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The c4 Repressors of Bacteriophages P1 and P7 Are Antisense RNAs

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Summary

The c4 repressors of P1 and P7 inhibit antirepressor synthesis and are solely responsible for heteroimmunity of the phages. We show that c4 is a new type of antisense RNA acting on a target, *ant* mRNA, that is transcribed from the same promoter. Interaction depends on complementarity of two pairs of short sequences encompassing the ribosome binding site involved in *ant* expression. We demonstrate that heteroimmunity of P1 and P7 is due to just two substitutions in each of the complementary sequences of c4 and *ant* mRNA. Based on P1-P7 sequence comparison and a mutant analysis, we propose a secondary structure model for c4 RNA, with the complementary regions in loops as important sites for antisense control.

Introduction

Naturally occurring antisense RNAs repress a variety of prokaryotic genes, mostly at the posttranscriptional level. In all antisense systems described so far, antisense and target RNAs are transcribed from different promoters. Except for the *micF* system of Escherichia coli (Mizuno et al., 1984), these promoters are in opposite orientation. The divergent transcription of antisense and target RNA leads to complementarity along the entire overlapping region (for reviews see Inouye, 1988; Simons and Kleckner, 1988). Among temperate phages, λ and P22 each code for an antisense RNA, which acts to repress the λ cII gene (Krinke and Wulff, 1987) and the P22 antirepressor gene (Liao et al., 1987; Wu et al., 1987), respectively. Here we show that two other temperate phages, P1 and P7, also use antisense RNAs to repress their antirepressor genes. This antisense RNA system, however, is novel since antisense and target RNAs are transcribed from the same promoter. A shortened version of the target RNA exerts its antisense RNA function by binding to sequences downstream in the same transcript.

The P1 antirepressor gene (*ant*) is located in the *imm*I region of the phage (for all but selected references see Yarmolinsky and Sternberg, 1988). The P1 *imm*I elements have been described previously (Heisig et al., 1989). *ant* encodes an antirepressor that must be repressed in a lysogen. Two antirepressor proteins, *ant*1 and *ant*2, are encoded by a single open reading frame, with the smaller protein initiating at an in-frame start codon. An open reading frame, *orf*x, of unknown function overlaps the start of *ant*1 and is preceded by a presumptive ribosome binding site (Figures 1 and 2). Intactness of *orf*x is a prerequisite for the expression of *ant*1 (Heisig et al., 1989). The c4 gene is located upstream of *orf*x and encodes the c4 repressor, which prevents expression of *ant*. This repressor has previously been suggested to be a 66-amino-acid protein (Baumstark and Scott, 1987) that binds to a site defined by the *vir*^{s} mutation. The immunity difference between the closely related, but heteroimmune, phages P1 and P7 has been mapped to the c4 gene. This means that the c4 repressor in a P1 or P7 lysogen inhibits *ant* expression only in homoimmune but not in heteroimmune superinfections (Chesney and Scott, 1975; Scott et al., 1977; Wandersman and Yarmolinsky, 1977). 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, *orf*x, and *ant*1/2 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).

Here we demonstrate that the c4 repressors of both phages are not proteins but regulatory RNAs containing two short sequence elements that are complementary to target sequences downstream in the same transcript. We show that complementarity between the short sequence elements and their targets is essential for c4 action and propose that c4 acts as a translational repressor of *orf*x, thereby also inhibiting *ant* expression. Immunity to superinfection by the homoimmune phage is explained by the ability of c4 RNA to act in *trans*, whereas heteroimmunity is due to just two base exchanges in each sequence element of P7 compared with P1.

Results

The P1 c4 Repressor Is an Antisense RNA and Not a Protein

We suspected that the c4 repressor is not a protein because of two results: all attempts to express a c4 protein using appropriate expression vectors failed; and a plasmid, pUM10a2, in which 7 bp of c4 had been exchanged without altering the hypothetical c4 amino acid sequence, no longer complemented P1Cmc4.32 for lysogeny, although the corresponding c4 wild-type plasmid, pUM10, did. We then asked whether the complete c4 open reading frame (Baumstark and Scott, 1987) would indeed be necessary for c4 activity. To that end, a series of 5′ and 3′ deletions of plasmids pUM10 and pUM13 were constructed (see Experimental Procedures and Figure 2) and tested for their ability to complement P1c4.32. Since complementation is dependent on transcription of the c4 gene (Baumstark and Scott, 1987), all the constructs contain either the heterologous T7φ10- or the P1-encoded P51a/51b tandem promoter. Complementation was possible with all derivatives containing at least nucleotides 4 to 326 (pUM10Δ6) or 242 to 410 (pTM13Δ3) (Figure 2). Finally, a construct, pTM13c4mini (Figure 2), containing only the 85 bp long overlapping c4 DNA sequence of the deletions Δ6 and Δ3

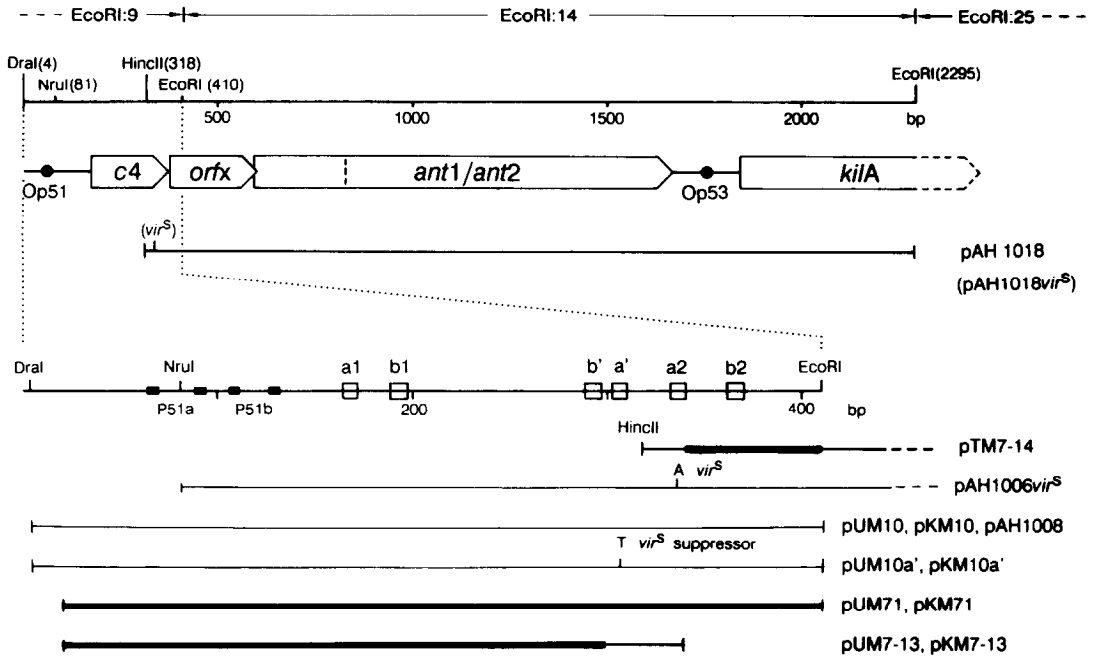

Figure 1. The *imm*I Region of P1

(Upper) The *imm*I region is located between the *c*1-controlled operators, Op51 and Op53 (black dots), and contains the genes *c*4, *orf*x, and *ant*1/*ant*2 (arrowheaded open bars). The *kil*A gene has been characterized by Hansen (1989). The sequence from the DraI site (4) in EcoRI:9 to the EcoRI:14/25 junction (2295) has been determined (Heisig et al., 1989; Hansen, 1989). Plasmids pAH1018 and pAH1018*vir*^{S} contain a P1 wild-type or P1*vir*^{S} DNA fragment (318 to 2295) inserted into pT7-6. From these plasmids the antirepressor proteins *ant*1 and *ant*2 (start marked by the dashed vertical line) and a *kil*A fusion protein named *kil*A* are overproduced upon induction of T7 RNA polymerase (Heisig et al., 1989) as described in Experimental Procedures.

(Lower) The *c*4 region (1 to 410) contains a tandem promoter (P51a/P51b) and complementary regions (open boxes) a1 through b2, which are explained in the legend to Figure 2. Horizontal thin, thick, and combined thin/thick lines represent P1, P7, and P1-P7 hybrid DNA, respectively, inserted into different vectors. Plasmid pTM7-14 is identical to pAH1018 except for the P7 sequence (340 to 410). Plasmid pAH1006*vir*^{S} contains the *imm*I region of P1*vir*^{S} starting from P51b (81 to 2295). The base substitution of the P1*vir*^{S} mutation (Heisig et al., 1989) and of the *vir*^{S} suppressor is indicated by A and T, respectively.

(nucleotides 242 to 326), was found to be sufficient for complementation. Since the *c*4mini region does not contain a complete open reading frame, these results ruled out the possibility that the *c*4 repressor is a protein and strongly suggested instead that the *c*4 gene directs the synthesis of a *trans*-acting regulatory RNA. If the *c*4 RNA acts by an antisense mechanism, it must contain a sequence complementary to a target RNA sequence. Interaction of complementary RNA sequences contained within the same or in separate molecules would then inhibit the expression of *ant*. Previously it was found that *ant* expression from plasmid pAH1018, which contains a P1*imm*I fragment downstream of the HincII site at position 318 (Figure 1), could be inhibited by *c*4 in *trans* (Heisig et al., 1989). Therefore, a possible target RNA sequence must be located downstream of the HincII site. Inspection of the sequence in question revealed two regions, a2 (7 bp, containing *vir*$^{S+}$) and b2 (8 bp, containing part of the presumptive ribosome binding site of *orf*x), which are complementary to sequences a′ and b′, respectively, within the *c*4mini region. No longer complementary domains between the *c*4mini region and the region downstream of the HincII site (position 318) until the start of *ant*2 (position 847) could be identified in a computer search. However, an additional set of two complementary elements, a1 and b1, the latter with a single mismatch, was found upstream of the *c*4mini region (Figure 2). The possible significance of these elements will be discussed later.

According to the antisense model proposed, constitutive synthesis of *ant* by the *vir*^{S} mutation located in a2 (Figure 2) results from disruption of a′/a2 base pairing. Therefore, *ant* synthesis might again be suppressible if complementarity is restored by a corresponding substitution in a′. We constructed such a *vir*^{S} suppressor plasmid, pKM10a′, and compared its properties with that of the corresponding wild-type plasmid pKM10 (Figure 1). Only plasmid pKM10a′ but not pKM10 is able to complement P1Cm*vir*^{S} for lysogeny up to 43°C (Table 1), whereas up to 37°C, both plasmids are active. Likewise, plasmid pAH1006*vir*^{S} (Figure 1), which expresses *ant* constitutively and therefore is normally not clonable, is stably maintained in E. coli only in the presence of pKM10a′, but not pKM10 (data not shown).

Heteroimmunity of P1 and P7 Is Due to Phage-Specific c4 RNAs

Provided that the *c*4 repression systems of P1 and P7 are basically the same, heteroimmunity of the phages might

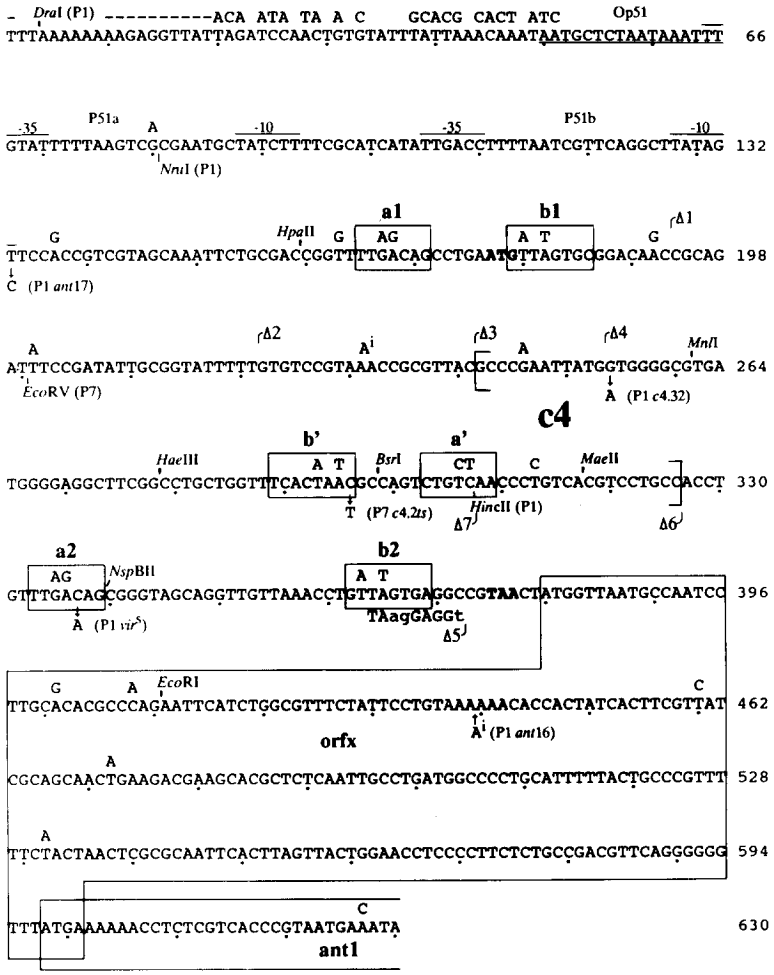

Figure 2. P1 and P7 DNA Sequence of the *c4* Regulatory Region with the Adjacent Genes *orfx* and *ant*1

The DNA sequence of P1 is shown. For P7, only differences from P1 are displayed above the P1 sequence. The only insertion relative to P1 is indicated by A^i. No P7 sequence information was obtained at the positions marked by dashes. Mutations are indicated below the sequences by vertical arrows (base substitutions) and A^i (inserted base). The insertion of an A in *orfx* (P1*ant*16) and the T→C substitution in P51b (P1*ant*17) eliminate *ant* expression (Heisig et al., 1989). Relevant restriction enzyme cutting sites are shown above and below the sequence followed by (P1) or (P7) if the recognition site is only present in P1 or P7 DNA. Complementary sequences (a′ versus a1 and a2; b′ versus b1 and b2) are boxed. Start and stop codons of the hypothetical *c4* open reading frame (Baumstark and Scott, 1987) are shown in bold letters. Endpoints of 5′ and 3′ deletions are indicated by greek deltas above and below the sequence, respectively. Brackets indicate the border of the P1c4mini region. The consensus sequence for binding of E. coli ribosomes is indicated 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 sequence of the operator, Op51, is underlined. Promoter regions are indicated by horizontal bars above the sequence. The *orfx* gene and the beginning of the *ant*1 gene are framed.

be due to differences between regions of their *c4* RNAs that are critically involved in antisense interactions. To locate the *c4* gene of P7 and to determine its sequence, we cloned subfragments of the 20 kb P7 BamHI:1 fragment known to contain the P7 *imm*I region (Iida and Arber, 1979). Recombinant plasmids, in which transcription originates from the vector, were then tested for their ability to complement P7*c4*.2ts for lysogeny at 40°C (data not shown). The smallest P7 restriction fragment found to be still active in complementation is a 139 bp EcoRV–NspBII fragment (position 201 to 339, Figure 2). DNA sequence analysis of a larger 610 bp P7 *imm*I fragment revealed a most striking feature: from an operator structure (starting at position 50, Figure 2), which is nearly identical to Op51 of P1 to the beginning of *ant*1 (position 630), the sequences of P7 and P1 are more than 95% homologous. However, in each of the complementary regions determined for P1, two bases are substituted in P7, resulting in a nucleotide sequence that exhibits the same pattern of complementary elements as found for P1. Moreover, the 139 bp EcoRV–NspBII fragment of P7 mentioned above overlaps the entire 85 bp P1c4mini region. These results clearly show that heteroimmunity of P1 and P7 is due to just four base exchanges that lead to the genome specificity of the phages' *c4* and target RNAs. We also identify a P7*orfx* gene (position 380 to 601, Figure 2) that has the same codon capacity as P1*orfx* and contains only five base substitutions. The first two of these (at positions 401 and 408) would lead to a Thr→Ala and Pro→Gln amino acid exchange, respectively, and the others would be silent, provided that *orfx* is translated.

c4 RNA Action Is Posttranscriptional

Synthesis of P1 antirepressor protein from plasmid pAH-1018 (see Figure 1) is inducible with the T7 RNA polymerase/promoter system (Tabor and Richardson, 1985) and is inhibited by *c4* encoded by a separate compatible plasmid (Heisig et al., 1989). To study *c4*-regulated antirepressor synthesis we used such a dual plasmid system in which one plasmid encodes the *c4* RNA and the other encodes the *c4* target RNA including *orfx* and *ant*1/*ant*2. Different plasmid combinations carrying *c4* gene constructs from P1 and P7 wild type or mutants as well as artificially altered *c4* regions were used. We expected that whenever the a′-b′ region of *c4* is complementary to the a2-b2 region of the target RNA, antirepressor synthesis should be re-

Table 1. Repression of *ant* Is Based on Complementarity of *c*4 and Target RNA

Combination of Plasmid and Plasmid/Phage	*c*4 RNA + Target: a1—b1—b′—a′—a2—b2 (b′ pairs with b2; a′ pairs with a2)	Assay: Relative Synthesis of *ant*1 (%)	Assay: Frequency of Lysogeny (Temperature, °C)
pKM10 plus:	P1 P1 U C A C U A A C — C U G U C A A P1 P1		
pAH1018	A G U G A U U G — G A C A G U U	6	–
pAH1018*vir*^{s}	· · · · · · · · — · · A · · · ·	51	–
pTM7-14	· · · · u · a · — · · · · · · ·	82	–
P1Cm*c*4.32	· · · · · · · · — · · · · · · ·	–	$\geq 10^{-3}$ (37)
P7*c*4.2ts	· · · · u · a · — · · · g a · ·	–	$<10^{-6}$ (40)
P1Cm*vir*^{s}	· · · · · · · · — · · A · · · ·	–	$<10^{-6}$ (43)
pKM10a′ plus:	P1 P1 U C A C U A A C — C U U U C A A P1 P1		
pAH1018*vir*^{s}	A G U G A U U G — G A A A G U U	6	–
pAH1018	· · · · · · · · — · · C · · · ·	36	–
P1Cm*vir*^{s}	· · · · · · · · — · · · · · · ·	–	$\geq 10^{-3}$ (43)
pKM71 plus:	P7 P7 U C A C a A u C — C U G c u A A P7 P7		
P7*c*4.2ts	A G U G u U a G — G A C g a U U	–	$\geq 10^{-3}$ (40)
P1Cm*c*4.32	· · · · A · U · — · · · A G · ·	–	$<10^{-6}$ (37)
pAH1018	· · · · A · U · — · · · A G · ·	51	–
pTM7-14	· · · · · · · · — · · · A G · ·	50	–
pKM7-13 plus:	P7 P7 U C A C a A u C — C U G U C A A P1 –		
pTM7-14	A G U G u U a G — G A C A G U U	5	–
pAH1018	· · · · A · U · — · · · · · · ·	41	–
P1Cm*c*4.32	· · · · A · U · — · · · · · · ·	–	$<10^{-6}$ (37)
P7*c*4.2ts	· · · · · · · · — · · · g a · ·	–	$<10^{-6}$ (40)

The underlined plasmids contain the *c*4 gene of P1 or P7 or a P1-P7 hybrid. They were tested for the ability to inhibit the expression of antirepressor from a second plasmid (Figure 3) and to complement a *c*4-defective P1 or P7 for lysogeny. Nucleotide sequences of the b′-a′ and b2-a2 regions of the *c*4 RNAs are shown in the center. Complementarity between the b′-a′ region (*c*4 plasmid to be tested, first row) and the b2-a2 region (*ant*-containing plasmid or phage, second row) is indicated by a vertical line. For other plasmids and phages (the following rows) only deviations from the corresponding b2-a2 nucleotide sequences are shown. P7 bases that deviate from the P1 nucleotide sequence are indicated by lowercase letters. The origin of the a1-b1 and a2-b2 region (P1 or P7) is only shown for the *c*4 plasmid to be tested. Normalized estimates of *ant*1 synthesis were obtained by scanning the gel (Figure 3) and defining *ant*1 induction from pAH1018/pKT101 uninduced and induced as 0% and 100%, respectively (Figure 3, lanes 2 and 3). Thus, induction of *ant*1 from pAH1018*vir*^{s}/pKT101 (Figure 3, lane 8) is 47%, suggesting that *ant*1 synthesis fluctuates by a factor of about 2. Frequency of lysogeny was determined as described in Experimental Procedures.

pressed. The effects of *c*4 plasmids on *ant*1 expression (Figure 3) and lysogenization frequencies of P1Cm*c*4.32, P1Cm*vir*^{s}, and P7*c*4.2ts were quantified and compared (Table 1). In bacteria carrying pAH1018 and only the vector plasmid pKT101, three proteins are induced: *ant*1, *ant*2, and *kil*A* (Figure 3, lanes 2 and 3). In the presence of pKM10*c*4, synthesis of *ant*1 is repressed, whereas that of *ant*2 and *kil*A* is not affected (Figure 3, lane 4). However, the presence of the *vir*^{s} suppressor plasmid pKM10a′ no longer allows a complete repression of *ant*1 (Figure 3, lane 5). The P7*c*4-containing plasmid pKM71 (Figure 1), which complements P7*c*4.2ts for lysogeny but differs from the P1*c*4 target RNA by 4 bp (Table 1), does not repress P1 *ant* synthesis (Figure 3, lane 6). The same negative result is obtained with plasmid pKM7-13 (Figure 3, lane 7), containing a P7b′-P1a′ hybrid *c*4 region (Figure 1). When pAH1018 is replaced by pAH1018*vir*^{s} in the test system (Figure 1), the effect of plasmids pKM10 and pKM10a′ is reversed: only the *vir*^{s} suppressor plasmid, which complements P1Cm*vir*^{s} for lysogeny, also represses *ant*1 synthesis (Figure 3, lanes 9 and 10). Most interestingly, P1 *ant* synthesis can be induced on pTM7-14 (Figure 3, lane 11). This plasmid contains a P1a2-P7b2 hybrid *c*4 target, a P7-P1 hybrid *orf*x gene, and a P1 *ant*1/2 gene (see Figure 1). Provided that *orf*x codes for a protein, the P7-P1 hybrid protein contains two amino acid exchanges. Nevertheless, this does not affect antirepressor synthesis; the latter can now only be repressed by pKM7-13, which is complementary to the hybrid *c*4 target RNA (Figure 3, last lane), but not by P1*c*4 or P7*c*4 (Figure 3, lanes 11 and 12), indicating that both regions a′ and b′ must find their complementary targets. These results clearly show that the genome specificity of *c*4 RNAs, as manifested by the ability to complement a phage for lysogeny, always corresponds to the target site specificity as measured by *ant*1 synthesis. In all experiments described, *ant*2 and *kil*A* are inducible, irrespective of whether or not *ant*1 is repressed. Therefore, *c*4 RNA does not inhibit transcription by T7 RNA polymerase, nor does it promote degradation of the entire *ant* mRNA. We propose that *c*4 RNA exerts its effect

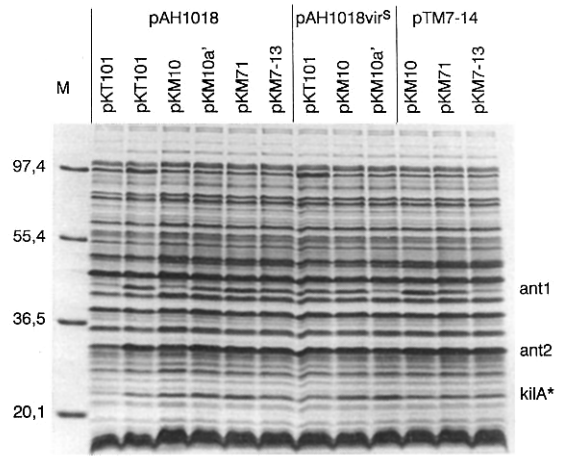

Figure 3. In Vivo Synthesis of P1 Antirepressor and Its Inhibition by c4

Bacteria carrying a plasmid with the *ant*1/*ant*2 genes (top line) and a second plasmid with the c4 gene (or the vector instead) (vertically written) were grown at 37°C. P1 antirepressor synthesis was induced for 2 hr at 37°C as described in Experimental Procedures, except for the sample in the lane next to the markers: pAH1018 + pKT101 were uninduced. After induction, bacteria were lysed, and crude extracts were subjected to 15% SDS–PAGE. Proteins were stained with Coomassie blue. Positions for the *ant*1, *ant*2, and *kilA** proteins (see Figure 1) are indicated. M, markers are in descending order (with molecular sizes in kilodaltons): phosphorylase B, glutamate dehydrogenase, lactate dehydrogenase, and trypsin inhibitor.

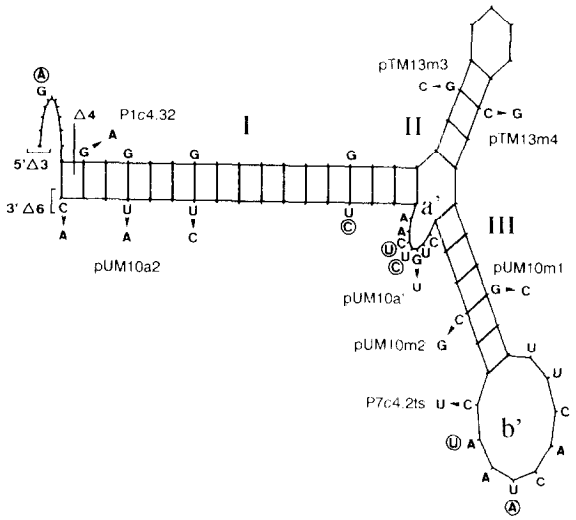

Figure 4. Secondary Structure Model of the c4 Antisense RNA

The sequence of P1c4mini (position 242 to 326, Figure 2) was folded using the FOLD program of Zuker and Stiegler (1981) with the energy parameters of Freier et al. (1986). Only relevant bases are shown. Positions where the sequence of P7 RNA differs from that of P1 are marked by circled letters. Naturally occurring and artificially induced mutations are indicated by arrowheads, followed by the name of the phage or plasmid. Loss of base pairing by single base substitutions in pUM10m1, pUM10m2, pTM13m3, and pTM13m4 is reversed by the corresponding double exchanges in pUM10m1,2 and pTM13m3,4 (not shown; for all constructions see Experimental Procedures). Note that pUM10a2 contains three base substitutions in the c4mini region. For the explanation of other symbols, see the legend to Figure 2.

by direct occlusion of the ribosome binding site of *orf*x, thereby also blocking translation of *ant*1.

A c4 RNA Secondary Structure Model

The deletion analysis described above implies that a functional c4 RNA molecule must extend beyond the complementary elements a′ and b′. The additional sequences not directly involved in the antisense-target-RNA interactions may be required to generate or stabilize a specific c4 RNA secondary structure. Using a computer folding program that provides minimum free energy structures for RNA (Zuker and Stiegler, 1981), the hypothetical structure of the c4mini RNA was generated (Figure 4). This structure is also predicted to be contained in the longer RNAs of all other functional P1 c4 derivatives described in this paper. To test the proposed model, we measured the effects of several mutations on complementation of P1Cm-c4.32. Disruption of the long stem domain I could account for the observed loss of c4 complementation with plasmids pUM10a2 and pTM13Δ4 and also for the c4 defect in P1c4.32. Certain mutations in stems II and III should also disrupt the secondary structure and inactivate c4, but complementation might be recovered by compensatory mutations that restore the stem. Indeed, if stem II is disrupted, as is the case in plasmids pTM13m3 and pTM-13m4, c4 complementation is completely lost, but it is fully recovered if the stem is restored, as in the double mutant pTM13m3,4. With regard to stem III, the single substitution in pUM10m1 also eliminates c4 complementation completely. A corresponding substitution in pUM10m2 still allows it, but the resulting lysogens grow poorly, indicating an impaired c4 activity. However, loss of c4 complementation of pUM10m1 can be reversed by restoring the stem, as demonstrated in the double mutant pUM10m1,2. Again, the resulting lysogen grows poorly. According to the model, regions a′ and b′ are present in single-stranded loops and therefore are accessible to pairing. Consequently, the mutation in pUM10a′, which alters the specificity, also maps to the a′ loop. Upon folding of P7 c4 RNA into the same conformation as P1 c4 RNA, all four base exchanges that lead to heteroimmunity map to the loop domains a′ and b′. In addition, in stem I a G-U is replaced by a G-C base pair. The P7c4.2ts mutation also maps to the b′ loop and is therefore assumed not to destabilize the c4 RNA secondary structure. Instead, interaction between c4.2ts and its target RNA appears to be only moderately affected because a C-G is replaced by the weaker U-G base pair. In summary, these results suggest that in vivo both P1 and P7 c4 RNA exhibit the structure presented in Figure 4.

Discussion

We have identified the c4 repressors of P1 and P7 as antisense RNAs. We propose that c4 exerts its function by an interaction of two sequences, a′ and b′, within c4, with two complementary sequences, a2 and b2, in the *ant* mRNA. By these means the putative ribosome binding site of *orf*x, which overlaps b2, is masked (Figure 2). Occlusion of a ribosome binding site has recently been demonstrated and shown to be sufficient to account for control in the IS10 antisense system (Ma and Simons,

1990). *orf*x has a coding capacity of 73 amino acids and overlaps *ant*1; the latter, on the other hand, has been proposed to be translationally coupled to *orf*x, because intactness of *orf*x is a prerequisite for *ant*1 expression (Heisig et al., 1989). Therefore, by blocking translation of *orf*x, that of *ant*1 is inhibited simultaneously. Alterations in the essential element a′-b′ or disruption of the *c*4 RNA secondary structure lead to a *c*4$^-$ phenotype; alterations in a2 or b2 cause constitutive expression of *ant*1 (Figure 3; Table 1) and should lead to virulence. In this connection, we point out that in our test systems repressibility of *ant*1 and *c*4 complementing activity are quantitatively different (Table 1). For example, the virulence of P1*vir*^{s} obviously depends on the relative copy numbers of *c*4 and target RNA. Thus, a *c*4$^+$ multicopy plasmid (pKM10) is able to complement one or two copies of P1*vir*^{s} for lysogeny up to 37°C; but when the *vir*^{s} target RNA is also supplied from a multicopy plasmid (pAH1018*vir*^{s}), the same *c*4$^+$ RNA no longer represses *ant*1 synthesis (Figure 3 and Table 1). Obviously, when *c*4$^+$ is supplied in excess, it can still interact with a target RNA containing only a single mismatch.

An important conclusion from our results is that very few base substitutions in the complementary regions a′/a2 and b′/b2 are sufficient to generate new immunity specificities. In particular, a virulent phenotype, P1*vir*^{s}, resulting from a single substitution, can be overcome by another single compensatory substitution. These examples support the assumption that a high rate of diversification may be the primary selective advantage of antisense regulation (Simons and Kleckner, 1988). Conversely, it seems to be much more difficult to alter the sequence specificity of the phages' primary repressor, the *c*1 protein. Moreover, a multitude of *c*1-controlled operators in the phages' genomes would have to be altered in addition. While the features discussed above are not unusual for an antisense RNA, *c*4 RNA is novel because it is transcribed from the same promoter, P51a/b, as its target, *orf*x-*ant* RNA. This could explain why complementarity between *c*4 and its target is restricted to two regions containing only 7 and 8 nucleotides, respectively. The higher number of complementary bases found in most other systems does not always seem to be critically involved in antisense interaction (Simons and Kleckner, 1988) and could merely result from divergent transcription. In contrast, in the *c*4 system, only the interacting bases must be complementary. The fact that antisense and target RNA share the same promoter structure raises the question of how expression of *c*4 and *ant* is separately controlled. Preliminary transcription experiments indicate that in the prophage state the vast majority of *imm*I transcripts terminate before reaching *orf*x (data not shown). These transcripts could serve as the pool of *c*4 repressor needed to act on a superinfecting phage. The antisense RNA would thus arise from a shortened version of its target transcript. *c*4 also acts in *cis*, since plasmids carrying a functional *c*4 gene upstream of the *orf*x-*ant* genes also do not express *ant*, even when the intact P1 *imm*I region is transcribed from the T7φ10 promoter (Heisig et al., 1989). However, since in a wild-type prophage transcription of *c*4 is a prerequisite for *ant* expression, it is logical to ask how *ant* expression is ever accomplished. In the prophage state, *c*4 a′-b′ must interact with a2-b2 in front of *orf*x. As illustrated in Figure 2, the latter sequences are also present upstream of the *c*4mini region and are named a1 and b1. It is therefore tempting to speculate that 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, thereby unmasking the ribosome binding site of *orf*x (Figure 2). This model requires an as yet unknown trigger that would activate a1/b1 to compete with a2/b2 for a′/b′ base pairing. It also predicts that mutations in a1 and b1 should be *ant*$^-$ phenotypically. Owing to its differences from the antisense paradigm, the possibility that *c*4 is a regulatory RNA has long been overlooked. Maybe there are further antisense RNAs of the same type yet to be discovered.

Experimental Procedures

Bacterial and Phage Strains

Bacteria (with relevant markers) used were C600 *supE44* (Appleyard, 1954), DW101 *sup*$^+$ (Walker and Walker, 1975), and JM83F′ *sup*$^+$ strep^{r}tet^r (Yanisch-Perron et al., 1985; Heisig et al., 1989). Phage used were P1Cm*c*4.32, P1Cm*vir*^{s} (Scott, 1968), P7*c*4.2ts, P7*c*1.48ts (Scott et al., 1977), and mGP1-2 (S. Tabor and C. C. Richardson, personal communication).

Plasmid Constructions

The cleavage sites of the restriction enzymes used for creating P1 and P7 DNA fragments are shown in Figures 1 and/or 2. The exceptions are HindIII, PvuII, and KpnI, the cleavage sites of which are in the vector DNA. Numbers in parentheses indicate the P1 and P7 nucleotide positions shown in Figures 1 and/or 2. Plasmids are named such that the first capital letter K, T (or A), and U refers to the vector used: pKT101 (Bagdasarian and Timmis, 1982), pT7-6 (S. Tabor and C. C. Richardson, personal communication), and pUC8 (Vieira and Messing, 1982) or pUC19 (Yanisch-Perron et al., 1985), respectively. Restriction enzymes used for cleavage of the vector DNA are shown in parentheses.

P1 Recombinant Plasmids

Plasmids pAH1018, pAH1018*vir*^{s} (318 to 2295), and pAH1008 (4 to 410) have been described previously (Heisig, 1989; Heisig et al., 1989).

pAH1006*vir*^{s}: NruI–EcoRI fragment (81 to 2295) of P1*vir*^{s} cloned into pT7-6 (SmaI, EcoRI).

pUM10: EcoRI–HindIII fragment (4 to 410) of pAH1008 cloned into pUC19 (EcoRI, HindIII).

pUM13: HpaII–EcoRI fragment (160 to 410) of pAH1008 cloned into pUC8 (AccI, EcoRI).

pUM10a2: MaeII–NspBII fragment (318 to 339) of pUM10 is replaced by a 22 bp synthetic MaeII–NspBII fragment:

5′-C G c C C a G C a A C a T G T c T t A C c G-3′
3′-g G G t C G t T G t A C A g A a T G g C-5′.

pUM10a′: BsrI–HincII fragment (299 to 307) of pUM10 is replaced by a 9 bp synthetic BsrI–HincII fragment (G→T at position 305).

pUM10m1: HaeIII–BsrI fragment (279 to 298) of pUM10 is replaced by a 20 bp synthetic HaeIII–BsrI fragment (G→C at position 285).

pUM10m2: BsrI–HincII fragment (299 to 307) of pUM10 is replaced by a 9 bp synthetic BsrI–HincII fragment (C→G at position 299).

pUM10m1,2: BsrI–HincII fragment (299 to 307) of pUM10m1 is replaced by the same synthetic BsrI–HincII fragment used to generate pUM10m2.

pKM10 (pKM10a′): PvuII–EcoRI fragment (4 to 410) of pUM10 (pUM10a′) cloned into pKT101 (EcoRV, EcoRI).

*c*4 Deletion Derivatives

5′ deletions were engineered by linearization of pUM13 with HindIII and subsequent Bal31 digestion followed by EcoRI cleavage and cloning

of the fragments into pT7-6 (EcoRI, SmaI). In these derivatives, *c4* is transcribed from the T7φ10 promoter of pT7-6 (pTM13Δ series).

3′ deletions were engineered by linearization of pUM10 with EcoRI and subsequent Bal31 digestion followed by HindIII cleavage and cloning of the fragments into pUC19 (HindII, HindIII); here, *c4* is transcribed from the P51a,b promoter of P1 (pUM10Δ series).

pTM13*c4*mini (242 to 326) was generated by replacing the 103 bp HincII–EcoRI fragment of pTM13Δ3 by the 54 bp HincII–EcoRI fragment of pUM10Δ6.

pTM13m3: MnlI–HaeIII fragment (262 to 278) of pTM13*c4*mini is replaced by a 17 bp synthetic MnlI–HaeIII fragment (G→C at position 272).

pTM13m4: HaeIII–BsrI fragment (279 to 298) of pTM13*c4*mini is replaced by a 20 bp synthetic HaeIII–BsrI fragment (C→G at position 279).

pTM13m3,4: HaeIII–BsrI fragment (279 to 298) of pTM13m3 is replaced by the same synthetic HaeIII–BsrI fragment used to generate pTM13m4.

P7 Recombinant Plasmids

pUM71: Bal31–EcoRI fragment (20 to 410) of P7*c*1.48ts DNA cloned into pUC19 (HincII, EcoRI).

pKM71: PvuII–EcoRI fragment (20 to 410) of pUM71 cloned into pKT101 (EcoRV, EcoRI).

P7-P1 Hybrid Plasmids

pTM7-14: the P1 NspBII–EcoRI fragment (340 to 410) of pAH1018 is replaced by the corresponding P7 fragment (Figure 1).

pUM7-13: the P7 HindIII–BsrI fragment (20 to 298) of pUM71 and the P1 BsrI–NspBII fragment (299 to 339) of pUM10 were cloned into pUC19 (HindIII, HindII) (Figure 1).

pKM7-13: the PvuII–EcoRI fragment (20 to 339) of pUM7-13 was cloned into pKT101 (EcoRV, EcoRI).

DNA manipulations were performed following methods compiled by Sambrook et al. (1989). P1 and P7 DNA were prepared as described (Heisig et al., 1989). Oligonucleotides were prepared by an automated DNA synthesizer (Applied Biosystems model 380A) and purified by high performance liquid chromatography. Complementary oligonucleotides were annealed prior to cloning.

DNA Sequencing

Sequence analysis of CsCl-purified plasmids using the dideoxy method (Sanger et al., 1977) was performed to establish the sequence of the P7 *c4-ant* region, to determine deletion endpoints, and to confirm sequences of P1-P7 recombinant and P1 mutant plasmids. The P7 wild-type sequence depicted in Figure 2 was obtained from both strands.

c4 Complementation Test

Upon infection with P1C*mc*4.32 or P1C*mvir*^{s} of DW101 carrying a recombinant plasmid, complementation of *c*4.32 and suppression of *vir*^{s} yield chloramphenicol-resistant P1 lysogens at 37°C and 43°C, respectively (Devlin et al., 1982; this paper). Bacteria were incubated at 30°C for pT7-6–derived plasmids in which pGP1-2 is the source of T7 RNA polymerase (Tabor and Richardson, 1985). At 30°C the basal level of T7 RNA polymerase is sufficient to allow detectable expression of genes downstream from T7φ10 (Baumstark and Scott, 1987). Upon infection with P7*c*4.2ts of C600, which carries a recombinant plasmid, complementation of *c*4.2ts yields ampicillin- and heat-resistant P7 lysogens at 40°C. Lysogenization frequency is the number of lysogenic colonies divided by the total number of cells used for infection. Frequencies of lysogenization $\geq 10^{-3}$ and $<10^{-6}$ are considered *c*4 complementation positive and negative, respectively.

c4 Inhibition of Antirepressor Synthesis

Synthesis of P1 antirepressor protein by the pT7-6/mGP1-2 coupled system and its inhibition by *c*4 repressor supplied in *trans* were tested as described previously (Heisig et al., 1989). In brief, JM83F′ bacteria carrying a plasmid, in which the *ant*1/*ant*2 genes are transcribed from the T7φ10 promoter, are infected with mGP1-2 containing the T7 RNA polymerase gene under control of the inducible *lac* promoter. The *c*4 gene product is supplied from a compatible pKM plasmid.

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GenBank Accession Number

The accession number for the P7 sequence described in the paper is M35139.