MUTATIONS THAT IMPROVE THE *ANT* PROMOTER OF SALMONELLA PHAGE P22

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ABSTRACT

Mutations that increase the activity of the promoter for the antirepressor gene of phage P22 were isolated by pseudoreversion of four severe promoter-down mutations. The sequence changes in these pseudorevertants include single base pair substitutions, single base pair deletions, tandem double base pair deletions and multisite mutations. The single base pair substitutions change nonconsensus base pairs to consensus base pairs at positions −14 and −8. The other mutations provide support for the idea that the length of the spacer region between the conserved −35 and −10 hexamers is an important determinant of promoter strength. Deletions of one or two base pairs in the spacer region apparently activate an alternate −10 hexamer by shifting it from a spacing of 19 base pairs to a spacing of 18 or 17 base pairs, respectively.

COMPARISON of a large number of promoter DNA sequences from *Escherichia coli*, *Salmonella typhimurium* and their phages has shown that most promoters are homologous in two regions situated about 10 and 35 base pairs (bp) prior to the start point of transcription (reviewed by HAWLEY and MCCLURE 1983; see Figure 2). The nature of the contribution of each region to promoter function has yet to be determined. These conserved regions are interrupted by a spacer region that is poorly conserved in sequence but highly conserved in length; the distance between the conserved hexamers 5′-TTGACA-3′ (−35 region) and 5′-TATAAT-3′ (−10 region) is 16–18 bp for 90% of the promoters compiled by HAWLEY and MCCLURE (1983).

The isolation and sequence analysis of promoter mutations underscores the importance of the −10 and −35 regions and the spacing between them. Most mutations that decrease promoter activity ("promoter-down" mutations) are changes that decrease homology of a promoter with the consensus sequence; most mutations that increase promoter activity ("promoter-up" mutations) are changes toward consensus. Effects of mutations that change the length of the spacer region suggest that optimal promoter activity corresponds to a 17-bp spacer length (see HAWLEY and MCCLURE 1983).

To elucidate the sequence determinants of promoter activity, we have iso-

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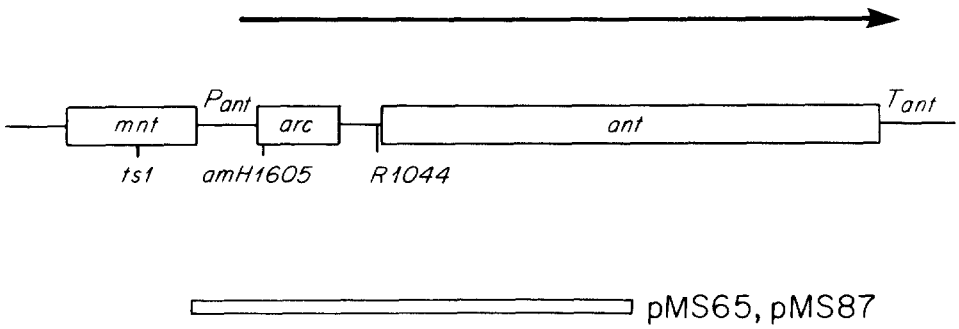

FIGURE 1.—Map of the antirepressor region. Genes and point mutations are drawn to physical scale as established by DNA sequence analysis (SAUER *et al.* 1983). The arrow represents the P_{ant} transcript. The open bar below the map shows the extent of the *Sau*3A1 fragment cloned in plasmids pMS65 and pMS87.

lated and characterized a large number of mutations in the bacteriophage P22 *ant* promoter (P_{ant}), a strong promoter extensively homologous with consensus (see Figure 2). As illustrated in Figure 1, P_{ant} directs the coordinate transcription of two genes: *arc* (antirepressor control) and *ant* (antirepressor) (SUSSKIND and YOUDERIAN 1982). Antirepressor protein inhibits P22 c2 repressor (analogous to λ cI repressor), the protein that directly represses lytic phage development in the lysogenic state. Two repressors control initiation of transcription from the *ant* promoter: the *mnt* gene product represses P_{ant} during lysogeny (BOTSTEIN *et al.* 1975; LEVINE *et al.* 1975) and the *arc* gene product turns down P_{ant} during lytic infection (SUSSKIND 1980). This cluster of regulatory genes lies in the immunity I (*immI*) region of the P22 genome.

Our genetic analysis of P_{ant} makes use of selections against phages that synthesize too much or too little antirepressor. First, *ant* promoter-down mutants ($P_{ant}\downarrow$ mutants) were selected as pseudorevertants of P22 *mnt-ts arc-am* (YOUDERIAN, BOUVIER and SUSSKIND 1982). During nonpermissive lytic infection, this phage overproduces antirepressor protein. Overproduction of antirepressor interferes with the synthesis of other phage proteins and prevents the production of progeny phage (the Arc^- lethal phenotype; SUSSKIND 1980). Most revertants of *mnt-ts arc-am* phage that grow on sup^0 cells at high temperature are pseudorevertants; many have acquired *ant* promoter-down mutations that decrease the rate of antirepressor synthesis to varying degrees (SUSSKIND and YOUDERIAN 1982; YOUDERIAN, BOUVIER and SUSSKIND 1982).

Some $P_{ant}\downarrow$ mutations have a severe effect on *ant* promoter activity and confer the Ant^- phenotype; phages carrying these mutations cannot produce sufficient antirepressor to grow in P22 $c2^+$ mnt^- ($immI_\Delta$) lysogens. [In the presence of c2 repressor made by the prophage, superinfecting phage must synthesize antirepressor in order to grow lytically (BOTSTEIN *et al.* 1975).] Hence, revertants of severe $P_{ant}\downarrow$ mutants that regain promoter activity may be selected by virtue of their ability to grow on a P22 $immI_\Delta$ lysogen (restoration of the Ant^+ phenotype). In a previous report, we described the isolation of three Ant^+ revertants of the severe promoter-down mutant, R204, and demonstrated that one of these (R204-R1) is a pseudorevertant, since R204-

TABLE 1

Bacterial strains

Strain	Genotype	Reference or source
DB7000	*leuA-am414 sup*^{0}	SUSSKIND, WRIGHT and BOTSTEIN (1971)
MS1363	*leuA-am414 supE*	SUSSKIND (1980)
DB7283	*leuA-am414 sup*^{0}(P22 *sieA44 Ap2del7283*)	WEINSTOCK, SUSSKIND and BOTSTEIN (1979)
MS1367	*leuA-am414 supE*(P22 *sieA44 Ap2del7283*)	SUSSKIND (1980)
MS1655	MS1363/pKM101 *bla*::Tn*5*	This work
MS1656	MS1367/pKM101 *bla*::Tn*5*	This work
MS1890	DB7283/pKM101 *bla*::Tn*5*	This work
MS1868	*leuA-am414 sup*^{0} r^- m^+	This work
MS1883	*leuA-am414 supE* r^- m^+	This work
MS1585	*leuA-am414 sup*^{0}(P22 *sieA44 Ap68tpfr49*)	This work
MS1582	*leuA-am414 supE*(P22 *16-amH*1455 sieA44 Ap68tpfr49)	This work

R1 phage synthesize antirepressor at a rate intermediate between that of their $P_{ant}\downarrow$R204 parent and P_{ant}+ phage (SUSSKIND and YOUDERIAN 1982). In this paper, we describe the isolation of Ant$^+$ revertants of several different P22 $P_{ant}\downarrow$ mutants. Many of these revertants are pseudorevertants that retain the original promoter-down mutation and have acquired *ant* promoter-up, or $P_{ant}\uparrow$, mutations that (at least partially) restore promoter activity.

MATERIALS AND METHODS

Bacteria: Derivatives of *S. typhimurium* strain LT2 are listed in Table 1. The prophage in DB7283 and MS1367 has a substitution, Ap2del7283, that deletes the *immI* region. The prophages in MS1585 and MS1582 carry Ap68tpfr49 (YOUDERIAN and SUSSKIND 1980), a deletion of genes *arc*, *ant*, *9* and *a1*. MS1655, MS1656 and MS1890 were constructed by transfer of the conjugative plasmid pKM101 *bla*::Tn*5* (WALKER 1978) to MS1363, MS1367 and DB7283, respectively, by selection for kanamycin resistance. MS1868 was constructed by transducing DB7000 with P22 *int3 HT12/4* (KLECKNER *et al.* 1975) grown in DB5532 (an r^- m^+ strain obtained from D. BOTSTEIN) and screening for colonies that are sensitive to unmodified P22 (grown in r^- m^- strain DB5301; SUSSKIND and BOTSTEIN 1980). MS1883 was constructed by transducing MS1868 to prototrophy with P22 *int*3 grown in MS1363.

Phage: Phages used in this study carry the mutations *sieA44* (SUSSKIND, WRIGHT and BOTSTEIN 1971), *mnt-ts1* (GOUGH 1968) and *arc-amH1605* (SUSSKIND 1980). For simplicity, we refer to these phages by their *ant* promoter genotypes. The severe $P_{ant}\downarrow$ mutants R204 (SUSSKIND 1980), RU267, RU523 and RE56 (YOUDERIAN, BOUVIER and SUSSKIND 1982) are the parents of the pseudorevertants described below.

R1044, a spontaneous pseudorevertant of P22 *mnt-ts arc-am* (one of the group II revertants described by YOUDERIAN, BOUVIER and SUSSKIND 1982), has a down-mutation in the ribosome-binding site for *ant* (M. M. SUSSKIND, unpublished results). $P_{ant}\uparrow$ phages were crossed with R1044 phage to obtain $P_{ant}\uparrow$ R1044 recombinants, which are distinguished from parental types by plaque morphology (see RESULTS for a discussion of the effects of antirepressor levels on plaque morphology). Recombinants were backcrossed with *mnt-ts arc-am* phage to confirm that both $P_{ant}\uparrow$ and R1044 types were regenerated.

All phages used in label experiments carry the clear-plaque mutation *c1-7* (LEVINE and CURTISS 1961), which prevents lysogeny and ensures a lytic response upon infection. To cross *c1-7* into our mutants without crossing out *immI* markers, a *c1-7* phage deleted for *immI* was used.

General: Media and general phage techniques are described by SUSSKIND, WRIGHT and BOTSTEIN (1971). Supplemented M9 medium is M9CAA with the casein hydrolysate omitted, supplemented with 20 μg/ml of L-histidine and L-leucine and 1 μg/ml of biotin. Phage dialysis buffer is 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM $MgSO_4$.

Enzymes and chemicals: Restriction endonucleases, *E. coli* DNA polymerase I large fragment and T4 DNA ligase were purchased from Bethesda Research Laboratories or New England Biolabs. Hydroxylamine hydrochloride was purchased from Fisher. ^{35}S-methionine and α-^{32}P-dTTP were from New England Nuclear or Amersham, and dGTP was from PL Biochemicals.

Reversion of $P_{ant}\downarrow$ mutants: Independent spontaneous revertants (designated "R") of P22 $P_{ant}\downarrow$R204 and RU267 were isolated from single-plaque stocks by plating on MS1367 at 40°.

Hydroxylamine-induced revertants (designated "RH") of $P_{ant}\downarrow$R204 were isolated by incubating phage stocks with hydroxylamine at room temperature (HALL and TESSMAN 1966) and plating on MS1367 at 30°. After mutagenesis for 6 hr, phage survival was 60% and the frequency of revertants increased 40-fold.

$P_{ant}\downarrow$R204, RU523 and RE56 phage were mutagenized with ultraviolet (UV) light by irradiation to a fluence of 900 to 3600 ergs/mm^2. Revertants (designated "RU") were selected at 30° on UV-irradiated (75 ergs/mm^2) MS1656 or MS1890. The plasmid pKM101 in these strains enhances both reactivation of UV-irradiated phage and mutagenesis due to error-prone repair (WALKER 1978). Phage survival (assayed on UV-irradiated MS1655) ranged from 0.3 to 0.9%, and the increase in the frequency of revertants ranged from 16-fold to 6000-fold.

Revertants were classified according to their ability to grow and their plaque morphology on DB7000, DB7283, MS1363, MS1367, MS1582 and MS1585 at low (30°) and high temperature (37° or 40°) (see RESULTS).

DNA sequencing: DNA sequences of the P_{ant} region of revertants were determined by the chemical method of MAXAM and GILBERT (1980). Purification of phage DNA and preparation of end-labeled *Eco*RI-*Ava*II fragments containing P_{ant} were as described previously (YOUDERIAN, BOUVIER and SUSSKIND 1982).

Plasmids: Plasmids pMS65 and pMS87 were constructed by ligation of a purified *Sau*3A1 fragment (818 bp; see Figure 1) from $P_{ant}\downarrow$R204 $P_{ant}\uparrow$R8 and $P_{ant}\downarrow$RU523 $P_{ant}\uparrow$RU4, respectively, into the *Bam*HI site of pBR322 (BOLIVAR *et al.* 1977). General procedures used to construct plasmids, select transformants in *E. coli* HB101 (BOLIVAR *et al.* 1977) and purify plasmid DNA have been described (YOUDERIAN, BOUVIER and SUSSKIND 1982).

Crosses between plasmids and phages: Salmonella strain MS1883 was transformed with plasmids pMS65 and pMS87 using the procedure of LEDERBERG and COHEN (1974). MS1883/pMS65 and MS1883/pMS87 were grown to 6 × 10^8 cells/ml in M9CAA containing 50 μg/ml of ampicillin and infected with a multiplicity of five phage per cell. To stimulate recombination with plasmid pMS87, phage were irradiated with UV (900 ergs/mm^2) prior to infection. After 20 min at 25° to allow adsorption of phage, the infected cultures were diluted 25-fold in M9CAA plus ampicillin and incubated 2.5 hr at 30°. Lysates were treated with chloroform, incubated with purified P22 tail protein (gift of P. BERGET) and plated on a *supE*(P22 *imm*I_Δ) lysogen (MS1367) at 30° to select Ant$^+$ recombinants.

Assay of antirepressor synthesis in vivo: Phage stocks used in label experiments were dialyzed against two changes of 100 volumes of phage dialysis buffer to remove contaminating methionine that otherwise interferes with incorporation of the methionine label. DB7000 cells were grown in supplemented M9 to a concentration of 2 × 10^8/ml. Unirradiated cells were diluted two-fold in supplemented M9 containing phage (multiplicity of infection, ten phage per cell) and shaken at 37°. At 40 min after infection, 50 μl of cells were mixed with 5 μl of ^{35}S-methionine (~50 μCi; ~1000 Ci/mmol), and incubated at 37° for 2–3 min. Twenty-five microliters of 3× sample buffer (LAEMMLI 1970) were added to each sample, and samples were immediately transferred to 90° for 2 min. Incorporation into trichloroacetic acid-insoluble material was measured for each sample to determine that incorporation was uniform for different infections in the same experiment.

Discontinuous SDS-polyacrylamide slab gels were prepared and run as described by STUDIER (1972) and MAIZEL (1972) using the recipes of LAEMMLI (1970). Gels were run at a constant current of 30 mA. Following electrophoresis, gels were fixed in 7.5% acetic acid, 30% methanol, dried and autoradiographed using Kodak XAR-5 film.

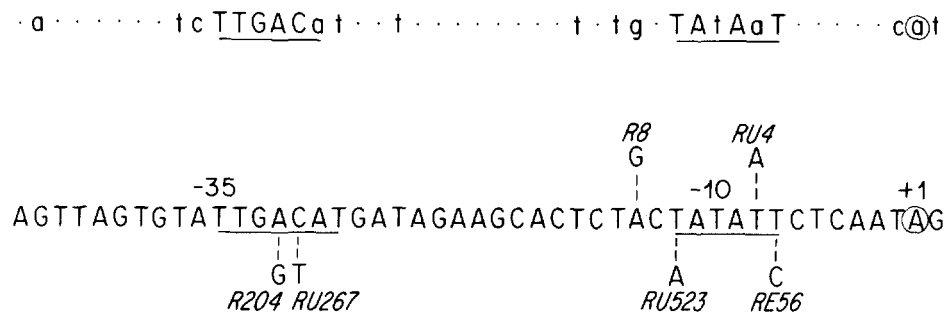

FIGURE 2.—P_{ant} substitution mutations. In the lower part of the figure, the sequence of wild-type P_{ant} is shown. Below it are the four parental $P_{ant}\downarrow$ mutations, and above it are the $P_{ant}\uparrow$ substitution mutations. The top line shows the consensus promoter sequence of HAWLEY and MCCLURE (1983). Highly conserved bases (occurring in >54% of wild-type promoters) are indicated by capital letters and weakly conserved bases (occurrence >39%) are indicated by lower case letters. The −35 and −10 hexamers are underlined and the start point of transcription is circled.

RESULTS

Isolation and genetic analysis of promoter-up mutants: Promoter-up mutations were obtained by reversion of four different severe $P_{ant}\downarrow$ mutations. Two of these are located in the −35 region (R204, −32, A:T → G:C and RU267, −31, C:G → T:A) and two are in the −10 region (RU523, −12, T:A → A:T and RE56, −7, T:A → C:G) (Figure 2). All are changes that decrease the homology of the *ant* promoter sequence with the consensus promoter sequence.

As explained above, Ant^+ revertants of severe $P_{ant}\downarrow$ mutants can be selected by virtue of their ability to grow on P22 $immI_\Delta$ lysogens. Three types of revertants might be expected: (1) true revertants, in which the wild-type P_{ant} sequence is restored; (2) primary-site pseudorevertants, in which the $P_{ant}\downarrow$ mutation changes to another mutant allele; and (3) secondary-site pseudorevertants, in which the $P_{ant}\downarrow$ mutation is retained and an additional mutation is acquired that results in increased promoter activity. All four parental $P_{ant}\downarrow$ phages were derived by pseudoreversion of P22 *mnt-ts arc-am* and retain both parental mutations. True revertants of these *mnt-ts* $P_{ant}\downarrow$ *arc-am* phages will have the genotype *mnt-ts arc-am* and will regain the Arc^- lethal phenotype (inability to grow on sup^0 cells at high temperature). The same expectation applies to any pseudorevertant that restores *ant* promoter activity to wild-type or higher levels. In contrast, some pseudorevertants are expected to behave like R204-R1, a pseudorevertant that has partially restored promoter activity. This phage synthesizes antirepressor at an intermediate rate, sufficient to confer the Ant^+ phenotype, yet insufficient to confer the Arc^- lethal phenotype (SUSSKIND and YOUDERIAN 1982).

To obtain a broad spectrum of revertants, most revertants were selected by plating on a *supE*(P22 $immI_\Delta$) lysogen. Since the host *supE* allele suppresses the *arc-am* mutation, this host is permissive for revertants with the Arc^- conditional-lethal phenotype. Revertants were also selected on a sup^0(P22 $immI_\Delta$) lysogen; this procedure selects against true revertants, the predominant type

obtained with a *supE* lysogen, and enriches for pseudorevertants that only partially restore P_{ant} activity.

More than 50 independent revertants of each of the four parental $P_{ant}\downarrow$ phages were isolated. These revertants were divided into phenotypic classes according to their plaque morphology on host strains with different *sup* alleles (to vary Arc activity) and at different temperatures (to vary Mnt activity). Plaque morphology is a sensitive indicator of differences in the rate of antirepressor synthesis, which varies depending on *ant* promoter strength and on the amount and activity of the Arc and Mnt repressors. Phages that synthesize little or no antirepressor form turbid plaques; phages that synthesize higher levels of antirepressor form semiclear or clear plaques (because intermediate levels of antirepressor interfere with establishment of lysogeny); phages that dramatically overproduce antirepressor form pinpoint clear plaques or no plaques at all, due to the Arc^- lethal phenotype. This phenotypic analysis enabled us to deduce that particular revertants were likely to be pseudorevertants and others were likely to be true. In addition, we were able to discern that some pseudorevertants were different from one another.

Revertants were also tested for virulence (ability to grow lytically in immune, P22 $c2^+$ mnt^+ lysogens MS1582 and MS1585). The vast majority of revertants are not virulent, indicating that synthesis of antirepressor by these revertants is still subject to control by Mnt repressor produced by the prophage. This is as expected, since the binding site for Mnt repressor (SAUER *et al.* 1983; YOUDERIAN *et al.* 1983; P. YOUDERIAN, unpublished results; A. VERSHON and R. T. SAUER, unpublished results) does not overlap the sequences thought to contribute to promoter activity, so that a single mutation is unlikely to activate P_{ant} and relieve Mnt repression. Virulent revertants, which presumably have multiple mutations in the *ant* promoter-operator region or mutations that create new (Mnt insensitive) promoters for transcription of *ant*, were not examined further.

Nucleotide changes of promoter-up mutants: DNA sequence changes of 41 revertants were determined. The choice of mutants to sequence was based on the phenotypic analysis described above. As summarized in Table 2 and Figures 2 and 3, the majority of sequenced revertants are pseudorevertants that retain the parental $P_{ant}\downarrow$ mutation and acquire an additional $P_{ant}\uparrow$ mutation. These $P_{ant}\uparrow$ suppressor mutations include single base pair substitutions, single base pair deletions, tandem double base pair deletions and more complex (multisite) changes. The most frequent mutation, an A:T $\rightarrow$ G:C transition at position -14, was obtained from three different severe promoter-down mutants (Table 2; Figure 2). For convenience, this mutation will be referred to as $P_{ant}\uparrow$R8, and representative revertants carrying it (R204-R8, RU267-R5 and RU523-RU29) will be referred to as R204-R8, RU267-R8 and RU523-R8. Another single base pair substitution, $P_{ant}\uparrow$RU4 (-8, T:A $\rightarrow$ A:T), was obtained from RU523 (Figure 2). Six different deletions of one or two base pairs in the spacer region were obtained by reversion of RU523 and RE56 (Figure 3).

None of the sequenced pseudorevertants has the Arc^- lethal phenotype (all

TABLE 2

Sequenced promoter-up mutations

	$P_{ant}\downarrow$ parent:	R204 −32, A → G			RU267 −31, C → T	RU523 −12, T → A	RE56 −7, T → C
	Mutagen:	S	H	U	S	U	U
	Selection:	*supE*	*supE*	*supE*	*supE*	*sup*^{0}	*supE*
Substitutions							
True ($P_{ant}\downarrow \rightarrow P_{ant}^+$)		3	1	7	1	1	1
−14, A → G		4	3	5	1	2	0
−8, T → A		0	0	0	0	2	0
Deletions							
−25, A → Δ		0	0	0	0	1	0
−22, A → Δ		0	0	0	0	1	0
−20, C → Δ		0	0	0	0	1	0
−17, T → Δ		0	0	0	0	1	0
−14 and −13, AC → Δ		0	0	0	0	1	0
−13 and −12, CT → Δ		0	0	0	0	0	1
Multisite							
−9 to −6, ATCC → CT		0	0	0	0	0	1
−25, A → G and −22, A → Δ		0	0	0	0	3	0
Total		7	4	12	2	13	3

Only bases in the top (*ant* coding) strand are indicated. At the right are tallied the numbers of independent isolates of each mutation among spontaneous revertants (S) and revertants induced by hydroxylamine (H) and UV with pKM101 (U).

are able to form plaques on *sup*^{0} cells at 37°). Several revertants that fail to grow on *sup*^{0} cells at 37° were sequenced, but these were found to be true revertants.

We previously reported the isolation and characterization of three spontaneous revertants of $P_{ant}\downarrow$R204, R204-R1, R204-R2 and R204-R3. Sequence analysis shows that R204-R2 and R204-R3 are true revertants, whereas R204-R1 is identical with R204-R8. These findings are consistent with the evidence that R204-R2 and R204-R3 fully restore promoter activity, whereas R204-R1 only partially restores activity (SUSSKIND and YOUDERIAN 1982).

Separating $P_{ant}\uparrow$ mutations from parental $P_{ant}\downarrow$ mutations: The $P_{ant}\uparrow$R8 and RU4 mutations, which substitute consensus base pairs for nonconsensus base pairs, might be expected to increase promoter activity when the promoter sequence is otherwise wild type. To test this prediction, recombinant phages carrying $P_{ant}\uparrow$R8 or RU4 in isolation were constructed. The $P_{ant}\uparrow$ mutations were separated from parental $P_{ant}\downarrow$ mutations by crossing phages with plasmids carrying the promoter region of $P_{ant}\downarrow$ $P_{ant}\uparrow$ revertants.

To isolate the $P_{ant}\uparrow$R8 mutation (Figure 4A), a small restriction fragment containing the promoter region of P22 $P_{ant}\downarrow$R204 $P_{ant}\uparrow$R8 was subcloned in pBR322. A Salmonella *supE* host carrying this plasmid was superinfected with P22 $P_{ant}\downarrow$RU523 phage. During this permissive infection, RU523 phage can recombine with the homologous *ant* promoter region carried by the plasmid and rescue the RU523$^+$ allele. Recovery of this allele from the plasmid requires at least two crossover events, one between RU523 and the left end point of

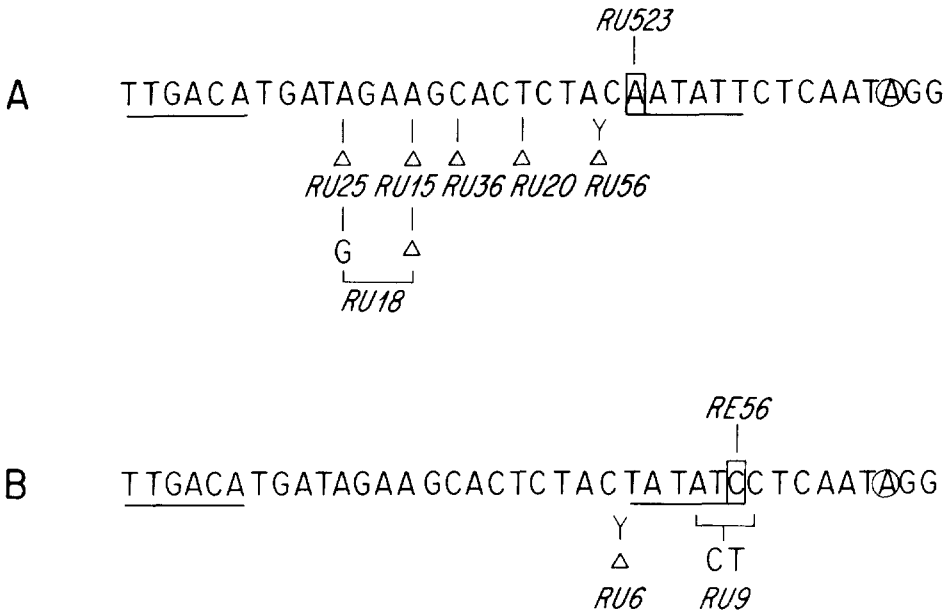

FIGURE 3.—Deletion and multisite mutations in pseudorevertants of RU523 and RE56. Bases are underlined and circled as in Figure 2, except that the parental $P_{ant}\downarrow$ mutation is boxed. Below each promoter sequence are shown the changes in Ant^+ pseudorevertants. Deletions are denoted by "Δ".

the subcloned fragment of P22 DNA and one between RU523 and the right end point of the fragment. Since crossovers to the left are restricted to a region only 63 bp long, a substantial fraction of these crossovers should occur within the 18-bp subinterval between $P_{ant}\downarrow$R204 and $P_{ant}\uparrow$R8, resulting in Ant^+ progeny phage carrying the $P_{ant}\uparrow$R8 mutation in isolation.

Progeny of this cross were plated at 30° on a *supE*(P22 $imm I_{\Delta}$) lysogen (MS1367) to select Ant^+ recombinants. Since the parental phage cannot grow under these conditions, the desired $P_{ant}\uparrow$ recombinant should represent a substantial fraction of the plaque-forming progeny on this host. Recombinants carrying the $P_{ant}\uparrow$R8 mutation were found to represent 8% of the Ant^+ progeny and could be distinguished from $P_{ant}\downarrow$R204 $P_{ant}\uparrow$R8 recombinants by their plaque morphology on MS1367 and by their inability to grow on sup^0 hosts (Arc^- lethal phenotype). The DNA sequence of the promoter region of one of these recombinants confirmed its expected genotype.

A similar strategy was used to isolate the $P_{ant}\uparrow$RU4 mutation by recombination between a plasmid containing the $P_{ant}\downarrow$RU523 $P_{ant}\uparrow$RU4 promoter and a superinfecting phage carrying $P_{ant}\downarrow$RE56 (Figure 4B). In this cross, homology to the left of $P_{ant}\downarrow$RE56 is limited to 68 bp, but the desired crossover must occur within a 4-bp subinterval (between the $P_{ant}\downarrow$RU523 and $P_{ant}\uparrow$RU4 mutations). Although $P_{ant}\uparrow$RU4 phage represented only about 1% of all Ant^+ recombinants, they could be distinguished from $P_{ant}\downarrow$RU523 $P_{ant}\uparrow$RU4 recombinants by their acquisition of the Arc^- lethal phenotype. DNA sequence analysis of two recombinants confirmed that they carry $P_{ant}\uparrow$RU4 in isolation.

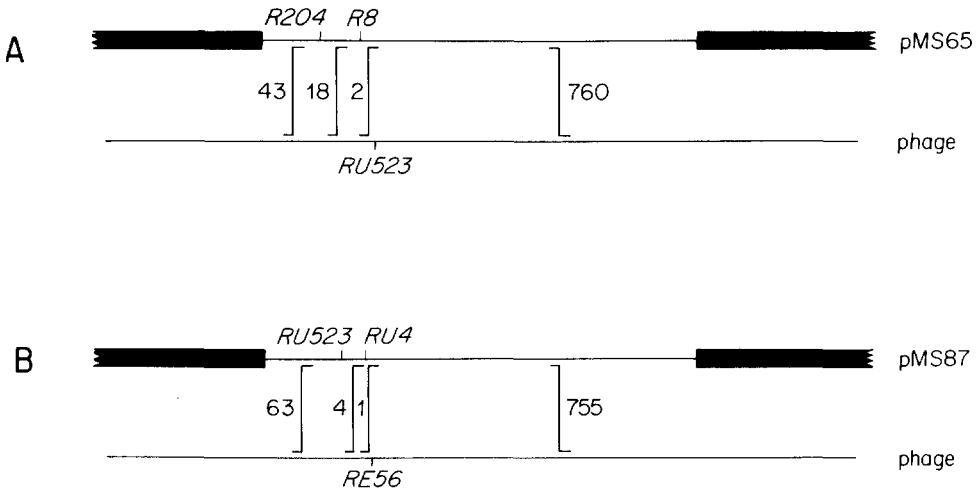

FIGURE 4.—Isolation of $P_{ant}\uparrow$ mutations by crosses between plasmids and phages. P22 $P_{ant}\downarrow$ phage were crossed with plasmids carrying inserts of P22 DNA (thin lines) as described in MATERIALS AND METHODS. Crossovers that generate Ant^+ progeny are drawn; numbers indicate distances available for each crossover (the number of phosphates between nonhomologous base pairs).

$P_{ant}\uparrow$R8 and $P_{ant}\uparrow$RU4 phage do not grow on *sup*^{0} hosts at 30°, whereas P_{ant}+ phage form pinpoint plaques at reduced efficiency under this condition (SUSSKIND 1980). This finding, indicating that the Arc^- lethal phenotype is accentuated by these mutations, suggests that the $P_{ant}\uparrow$ promoters are more active than the wild-type promoter.

Effect of $P_{ant}\uparrow$ substitution mutations on promoter activity: To examine the effects of the $P_{ant}\uparrow$ R8 and RU4 substitution mutations on promoter activity *in vivo*, we examined the rate of antirepressor synthesis during infection of a *sup*^{0} host with various P22 P_{ant} mutants at 37°. (Since all of these phages carry *arc-am* and *mnt-ts* mutations, the Arc and Mnt repressors are inactive under these conditions.) We might naively expect $P_{ant}\downarrow$ $P_{ant}\uparrow$ pseudorevertants to synthesize antirepressor at rates intermediate between those of $P_{ant}\downarrow$ and P_{ant}+ phages and $P_{ant}\uparrow$ phages to synthesize antirepressor at rates greater than that for P_{ant}+ phage.

As shown in Figure 5A, P22 phages carrying the severe promoter-down mutations R204 (lane c), RU267 (lane e) and RU523 (lane g) synthesize no detectable antirepressor, whereas P_{ant}+ phage (lane b) synthesizes antirepressor at a high rate. It is also evident that P_{ant}+ phage underproduce P22 late proteins, whereas $P_{ant}\downarrow$ phages synthesize the late proteins at normal rates. The $P_{ant}\downarrow$ $P_{ant}\uparrow$ pseudorevertants R204-R8 (lane d), RU523-R8 (lane h) and RU523-RU4 (lane i) synthesize more antirepressor than their $P_{ant}\downarrow$ parents but less antirepressor than P_{ant}+ phage. These pseudorevertants also produce normal levels of P22 late proteins. These patterns of protein synthesis are as expected, since these phages have the Ant^+ phenotype but not the Arc^- lethal phenotype. The rate of antirepressor synthesis is different for each of these revertants (R204-R8 > RU523-RU4 > RU523-R8), in agreement with differences in their

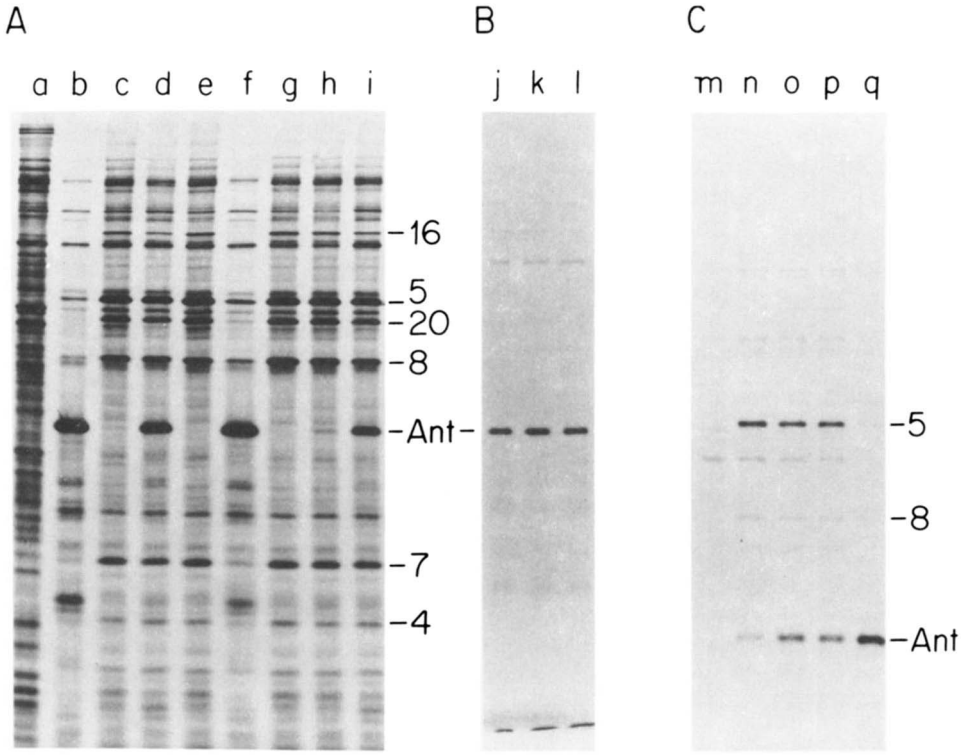

FIGURE 5.—Effects of $P_{ant}\uparrow$ substitution mutations on promoter activity *in vivo*. Proteins pulse labeled with ^{35}S-methionine 39–41 min after infection of DB7000 (*sup*^{0}) cells were analyzed by SDS-polyacrylamide gel electrophoresis as described in MATERIALS AND METHODS. Lanes a and m are uninfected cell controls. The infecting phages were: b, P_{ant}+; c, R204; d, R204-R8; e, RU267; f, RU267-R8; g, RU523; h, RU523-R8; i, RU523-RU4; j, R8; k, RU4; l, P_{ant}+; n, P_{ant}+ R1044; o, R8 R1044; p, RU4 R1044; q, P_{ant}+. Several P22 late proteins are designated by the corresponding gene number. In A, the film is overexposed to show antirepressor protein produced by RU523-R8.

plaque morphology on this *sup*^{0} host at 37° (clear, semiclear and turbid, respectively).

The pseudorevertant RU267-R8 (lane f) appears to synthesize as much antirepressor as P_{ant}+ phage yet produces slightly higher levels of the P22 late proteins. Whereas P_{ant}+ phage do not form plaques on this *sup*^{0} host at 37°, RU267-R8 phage form pinpoint clear plaques, indicating that the Arc$^-$ lethal phenotype is not fully suppressed. Therefore, this phage is expected to synthesize slightly less antirepressor than P_{ant}+ phage, a difference that is apparently too subtle to detect by gel autoradiography of pulse-labeled proteins. Clearly, the RU267-R8 promoter is similar to the P_{ant}+ promoter in activity and is more active than the other three $P_{ant}\downarrow$ $P_{ant}\uparrow$ promoters.

To our initial surprise, Figure 5B shows that $P_{ant}\uparrow$R8 (lane j), $P_{ant}\uparrow$RU4 (lane k) and P_{ant}+ (lane 1) phages synthesize antirepressor at approximately the same rate. Densitometric analysis of the autoradiogram presented in Figure 5B re-

veals that, for all three genotypes, the labeled band corresponding to antirepressor accounts for about 90% of total incorporated label. Thus, most of the protein synthetic capacity of *sup*^{0} cells infected with *mnt-ts* P_{ant}+ *arc-am* phage is dedicated to the production of antirepressor, and any further increase in the rate of antirepressor synthesis due to the $P_{ant}\uparrow$ mutations cannot be detected.

To show that these mutations increase P_{ant} activity in otherwise wild-type promoters, we combined each $P_{ant}\uparrow$ allele with a mutation in the *ant* ribosome-binding site, R1044, which changes the *ant* SHINE and DALGARNO (1974) sequence from 5′-GGAG-3′ to 5′-GTAG-3′ (M. M. SUSSKIND, unpublished results). As shown in Figure 5C, the R1044 mutation decreases the rate of antirepressor synthesis by P_{ant}+ phage about five-fold (compare lanes n and q). When P_{ant}+ and $P_{ant}\uparrow$ phages carrying R1044 are compared, it is evident that $P_{ant}\uparrow$R8 (lane o) and $P_{ant}\uparrow$RU4 (lane p) phages synthesize about twice as much antirepressor as the isogenic P_{ant}+ control (lane n). We conclude that $P_{ant}\uparrow$R8 and $P_{ant}\uparrow$RU4 increase the intrinsic activity of the *ant* promoter, both in combination with $P_{ant}\downarrow$ alleles and in isolation.

Effect of deletion mutations on promoter activity: In the experiment shown in Figure 6, the rate of synthesis of antirepressor during infection of *sup*^{0} cells at 37° was examined for the revertants with deletion or multisite mutations (see Figure 3). The rate of antirepressor synthesis is too low to detect (or barely detectable) for six revertants of RU523 with single base pair deletions in the spacer region (lanes d–h). In contrast, much higher levels of antirepressor (within two- to six-fold of the P_{ant}+ control) are produced by RU523-RU56 (lane i) and RE56-RU6 (lane l), which have deletions of 2 bp, and by RE56-RU9 (lane j), which has a rearrangement, a net deletion of 2 bp, in the −10 region.

DISCUSSION

$P_{ant}\uparrow$ substitution mutations: Our results demonstrate that two substitution mutations, $P_{ant}\uparrow$R8 and $P_{ant}\uparrow$RU4, increase *ant* promoter activity *in vivo*. These mutations are suppressors of promoter-down mutations and increase P_{ant} activity when the promoter sequence is otherwise wild type.

The $P_{ant}\uparrow$R8 mutation is an A:T → G:C transition at position −14 that increases the homology of the *ant* promoter with the consensus promoter sequence. The reverse transition at this position (−14, G:C → A:T) has been isolated and shown to be a promoter-down mutation in the *E. coli arg* promoter (PIETTE *et al.* 1982) and the λ P_{re} (ROSENBERG *et al.* 1978) and P_{rm} (ROSEN *et al.* 1980) promoters. The $P_{ant}\uparrow$RU4 transversion (−8, T:A → A:T) also increases P_{ant} homology with consensus. This mutation is analogous to the difference between the *E. coli lac* P^s and UV5 promoters, which accounts for a 5-fold increase in promoter activity *in vivo* (GILBERT 1976). The $P_{ant}\uparrow$R8 and RU4 sequence changes lend further support to the hypothesis that the consensus promoter sequence corresponds to maximal promoter strength (see HAWLEY and MCCLURE 1983).

These $P_{ant}\uparrow$ mutations are clearly not allele-specific suppressors. The results

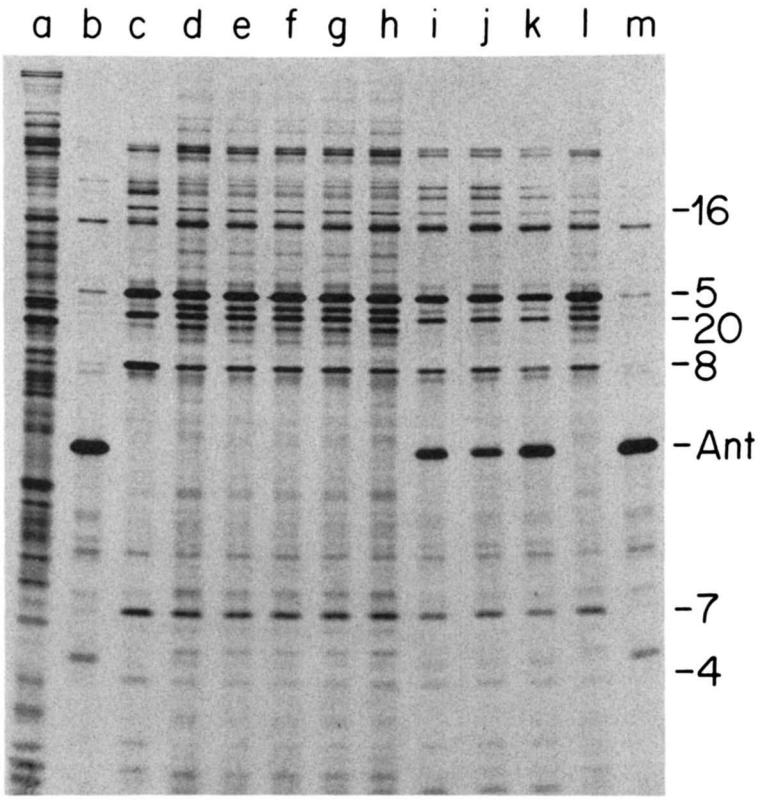

FIGURE 6.—Effects of deletion and multisite mutations on promoter activity *in vivo*. Procedures were the same as those in the experiment shown in Figure 5. a, Uninfected cell control. The infecting phages were: b and m, P_{ant}+; c, RU523; d, RU523-RU25; e, RU523-RU18; f, RU523-RU15; g, RU523-RU36; h, RU523-RU20; i, RU523-RU56; j, RE56-RU9; k, RE56-RU6; l, RE56.

described here show that P_{ant}↑R8 increases promoter activity in combination with three different P_{ant}↓ mutations and in isolation; similarly, P_{ant}↑RU4 has a promoter-up phenotype in combination with P_{ant}↓RU523 and in isolation (Figure 5). By recombining P_{ant}↓ and P_{ant}↑ mutations, we find that P_{ant}↑R8 and P_{ant}↑RU4 suppress nine different P_{ant}↓ mutations (D. GRAÑA AND M. M. SUSSKIND, unpublished results). Assays of rates of antirepressor synthesis *in vivo* indicate that promoters with different combinations of P_{ant}↓ and P_{ant}↑ alleles vary considerably in activity (see Figure 5). Further analysis of the strength of these promoters *in vivo* and *in vitro* is in progress to elucidate the quantitative effects of each allele on promoter strength and the nature of the mutually epistatic interaction between promoter-down and promoter-up mutations.

The spacer region: Comparison of more than 150 prokaryotic promoter sequences shows that the region of DNA between the conserved −35 and −10 regions is highly conserved in length. Mutations in other promoters that increase or decrease the length of the spacer region indicate that the consensus spacer length of 17 bp corresponds to optimal promoter activity. Although the wild-type *ant* promoter has the consensus 17-bp spacing, reversion of

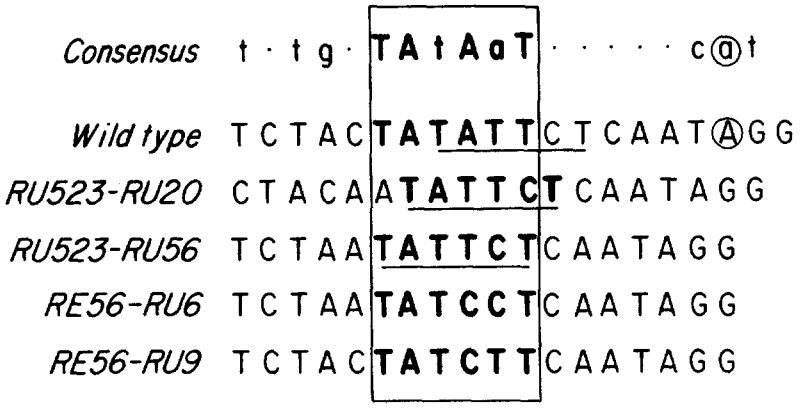

FIGURE 7.—Comparison of the −10 regions of revertants with deletion or multisite mutations. All four mutant promoters are identical with the wild type in the region preceding the sequences shown. The hexamer 5′-TATTCT-3′ (spaced at 19 bp in the wild-type promoter) is underlined. For each promoter, the hexamer presumed to be active is indicated by boldface letters. Hexamers within the window have the consensus spacing of 17 bp. The sequence shown for RU523-RU20 also applies to the other revertants of RU523 (RU36, 15, 18 and 25), but those promoters also differ from wild type in the preceding 4–8 bp.

P_{ant}↓RU523 yields pseudorevertants with single or tandem double base pair deletions in the spacer region (Figure 3). Hence, these suppressor mutations change the length of the spacer region away from the consensus length yet result in increased promoter activity.

An explanation for this unexpected result is that this class of mutant promoter utilizes a different −10 hexamer. The −10 region of the wild-type *ant* promoter sequence has three overlapping hexamers that are extensively homologous with the −10 consensus sequence: 5′-TACTAT-3′, spaced 14 bp from the −35 hexamer; 5′-TATATT-3′, spaced at 17 bp; and 5′-TATTCT-3′, spaced at 19 bp. The analysis of *ant* promoter-down mutations implies that the active hexamer in wild-type P_{ant} is the one spaced at 17 bp. For example, of 11 single bp substitution mutations in this 11-bp region, none is in the bases unique to the hexamers spaced at 14 and 19 bp, and six mutations leave unchanged one or the other of these alternate hexamers. Moreover, mutations that *improve* either alternate hexamer, but weaken the hexamer spaced at 17 bp, have a promoter-down phenotype (YOUDERIAN, BOUVIER and SUSSKIND 1982). It seems likely, however, that the hexamer spaced at 19 bp in the wild-type promoter (5′-TATTCT-3′) is activated when shifted to a new spacing of 18 bp (by any of four different single bp deletions) or 17 bp (by the 2-bp deletion in RU523-RU56) (Figure 7). Consistent with this idea, preliminary results of transcription initiation experiments *in vitro* (MCCLURE 1980) suggest that the RU523-RU56 promoter preferentially utilizes a start point of transcription located 2 bp downstream of the wild-type start point (D. HAWLEY, P. YOUDERIAN, M. M. SUSSKIND and W. R. MCCLURE, unpublished results).

As illustrated in Figure 7, the revertants of RE56 can be explained by similar considerations. The 2-bp deletion in RE56-RU6 shifts the sequence 5′-TATCCT-3′ (the alternate hexamer carrying the RE56 mutation) to a spacing

of 17 bp, and the multisite mutation in RE56-RU9 (a net 2-bp deletion) creates the sequence 5′-TATCTT-3′ spaced at 17 bp. These hexamers, like the one shifted by the revertants of RU523, differ from consensus only at the fourth and fifth positions.

Figure 6 shows that the revertants with 2-bp deletions synthesize much higher levels of antirepressor than the revertants with 1-bp deletions. These findings support the hypothesis that a spacer length of 17 bp is more favorable than one of 18 bp, although differences in sequence, particularly at the weakly conserved positions preceding the −10 hexamer, may also be important. It is also intriguing that the rate of antirepressor synthesis apparently varies for the three revertants with 2-bp deletions, since RU523-RU56 and RE56-RU6 differ only at the fourth position of the −10 hexamer, whereas RE56-RU6 and RE56-RU9 differ at the fifth position and in the base pair preceding the hexamer (Fig. 7). Precise quantitation of the strength of these promoters is necessary, however, to establish the effects of these differences.

The P_{ant} region is also thought to contain a divergent, overlapping promoter for leftward transcription of the *mnt* gene (SAUER *et al.* 1983). Thus, positions −2 to −7 of P_{ant} are proposed to be the −35 heaxamer of P_{mnt} (5′-TTGAGA-3′), and positions −25 to −30 of P_{ant} are proposed to be the −10 hexamer of P_{mnt} (5′-TATCAT-3′). Transcription experiments *in vitro* (D. HAWLEY and W. R. MCCLURE, unpublished results) provide evidence that P_{mnt} is active only when P_{ant} is damaged by mutation or is repressed by Mnt protein. These findings suggest that competition from P_{ant} decreases P_{mnt} activity. If competition from P_{mnt} limits P_{ant} activity, mutations that increase P_{ant} activity might be expected to include mutations that damage P_{mnt}. All of the $P_{ant}\uparrow$ mutations we have isolated can be explained in terms of their effects on the rightward promoter sequence; whether their effects on the leftward promoter sequence are also relevant can only be assessed when the interaction between P_{ant} and P_{mnt} is more clearly defined.

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