not shown).

Figure 6. Northern blot analysis of P1-infected *E. coli* ρ mutants. SA1030 (ρ^+) and AD1600 (ρ^-) cells were grown at 28°C to $A_{600} = 0.2$ and infected with P1wt, P1vir^S, and P1vir^Sorfx16 at a m.o.i. of 2 for 40 min at 42°C. Chloramphenicol (Cm) at a final concentration of 50 μ g/ml was added (+) shortly before P1vir^S infection. Total RNA (10 μ g) was subjected to 1.5% agarose glyoxal gel electrophoresis, and the RNA was hybridized to the *ant* probe (Fig. 1). The same markers as in Fig. 3A were used (not shown).

Discussion

We have shown that *immI* gene expression of bacteriophages P1 and P7 is controlled by a unique combination of regulatory mechanisms. The key element, *c4* antisense RNA, is an unusual repressor, which is transcribed from the same promoter as *ant* mRNA. Therefore *c4* RNA is a shortened version of its target (Citron and Schuster 1990) containing only 77 ± 1 bases (Fig. 2). Its 5' end is generated by processing, probably by the *E. coli* RNase P processing system for tRNAs; the 3' end may also be generated by an endonucleolytic cleavage or simply by termination (Citron and Schuster 1992). It is not known if and how the formation of mature *c4* RNA is itself regulated. We assume that *c4* RNA synthesis is influenced by the *c1*-controlled operator Op51 (Fig. 2). But, so far, we only know that in P1-infected bacteria or lysogens most of the RNA molecules starting at the promoters P51a and b end up as *c4* RNA.

We have provided evidence that *c4* RNA directly inhibits translation of *orfx*. Using a T7 RNA polymerase-dependent protein expression system in which the normal transcriptional regulation of *E. coli* RNA polymerase is bypassed, we have also demonstrated that translation of *ant1* is coupled to translation of *orfx*. *ant* mRNA is only detectable in vivo when *c4* cannot act and translation of *orfx* is not disturbed. In ρ^- bacteria, however, the *orfx* requirement can be circumvented. Thus, by blocking *orfx* translation, *c4* RNA indirectly represses elongation of *ant* mRNA.

Our results do not exclude the possibility that *c4* RNA also affects the stability of its target *ant* mRNA. This possibility seems to be rather unlikely, however, for the following reasons. A *c4*-mediated degradation of *immI* transcripts synthesized by T7 RNA polymerase is not

observed. About equal amounts of Ant2 protein are produced in the presence and absence of *c4* (Fig. 4, lanes 3, 4). This indicates that *c4* represses *ant1* translation but does not result in degradation of the whole *ant* mRNA. Furthermore, in P1wt-infected *E. coli* ρ^+ and ρ^- bacteria, about the same amount of *c4* RNA is found (data not shown). This RNA, however, does not wipe out the abundance of *ant* mRNA observed in ρ^- bacteria (Fig. 6, second lane). An effect of *c4* on the stability of *ant* mRNA of P1wt is difficult to measure because in *E. coli* ρ^+ bacteria, the latter is not synthesized in the presence of an active *c4*. Therefore we used the following approach: In bacteria infected with either P7c4.2ts or P1vir^S, *ant* mRNA synthesis was allowed to proceed for 20 min at 42°C, rifampicin was then added to block further synthesis, and the temperature was lowered to 30°C. Under these conditions, the ability of P7c4.2ts RNA to interact with its target RNA is restored, whereas an interaction of P1 *c4* and *vir^S* target does not take place. The amount of *ant* mRNA was then measured at intervals over a period of 20 min. P7 and P1 *ant* mRNA were degraded at about the same rate ($\tau \approx 8$ min) with the exception that degradation of the P1 *ant* mRNA started only after an initial delay of ≈ 3 min (data not shown). These results exclude a *c4*-mediated degradation of the intact *ant* mRNA. However, they do not exclude the possibility that *c4* is involved in the degradation of the ρ -mediated *ant* termination product. The failure to detect such a product in the phage infection experiments (Figs. 3B and 6) points to such a possibility.

Individual regulatory elements found in the P1/P7 antisense RNA system are well known: Translational repression has been postulated for several antisense RNAs (for review, see Simons and Kleckner 1988; Takayama and Inouye 1990; Eguchi et al. 1991) and has been shown to be sufficient to account for control in the IS10 antisense system (Ma and Simons 1990). The motif of antisense regulation via translational coupling has also been found in two other systems, the *repZ* gene expression of plasmid ColIb-P9 (Asano et al. 1991), and the *hok/sok* locus of plasmid R1 (Thisted and Gerdes 1992). Transcriptional repression via antisense RNA has so far been demonstrated in only two cases (Okamoto and Freundlich 1986; Novick et al. 1989). In both systems, binding of the antisense RNA leads directly to premature termination of target RNA transcription by forming a terminator-like structure. In contrast, we describe for the first time a unique antisense translational repressor, which utilizes ρ -dependent termination to achieve an indirect transcriptional control. One might also expect to find other antisense systems that combine the common mechanisms of translational repression and ρ -dependent termination, because it seems more economical to terminate a superfluous transcript than to synthesize and degrade it.

Materials and methods

Bacterial and phage strains

Bacteria (with relevant markers) used were C600 *supE44* (App-

leyard 1954), CB454 *ΔlacZ galK recA56* (Schneider and Beck 1987), JM83F' Tet^r (Yanisch-Perron et al. 1985; Heisig et al. 1989), SA1030 ρ⁺ *ilvY::Tn10 galP3*, AD1600 *pts15 ilvY::Tn10 galP3* (Das et al. 1976), and SCS1 *supE44 recA1* (Stratagene, Inc.) and the P1Cm and P7c4.2ts lysogen of C600. Phages used were mGP1-2 (S. Tabor and C.C. Richardson, pers. comm.), P7c4.2ts (Scott et al. 1977), P1Cm (Rosner 1972), P1Cm^{vir} (Scott 1968), and P1Cm^{vir}*orfX16*. The latter, Ant-defective mutant was originally called *ant16* (Sternberg 1979).

Recombinant plasmids

The cleavage sites of the restriction enzymes used for creating P1 and P7 DNA fragments are shown in Figures 1 and 2. Numbers in parentheses indicate the P1 or P7 nucleotide positions shown in Figures 1 and 2. Plasmids are named such that the first uppercase letter, N, T, or U, refers to the vector used: pNM480 (Minton 1984), pT7-6 (S. Tabor and C.C. Richardson, pers. comm.), and pUC19 (Yanisch-Perron et al. 1985), respectively. Restriction enzymes used for cleavage of the vector DNA are shown in parentheses. Plasmids pGP1-2 (Tabor and Richardson 1985), pAH1018 (Heisig et al. 1989), pAH1018*orfX16* (Heinrich 1991), and pKM10 (Citron and Schuster 1990) have been described previously. Plasmid pAC8 is a *coi*-defective derivative of pAM8 (Velleman 1989). It contains the ColD replicon and Tn903 (Km^r), and carries the P1 *c1* and *bof* gene. It was used to supply *c1* repressor and *bof* corepressor. Plasmids pGP1 and pGP7 contain the *PvuII* fragment of pUM10 (P1, 4–410) and pUM71 (P7, 20–410), respectively, (Citron and Schuster 1990), inserted into pGP1-2 (*EcoRI* made blunt-ended using Klenow polymerase). Recombinant plasmids were selected in which the *c4* gene is transcribed in opposite orientation to T7 RNA polymerase. pNMC1: *PvuII*–*NlaIV* fragment (P1, 308–505) of pAH1018 cloned into pNM480 (*SmaI*); pUA1ts: *Bal31*–*EcoRI* fragment (20–2292) of P7c4.2ts cloned into pUC19 (*HincII*, *EcoRI*); pUA2ts: *Bal31*–*EcoRI* fragment (20–410) of P7c4.2ts and *EcoRI* fragment (410–2292) of P1Cm cloned into pUC19 (*HincII*, *EcoRI*); pUA3ts: pUA2ts with A inserted into the cluster of 6 A (*orfX16*, positions 437–442); pUA12ts: pUA3ts with deletion of C at position 471 (*orfX16*, 1); pUA17ts: pUA2ts with deletion of AT at positions 640/641 by degrading protruding nucleotides of the *PvuI* restriction site; pUA18ts: pUA17ts with substitution of T→G at position 693 (*ant a*) and insertion of TC after position 736 (*ant b*); pUA10ts: pUA2ts with insertion of CT after position 798 by filling in protruding *AccI* restriction site; pTA12: *HincII*–*EcoRI* fragment (307–2292) of pUA12ts cloned into pT7-6 (*SmaI*, *EcoRI*).

β-Galactosidase assay

CB454 bacteria containing the indicated plasmids were grown to A₆₀₀ = 0.4 at 28°C. T7 RNA polymerase was then induced for 45 min at 42°C. β-galactosidase activity was determined by the method of Miller (1972). Units presented are an average of the results from six experiments. They are reported with their standard errors.

Site-directed mutagenesis

Site-directed mutagenesis of CsCl-purified plasmid DNAs was performed as described by Olsen and Eckstein (1990) using the oligodeoxyribonucleotides CTTCGTCTTCATTGCTGC (482–464), CATGAAATAGTTCGCAATTGCG (705–684), and CAGCCTGATATCTGTTCGG (744–726) for the construction of pUA12ts and pUA18ts, respectively. Mutations were con-

firmed by sequence analysis using the dideoxy method (Sanger et al. 1977).

Preparation of RNA

Bacteria were grown to mid-logarithmic phase at 30°C. Induction of *ant* mRNA synthesis (by inactivation of *c4ts*) was achieved by shifting the temperature to 42°C for 20 min. Total RNA was isolated by the hot phenol method of Aiba et al. (1981). RNA concentrations were measured by determining absorbance at 260 nm.

Northern blot analysis

Total RNA (30 μg for each sample if not otherwise noted) was subjected to glyoxal gel electrophoresis as described by Thurston et al. (1988). Transfer was performed with a Pressure Blotter (Stratagene) according to the instruction manual. Hybridization and washing was as described by Pollard et al. (1988). All hybridization probes were generated from DNA restriction fragments by the method of Feinberg and Vogelstein (1983, 1984) using a Promega kit.

Synthesis of antirepressor protein

Synthesis of P1 antirepressor protein by the pT7-6/mGP1-2-coupled system and its inhibition by *c4* repressor supplied in *trans* was performed as described previously (Heisig et al. 1989). In brief, JM83F' bacteria carrying a plasmid, in which the *orfX/ant1/ant2* genes are transcribed from the T7φ10 promoter, are infected with mGP1-2 containing the T7 RNA polymerase gene under control of the IPTG-inducible *lac* promoter. The *c4* gene product is supplied from the compatible plasmid pKM10.

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