The Effects of Mutations in the *ant* Promoter of Phage P22 Depend on Context

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ABSTRACT

Recombination was used to construct 22 two- or three-way combinations of down- and up-mutations in P_{ant}, a strong, near-consensus promoter of phage P22. The relative strengths of these promoters *in vivo* were assayed by fusing them to an *ant/lacZ* gene fusion and measuring β-galactosidase levels produced by lysogens carrying the fusions on single-copy prophages. The results of these assays show that the magnitude of the effect of a promoter mutation can vary considerably when its context is changed by the presence of another mutation. In addition, as P_{ant} approaches conformity with the consensus promoter sequence, the up-mutations decrease promoter strength, even though the same mutations increase promoter strength in the presence of a down-mutation. These context effects imply that individual consensus base pairs cannot be considered to contribute to promoter strength independently.

Most promoters from *Escherichia coli*, *Salmonella typhimurium* and their phages have two regions of conserved DNA sequence located approximately 10 and 35 bp upstream from the start point of transcription (HAWLEY and MCCLURE 1983; HARLEY and REYNOLDS 1987) (Figure 1). The most prominent features of the consensus promoter sequence are the "−35 hexamer" (5′-TTGACA-3′), the "−10 hexamer" (5′-TATAAT-3′), and the length of the spacer region between them (17 bp). Mutational analysis of several promoters has shown that these conserved features are important determinants of promoter activity (reviewed by HAWLEY and MCCLURE 1983). Substitution mutations that affect promoter strength usually alter base pairs in the −10 or −35 hexamers, but can also occur in a few of the weakly conserved base pairs outside the hexamers (most notably, at position −14). Mutations that decrease promoter activity ("down-mutations") are usually changes away from the consensus sequence, and mutations that increase promoter activity ("up-mutations") are usually changes toward consensus. Biochemical studies show that the conserved hexamers affect the rates of early steps in the interaction between the promoter and RNA polymerase, the formation of an initial "closed" complex and isomerization to a transcriptionally active "open" complex (reviewed by MCCLURE 1985).

A logical interpretation of these findings is that the consensus sequence is optimal for promoter strength, but it appears that this is not necessarily so. A synthetic, consensus promoter is less active *in vivo* than several natural promoters with one, two, or three nonconsensus base pairs in the hexamers (DEUSCHLE *et al.* 1986). Changing a natural promoter to make it conform to the consensus sequence in both hexamers optimizes the activity of the λ P_{RM} promoter (SZOKE, ALLEN and DEHASETH 1987), but not the *E. coli lpp* (INOUYE and INOUYE 1985), *E. coli lac* (MANDECKI *et al.* 1985), and λ P_L promoters (BUJARD *et al.* 1987). Bujard and coworkers have shown that the activity of a consensus or near-consensus promoter can be influenced by sequences downstream of the −10 hexamer, which affect the rate of a late step in transcription initiation (KAMMERER *et al.* 1986; see DISCUSSION).

In analyzing the relationship between promoter structure and function, one question that arises is whether individual consensus base pairs can be considered to contribute to promoter strength independently. This assumption underlies current attempts to formulate programs that predict promoter strength from sequence alone (MULLIGAN *et al.* 1984). A simple way to test this assumption is to compare the effects of single and double mutations in a single promoter, in order to determine whether the effect of one mutation varies when its context is changed by the presence of another mutation. In this report, we address this question by combining mutations in P_{ant}, a strong, near-consensus promoter of Salmonella phage P22 (Figure 1).

As illustrated in Figure 2, P_{ant} directs transcription of two genes, *arc* and *ant* (reviewed by SUSSKIND and YOUDERIAN 1983). The *ant* gene encodes antirepressor, an inhibitor of c2 repressor, the protein that directly represses genes essential for lytic growth. The *arc* gene encodes a repressor that turns off P_{ant} after the first few minutes of infection by P22. Another

repressor, encoded by the linked gene *mnt*, turns off P_{ant} during lysogeny. Expression of the *ant* gene is also negatively regulated by a small antisense RNA called *sar*, which is transcribed from P_{sar}, a leftward promoter in the beginning of the *ant* coding sequence (LIAO *et al.* 1987; WU *et al.* 1987).

Our genetic analysis of P_{ant} takes advantage of two different selections against phages that synthesize too much or too little antirepressor. (1) In Su^- cells infected with P_{ant}^+ *arc-am* phage, antirepressor is so grossly overproduced that synthesis of essential phage proteins is hampered and progeny phage are not produced (SUSSKIND 1980). The lethal effect of the *arc-am* mutation is suppressed by promoter-down ($P_{ant}\downarrow$) mutations. Figure 1 shows the sequence changes of ten of the 25 $P_{ant}\downarrow$ mutations we previously obtained as suppressors of the Arc^- lethal phenotype (YOUDERIAN, BOUVIER and SUSSKIND 1982). (2) Antirepressor is not normally required for lytic growth of P22, but is essential for growth of the phage upon superinfection of P22 $c2^+$ lysogens deleted for the *mnt-ant* region ($immI_\Delta$ lysogens). In such lysogens, the superinfecting phage must make enough antirepressor to inactivate the c2 repressor produced by the prophage. All of the $P_{ant}\downarrow$ mutations shown in Figure 1 severely reduce the rate of synthesis of antirepressor and confer the Ant^- phenotype (failure to grow in $immI_\Delta$ lysogens). By selecting Ant^+ revertants of a few of these $P_{ant}\downarrow$ mutants, we obtained the two promoter-up ($P_{ant}\uparrow$) mutations shown in Figure 1 (GRAÑA, YOUDERIAN and SUSSKIND 1985). Here we describe the construction and analysis of a set of two- and three-way combinations of these down- and up-mutations, and show that their effects depend on context.

MATERIALS AND METHODS

Bacteria: Derivatives of *S. typhimurium* LT2 are listed in Table 1.

Phage: All phages in this study carry *arc-amH1605* (Gln9 → *am*) (SAUER *et al.* 1983) and either *sieA44* and *mnt-ts1* (Val39 → Ala) (SAUER *et al.* 1983) or *Kn6*, a substitution that replaces *sieA* and *mnt* with a kanamycin resistance gene (WU *et al.* 1987) (Figure 2). $P_{ant}\downarrow$ mutations (YOUDERIAN, BOUVIER and SUSSKIND 1982) and $P_{ant}\uparrow$ mutations (GRAÑA, YOUDERIAN and SUSSKIND 1985) have been described. *R1009* and *RU1220* are single base pair changes in the *ant* transcription terminator, T_{ant} (Susskind, unpublished results); they are two of the Group III mutations obtained by pseudoreversion of P22 *mnt-ts arc-am* (YOUDERIAN, BOUVIER and SUSSKIND 1982).

Plasmids: Restriction fragments used for cloning are shown in Figure 2. For all P_{ant} genotypes, the relevant *Sau*3A1 fragment was purified from digests of *mnt-ts arc-am* phage DNAs and inserted in the *Bam*HI site of pMS99 (YOUDERIAN, BOUVIER and SUSSKIND 1982), generating the pMS200 series. In pMS88 and pMS101, the same *Sau*3A1 fragment carrying $P_{ant}\uparrow RU4$ or $P_{ant}\uparrow R8$, respectively, is inserted in the *Bam*HI site of pBR322. In pMS145 and pMS146, the *Eco*RI-*Ava*II fragment from *mnt-ts arc-am* phages carrying $P_{ant}\downarrow RE167$ or $P_{ant}\downarrow RU1099$, respectively, is

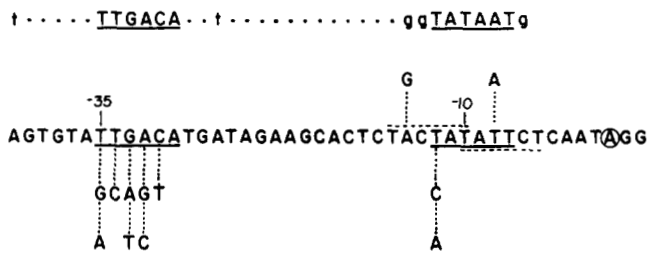

FIGURE 1.—P_{ant} mutations. $P_{ant}\uparrow$ and $P_{ant}\downarrow$ mutations are shown above and below, respectively, the sequence of wild-type P_{ant}. Allele designations for these mutations are listed in Table 2. The upper part of the figure shows the consensus promoter sequence of HARLEY and REYNOLDS (1987). Highly conserved bases are indicated by capital letters, and weakly conserved bases by lower case letters. The −35 and −10 hexamers are underlined, alternative −10 hexamers are marked by dashed lines, and the start point of transcription is circled.

inserted between the *Eco*RI and *Hin*dIII sites of pBR322; the *Ava*II and *Hin*dIII ends had been filled in by DNA polymerase I-large fragment.

Details of the multistep construction of pMS580 (Figure 3) are available upon request. The plasmid has the *Acc*I-*Eco*RI (*bla-ori*) fragment of pBR322 (bp 2247 to 2) (MANIATIS, FRITSCH and SAMBROOK 1982) and, clockwise from the *Eco*RI site, the following segments: (1) the $P_{lac}UV5$ promoter (bp 1187–1281 of the *E. coli lac* sequence); (2) 5′-CAGTCGA-3′; (3) the *arc* gene and 5′ end of *ant* (*Ava*II-*Hpa*I = bp 349–629 of *immI*) (SAUER *et al.* 1983), carrying *arc-amH1605* and P_{sar}-*subS* (a substitution that changes bp 609–612 from 5′-ATAG-3′ to 5′-CGAC-3′); (4) 5′-GGGGATCCC-3′; (5) bp 1311–6274 of *E. coli lac* (codon 10 of *lacZ* to the *Nru*I site in *lacA*; the *Eco*RI site in wild-type *lacZ* is not present); (6) the 3′ end of *ant* and T_{ant} (*Eco*RI-*Taq*I = bp 1354–1617 of *immI*); and (7) the P22 *attP* region (from *Bam*HI site 2 to the *Acc*I site in *int*) (LEONG *et al.* 1986). The resulting *ant/lacZ* gene fusion has codon 10 of *ant* joined by three synthetic codons to codon 10 of *lacZ*. The P_{sar}-*subS* substitution in codons 4 and 5 of *ant* inactivates P_{sar} and creates a *Sal*I site. The joint between segments (5) and (6) fuses part of *lacA* to the 3′ end of *ant* in the wrong frame; translation of the resulting *lacA/ant* fusion terminates before the normal end of *ant*. The joint between segments (6) and (7) is the deletion *9-delTB* (WU *et al.* 1987) (Figure 2). We maintain pMS580 in the presence of pMS421, a compatible $lacI^Q$ plasmid. To construct pMS421, *Eco*RI linkers were attached to the 1.7 kb $lacI^Q$ fragment generated by partial *Hin*cII digestion of pMC7 (CALOS 1978); this *Eco*RI fragment was subcloned in a plasmid that requires *lac* repressor for maintenance, and was then subcloned in the *Eco*RI site of pGB2 (CHURCHWARD, BELIN and NAGAMINE 1984).

Cross procedures: Crosses between phages were carried out in strain MS1363 at 30° as described by SUSSKIND, WRIGHT and BOTSTEIN (1971). Crosses between plasmids and phages were performed as described by GRAÑA, YOUDERIAN and SUSSKIND (1985), except that irradiation with ultraviolet was used to stimulate recombination only in the crosses shown in Figure 4. Crosses with pMS580 were done in strain MS2657 at 30°; other plasmid-phage crosses were done in MS1883 at 30°. After initial selection of recombinants (see below), phages were purified and high-titer stocks were prepared in strain MS1363 at 30°. Procedures for propagating P22 gene 9^- phages using exogenous P22 tail protein (p9; gift of P. BERGET) are described by YOUDERIAN and SUSSKIND (1980).

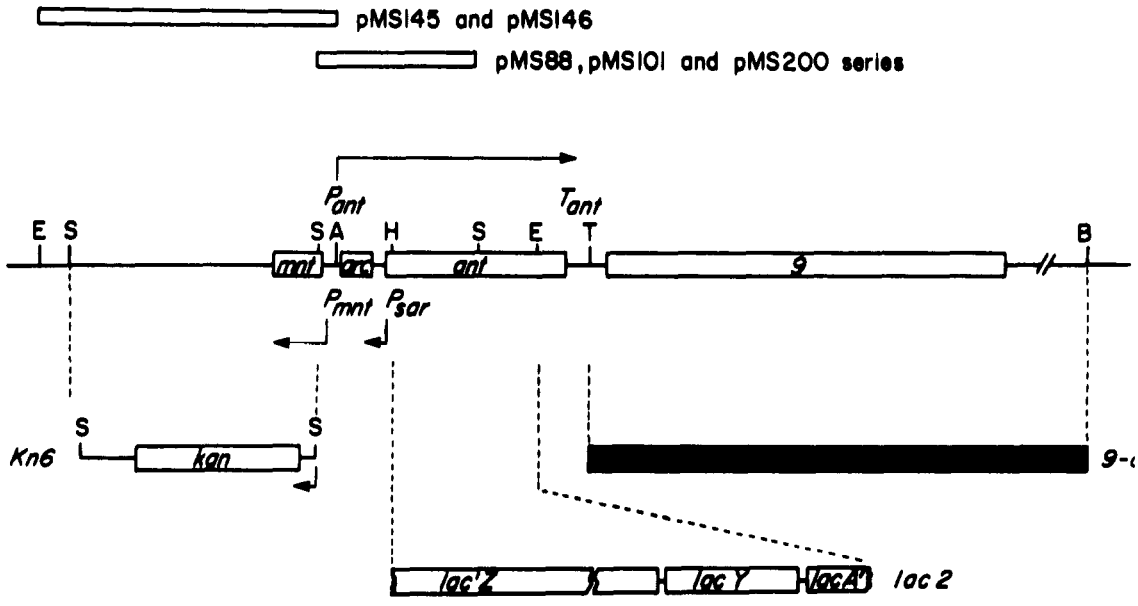

FIGURE 2.—The P22 *immI*-gene *9* region. The map is drawn to scale as established by DNA sequence analysis (SAUER *et al.* 1982, 1983, and unpublished data). Transcripts are indicated by arrows. Restriction sites used for cloning are: A, *Ava*II; B, *Bam*HI; E, *Eco*RI; H, *Hpa*I; S, *Sau*3A1; T, *Taq*I. Only relevant sites are shown. The bars above the map represent the *Eco*RI-*Ava*II and *Sau*3A1 inserts in plasmids. Below the map are shown the *Kn6* substitution, the *lac2* substitution, and the *9-delTB* deletion, all of which are present in P22 *ant/lacZ* strains. The *lac* segment is not drawn to scale. The *Kn6* segment should include (from right to left) bp 831–2264 of Tn*903* (OKA, SUGISAKI and TAKANAMI 1981), but bp 831-1051 are missing. This deletion is also present in pUC71K (VIEIRA and MESSING 1982), the source of the *kan* fragment used to construct *Kn6*.

TABLE 1

Bacterial strains

Strain	Genotype	Reference or source
DB7000	*leuA-414(am)*	SUSSKIND, WRIGHT and BOTSTEIN (1971)
MS1363	*leuA-414(am) supE*	SUSSKIND (1980)
DB7283	*leuA-414(am)* (P22 *sieA44 Ap2del7283*)	WEINSTOCK, SUSSKIND and BOTSTEIN (1979)
MS1367	*leuA-414(am) supE*(P22 *sieA44 Ap2del7283*)	SUSSKIND (1980)
MS1883	*leuA-414(am) supE* r^- m^+	GRAÑA, YOUDERIAN and SUSSKIND (1985)
MS2657	*leuA-414(am) supE* r^- m^+ *recA*/ pMS421	This work

Construction of P22 *ant/lacZ* phages: P22 *ant/lacZ* phages were constructed in two steps. First, each promoter genotype was combined with the *Kn6* substitution. This was accomplished by crossing plasmids carrying each promoter genotype (the pMS200 series) with *Kn6* phages in one of three ways: (1) In most cases, the parental phage was *Kn6* P_{ant}^+ *arc-am*, and recombinants carrying the promoter genotype of the plasmid were obtained by selecting for suppression of the Arc$^-$ lethal phenotype (growth on DB7000 at 37°). (2) In some cases, the parental phage was an Ant$^-$ *Kn6* $P_{ant}\downarrow$ *arc-am* mutant, and recombinants carrying the promoter genotype of the plasmid were obtained by selecting for the Ant$^+$ phenotype (growth on the $immI_\Delta$ lysogen MS1367 at 30°). (3) An Ant$^-$ *Kn6* $P_{ant}\downarrow$ *arc-am* T_{ant}-*RU1220* mutant was used to construct *Kn6* phages with the promoter genotypes $P_{ant}\uparrow$*R8*, $P_{ant}\uparrow$*RU4*, and $P_{ant}\uparrow$*R8*$\uparrow$*RU4*. Ant$^+$ recombinants were selected as described above. The T_{ant}-*RU1220* mutation, a suppressor of the Arc$^-$ lethal phenotype, was included in this cross because we thought that *supE* might not adequately suppress the Arc$^-$ lethal phenotype of *Kn6* $P_{ant}\uparrow$ *arc-am* phages.

The second step was to cross each of the resulting *Kn6* phages with the *ant/lacZ* plasmid pMS580 (Figure 3). Cross lysates were plated at 30° on MS1363 in the presence of P22 tail protein and XGal. Recombinants that had acquired the *lac* substitution and *9-delTB* deletion from the plasmid were recognized by their blue, fuzzy (tail-dependent) plaque morphology. As shown in Figure 3, these *lac* phages must inherit the P_{ant} genotype of their phage parents, as well as the *Kn6* substitution and the *arc-am* mutation. Restriction enzyme cleavage analysis of *lac* phage DNAs was carried out to show that they have the P_{sar}-*subS* mutation (a *Sal*I site) and, where relevant, that they lack the T_{ant}-*RU1220* mutation (a *Hin*dIII site). Finally, the P_{ant} region of each *lac* phage was sequenced to confirm its genotype.

β-Galactosidase assays: Lysogens of P22 *lac* phages in strain DB7000 were constructed and propagated without selection for the *Kn6* (Kan^r) or *lac* determinants. Assays of several independent lysogens of the same *lac* phage show no evidence of double lysogens. [Control experiments indicate that P22 c^+ att^+ int^+ phages carrying suitable drug resistance markers do not readily form unselected double lysogens unless the cell is made diploid for *ataA*, the *attB* site for P22 (M. M. SUSSKIND, unpublished results).]

Cultures (6 ml in 50 ml flasks, started by 1:200 dilution of overnights) were grown in M9CAA (SUSSKIND, WRIGHT

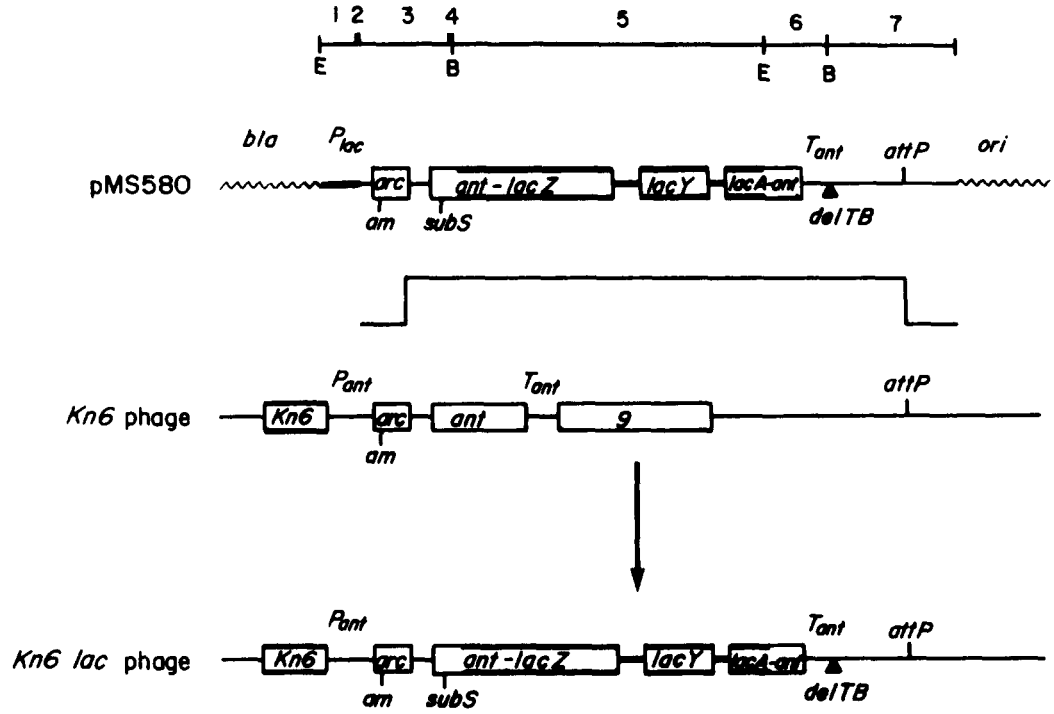

FIGURE 3.—Construction of P22 *ant/lacZ* phages. P22 *Kn6 arc-am* phages with various P_{ant} genotypes were crossed with pMS580 to construct a set of P22 *ant/lacZ* phages (see text). The figure is not drawn to scale. P22 DNA is thin, pBR322 DNA is wavy, and *kan* and *lac* DNA is thick. Above the map of pMS580, segments are numbered as in the text. E, *Eco*RI; B, *Bam*HI.

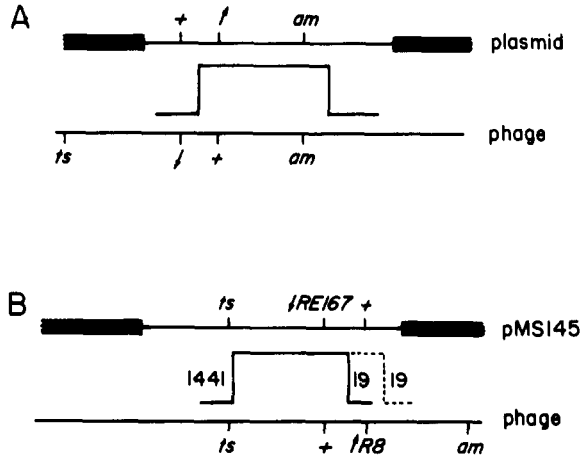

FIGURE 4.—Construction of $P_{ant}\downarrow\uparrow$ phages. Part A illustrates the construction of Ant$^+$ $P_{ant}\downarrow\uparrow$ phages by crossing P22 *mnt-ts* $P_{ant}\downarrow$ *arc-am* phages with $P_{ant}\uparrow$ *arc-am* plasmids pMS88 and pMS101. Thirteen down-up combinations were obtained in this way, including one (*RU483-R8*) in which the map order of the down- and up-mutations is reversed. Part B illustrates construction of three Ant$^-$ $P_{ant}\downarrow\uparrow$ phages by crossing P22 *mnt-ts* $P_{ant}\uparrow$ *arc-am* phages with *mnt-ts* $P_{ant}\downarrow$ plasmids pMS145 and pMS146. Crossovers are shown that generate recombinants that grow well under the plating conditions used to counterselect the parental phage (see text). In part B, numbers denote distances available for crossovers (number of phosphates between nonhomologous bases).

and BOTSTEIN 1971) at 37° to mid-log phase (OD_{600} = 0.3). Cultures were harvested, lysates prepared, and β-galactosidase activity assayed according to the protocol developed by D. BARRICK, K. VILLA-NUEVA, J. CHILDS, R. KALIL, T. SCHNEIDER, L. GOLD, C. LAWRENCE and G. STORMO (in preparation and personal communication). We kept constant the volumes of Z buffer (0.8 ml) and cells (0.2 ml) used to prepare lysates, as well as the volumes of lysate (125 μl) and *o*-nitrophenyl-β-galactopyranoside solution (50 μl) used in the assay. Reactions were carried out in microtiter plates and monitored by a computer-interfaced Titertek Multiskan MC plate-reader (Flow Laboratories) at ~5-min intervals for 30 min. Reaction rates were computed by linear regression and specific activities were calculated using software generously provided by T. SCHNEIDER, L. GOLD and G. STORMO.

Sets of 15–20 lysogens were assayed in parallel; $P_{ant}\downarrow\uparrow$ and $P_{ant}\downarrow$ strains carrying the same down-mutation were always included in the same set. One lysate of each culture was assayed in quadruplicate or two lysates of each culture were assayed in duplicate. The standard deviation of the four measurements was always ≤6% of the mean. This mean specific activity was converted to relative specific activity by dividing by the mean specific activity of the P_{ant}^+ control measured on the same microtiter plate. For each promoter genotype, relative specific activity was measured on two or three different occasions. The mean and standard deviation of these values are given in Table 2.

DNA sequence determination: The P_{ant} region of *mnt-ts arc-am* phages was sequenced by the method of MAXAM and GILBERT (1980) as described by YOUDERIAN, BOUVIER and SUSSKIND (1982). The P_{ant} region of *ant/lacZ* phage DNA's was sequenced by the method of SANGER, NICKLEN and COULSON (1977) using avian myeloblastosis virus reverse transcriptase.

RESULTS

Construction of $P_{ant}\downarrow\uparrow$ phages: To systematically investigate the consequences of combining down- and up-mutations, we constructed twenty $P_{ant}\downarrow\uparrow$ double mutants, each of which has one of the ten down-mutations and one of the two up-mutations shown in

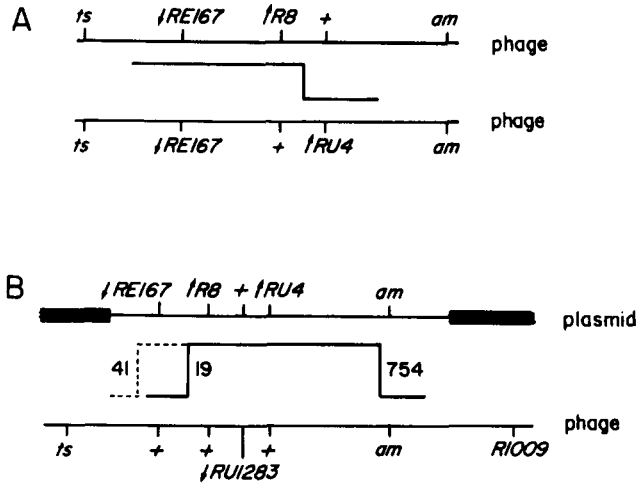

FIGURE 5.—Construction of $P_{ant}\uparrow\uparrow$ phage. Part A illustrates the construction of the triple promoter mutant *RE167-R8-RU4* by crossing P22 *mnt-ts* $P_{ant}\downarrow$*RE167*↑*R8 arc-am* with P22 *mnt-ts* $P_{ant}\downarrow$*RE167*↑*RU4 arc-am*. Part B illustrates the construction of a $P_{ant}\uparrow\uparrow$ phage by crossing P22 *mnt-ts* $P_{ant}\downarrow$*RU1283 arc-am* T_{arc}-*R1009* with a $P_{ant}\downarrow$*RE167*↑*R8*↑*RU4 arc-am* plasmid. $P_{ant}\downarrow$*RU1283* deletes positions −13 and −12; it also has a transition at position −6 (not shown; YOUDERIAN, BOUVIER and SUSSKIND 1982). Crossovers are shown that generate the two major types of Ant$^+$ recombinants.

Figure 1. We previously obtained four of these "down-up" combinations by reverting $P_{ant}\downarrow$ mutants, and used recombination between plasmids and phages to isolate each up-mutation in an otherwise wild-type promoter (GRAÑA, YOUDERIAN and SUSSKIND 1985). New down-up combinations were constructed by recombination in two types of plasmid-phage crosses. In these constructions, we took advantage of the following facts: (1) the *arc-amH1605* mutation, which is carried by all phages used in this study, makes P_{ant}^+ and $P_{ant}\uparrow$ phages defective for growth in Su$^-$ cells, especially at high temperatures (SUSSKIND 1980); and (2) all ten $P_{ant}\downarrow$ mutants can grow in nonlysogenic Su$^-$ cells, but cannot grow in $imm I_\Delta$ lysogens (YOUDERIAN, BOUVIER and SUSSKIND 1982).

Thirteen new $P_{ant}\downarrow\uparrow$ phages were constructed by crossing P22 *mnt-ts* $P_{ant}\downarrow$ *arc-am* phages with $P_{ant}\uparrow$ *arc-am* plasmids as illustrated in Figure 4A. Among the progeny of such crosses, $P_{ant}\downarrow\uparrow$ recombinants were selected by their ability to form normal plaques on the Su$^-$ P22 $imm I_\Delta$ lysogen DB7283 at 30°. Under these conditions, the parental $P_{ant}\downarrow$ phages cannot form plaques because they cannot make enough antirepressor, $P_{ant}\uparrow$ recombinants cannot form plaques because they make too much antirepressor, and P_{ant}^+ recombinants form poor plaques at reduced efficiency. We reasoned that the desired $P_{ant}\downarrow\uparrow$ recombinants (like the four $P_{ant}\downarrow\uparrow$ phages obtained by reversion) would grow well under these conditions if they synthesize intermediate levels of antirepressor—less than the lethal level, but enough to grow in $imm I_\Delta$ lysogens. [This is not a very demanding selection, since even a slight reduction in antirepressor synthesis suppresses the Arc$^-$ lethal phenotype, and quite low levels of antirepressor suffice for the Ant$^+$ phenotype (YOUDERIAN, BOUVIER and SUSSKIND 1982; GRAÑA, YOUDERIAN and SUSSKIND 1985).] Thirteen crosses of this kind yielded such recombinants at frequencies $\geq 10^{-5}$. In these cases, DNA sequence analysis of the promoter region of one recombinant from each cross confirmed that it carried the expected down- and up-mutations.

Attempts to construct the down-up combinations *RE167-R8*, *RE167-RU4*, and *RU1099-RU4* by the cross procedure described above were unsuccessful; recombinants that grew on the selective plate were rare, and those that were studied further were found to be P_{ant}^+ rather than $P_{ant}\downarrow\uparrow$. To construct these down-up combinations, the cross procedure illustrated in Figure 4B was used. This method does not demand that the down-up recombinant produce enough antirepressor to have the Ant$^+$ phenotype; the only assumption made is that the recombinant does not produce lethal levels of antirepressor. *RE167-R8* phage were obtained by crossing *mnt-ts* $P_{ant}\uparrow$*R8 arc-am* phage with a *mnt-ts* $P_{ant}\downarrow$*RE167* plasmid (pMS145) and selecting recombinants that are able to grow at 37° on the Su$^-$ nonlysogenic host DB7000. Neither the parental phage nor P_{ant}^+ recombinants form plaques under these conditions because of the Arc$^-$ lethal phenotype. Two recombinant phage types, carrying *RE167* or *RE167-R8*, were expected to appear on the selective plate at comparable frequencies. All of the recombinants that grew on DB7000 at 37° were found to have the Ant$^-$ phenotype. To distinguish $P_{ant}\downarrow$ recombinants from $P_{ant}\downarrow\uparrow$ recombinants, candidates were tested by crossing them with P22 *mnt-ts* $P_{ant}\downarrow$*R204 arc-am* phage to determine whether *R204-R8* recombinants could arise (recombinants that are able to form plaques on DB7283 at 30°). DNA sequence analysis of one candidate that passed this test confirmed its *RE167-R8* genotype. The same strategy was used to construct *RE167-RU4* and *RU1099-RU4*, by crossing P22 *mnt-ts* $P_{ant}\uparrow$*RU4 arc-am* phage with plasmids carrying $P_{ant}\downarrow$*RE167* (pMS145) or $P_{ant}\downarrow$*RU1099* (pMS146).

Construction of $P_{ant}\uparrow\uparrow$ phage: The Ant$^-$ phenotype conferred by the $P_{ant}\downarrow$*RE167* mutation is not suppressed by either $P_{ant}\uparrow$*R8* or $P_{ant}\uparrow$*RU4*. As diagrammed in Figure 5A, the Ant$^-$ down-up mutants *RE167-R8* and *RE167-RU4* were crossed with each other and found to yield Ant$^+$ recombinants at the frequency expected for the *RE167-R8-RU4* recombinant. DNA sequence analysis confirmed the genotype of one such recombinant. Therefore, the two promoter-up mutations together suppress the Ant$^-$ phenotype of $P_{ant}\downarrow$*RE167*.

The cross illustrated in Figure 5B was used to construct a phage that has both promoter-up muta-

TABLE 2

Relative activities of mutant promoters

Promoter-down mutation	Promoter-up mutation: None	*R8* (−14G)	*RU4* (−8A)	*R8-RU4* (−14G, −8A)
None	(100)	104.0 ± 3.0	83.6 ± 1.3	38.0 ± 2.5
RU369 (−35G)	10.6 ± 0.3	37.1 ± 0.8	30.6 ± 2.1	
RU1099 (−35A)	6.5 ± 1.2	20.6 ± 0.4	17.9 ± 1.6	
RU454 (−34C)	11.2 ± 0.9	40.2 ± 0.5	32.5 ± 0.2	
RE167 (−33A)	2.4 ± 0.1	13.0 ± 0.6	9.2 ± 0.3	24.4 ± 0.7
RU630 (−33T)	20.1 ± 0.1	66.6 ± 2.8	56.4 ± 1.1	
RU612 (−32C)	22.3 ± 1.4	68.5 ± 2.4	55.8 ± 2.0	
R204 (−32G)	8.7 ± 0.4	34.7 ± 0.7	27.9 ± 2.3	
RU267 (−31T)	17.4 ± 1.2	61.2 ± 4.3	50.1 ± 0.4	
RU483 (−12C)	12.3 ± 0.7	72.9 ± 1.6	77.2 ± 1.7	
RU523 (−12A)	3.6 ± 0.5	36.9 ± 0.9	46.5 ± 1.6	

The relative activities of the indicated promoters were determined by assaying β-galactosidase produced by lysogens of P22 *ant/lacZ* phages as described in MATERIALS AND METHODS. The percent activity is given relative to that of the wild-type promoter, which is 100% and equal to 2082 Barrick units (~2500 Miller units).

TABLE 3

Effect of promoter-up mutations

Promoter-down mutation	Promoter-up mutation: *R8* (−14G)	*RU4* (−8A)	*R8-RU4* (−14G, −8A)
None	1.0	0.84	0.38
RU369 (−35G)	3.5	2.9	
RU1099 (−35A)	3.2	2.8	
RU454 (−34C)	3.6	2.9	
RE167 (−33A)	5.4	3.8	10.2
RU630 (−33T)	3.3	2.8	
RU612 (−32C)	3.1	2.5	
R204 (−32G)	4.0	3.2	
RU267 (−31T)	3.5	2.9	
RU483 (−12C)	5.9	6.3	
RU523 (−12A)	10.3	12.9	

The effects of the up-mutations in various contexts were calculated from the data in Table 2 by dividing the activity of the promoter with the up-mutation(s) by the activity of the otherwise isogenic promoter without the up-mutation(s).

tions and does not have the P_{ant}↓*RE167* mutation. A plasmid carrying the *RE167-R8-RU4* promoter was crossed with P22 *mnt-ts* P_{ant}↓*RU1283 arc-am* T_{ant}-*R1009*. This phage has the Ant⁻ phenotype because it carries both *RU1283*, a mild promoter-down mutation in the −10 region, and *R1009*, a suppressor of the Arc⁻ lethal phenotype that changes the *ant* transcription terminator, thereby slightly decreasing the rate of synthesis of antirepressor (M. M. SUSSKIND, unpublished data). Ant⁺ progeny phage were selected by plating on the *supE*(P22 *immI*$_\Delta$) lysogen MS1367 at 30°. As expected, *R8-RU4* was the second most frequent type of Ant⁺ recombinant produced in this cross, and was easily distinguished from *RE167-R8-RU4*, the most frequent type, because the *R8-RU4* recombinant forms clearer plaques on MS1367. The T_{ant}-*R1009* mutation, which must be retained by all progeny phage, was included in this cross because we thought that without some sort of down-mutation, the *R8-RU4* "double-up" promoter might produce lethal levels of antirepressor, even in the presence of *supE* to suppress the *arc-am* mutation. We subsequently learned that the *R1009* mutation was unnecessary (see below).

Activity of P_{ant} mutant promoters *in vivo*: To assay the activities of these mutant promoters, we constructed a set of P22 phages in which various P_{ant} genotypes control expression of an *ant/lacZ* gene fusion, and measured the levels of β-galactosidase produced by lysogens of these phages in Su⁻ strain DB7000 (see MATERIALS AND METHODS). These lysogens do not produce Mnt repressor, Arc repressor, or *sar* RNA because the prophages carry a substitution that deletes *mnt*, an amber mutation in *arc*, and a substitution that inactivates the *sar* promoter. The results of these assays are shown in Table 2. The same data were used to prepare Table 3, which lists the effects of the up-mutations on promoter activity. Each P_{ant}↓↑ promoter is more active than the corresponding P_{ant}↓ promoter, but less active than P_{ant}^{+}. However, the down-up combinations show that the magnitude of the effect of a promoter mutation can vary considerably depending on what other mutation is present. Even more striking is the observation that the up-mutations do not increase promoter activity in the absence of a down-mutation. These context effects are discussed in detail below (see DISCUSSION). For the sake of simplicity, we will continue to call *R8* and *RU4* up-mutations, even though in certain situations they do not increase promoter activity.

These measurements of promoter strength using single-copy P_{ant}-*ant/lacZ* fusions (Table 2) are not in complete agreement with previous studies in which some of the same promoter genotypes were compared by assaying the rate of synthesis of antirepressor during phage infection (GRAÑA, YOUDERIAN and SUSSKIND 1985). The hierarchy of promoter strengths based on antirepressor assays is

$$R8 \simeq RU4 > \text{wild type} \simeq RU267\text{-}R8 > R204\text{-}R8 > RU523\text{-}RU4 > RU523\text{-}R8,$$

whereas in Table 2 the hierarchy of the same pro-

moters is

R8 ≃ wild type > *RU4* > *RU267-R8* > *RU523-RU4* > *RU523-R8* > *R204-R8*.

Even when the two assays agree that one promoter is stronger than another, the magnitude of the difference is frequently greater by the Ant assay than by the Lac assay. The reasons for these discrepancies are not known. One likely contributing factor is that the *ant* phages have the *sar* (antisense RNA) promoter, which is sensitive to the activity of the P_{ant} promoter; transcription from P_{ant} interferes with synthesis of full-length *sar* RNA (LIAO *et al.* 1987). Another obvious difference is that phage-infected cells and exponentially growing lysogenic cells are in quite different physiological states. The activities of some promoters are known to vary *in vivo* depending on cellular growth conditions (NOMURA, GOURSE and BAUGHMAN 1984; DEUSCHLE *et al.* 1986), and the hierarchy of various strong promoters varies *in vitro* depending on RNA polymerase concentration (KAMMERER *et al.* 1986). Hence, it is conceivable that the hierarchy of P_{ant} promoters in P22-infected cells differs from that in lysogens. We determined the nucleotide sequence of the P_{ant} region of each *lac* phage to confirm that no mix-ups had occurred in their construction. In addition, the entire region between *kan* and *lac* of the P_{ant}^{+}, P_{ant}↑*R8*, P_{ant}↑*RU4*, and P_{ant}↑*R8*↑*RU4* *lac* phages was sequenced to confirm that only the expected mutations are present. These four *lac* phages were also back-crossed to replace *lac* sequences with *ant* and gene *9*. The resulting *Kn6 arc-am* recombinants carrying P_{ant}^{+}, P_{ant}↑*R8*, or P_{ant}↑*RU4* have the Arc^{-} conditional-lethal phenotype, whereas the resulting *Kn6* P_{ant}↑*R8*↑*RU4* *arc-am* recombinant forms small plaques on Su^{-} strain DB7000 at 30°. This finding that the Arc^{-} lethal phenotype is suppressed by the *R8-RU4* genotype suggests that the double-up promoter is less active than the wild-type promoter with respect to antirepressor synthesis as well as β-galactosidase synthesis.

DISCUSSION

Context effects in the P_{ant} down-up promoters: Assays of the activities of 20 promoters with pairwise combinations of down- and up-mutations show that P_{ant}↑*R8 and* P_{ant}↑*RU4* partially suppress each of ten severe down-mutations. Hence, these promoter-up mutations appear to be global suppressors of promoter-down mutations. However, it is also clear that the efficiency of suppression by each up-mutation depends on which down-mutation is present. For seven down-mutations in the −35 hexamer, P_{ant}↑*RU4* increases promoter activity 2.5–3.2-fold, and P_{ant}↑*R8* increases promoter activity 3.1–4-fold. The "up-effect" of each up-mutation is slightly larger for P_{ant}↓*RE167*, an eighth mutation in the −35 hexamer, and is larger still (6–13-fold) for two down-mutations at position −12. P_{ant}↑*R8* is slightly more effective than P_{ant}↑*RU4* in suppressing mutations in the −35 hexamer, whereas *RU4* is slightly more effective than *R8* in suppressing mutations at position −12. Thus, the magnitude of the effect of each up-mutation (or of each down-mutation) depends on the context in which it is placed.

The cause of these context effects is not known, but two trends are apparent in these results. First, two of the down-mutations that are suppressed unusually well, *RE167* and *RU523*, are also the two severest down-mutations in this set. The significance of this observation is unclear, but it is interesting that the up-effect of the up-mutations seems to be maximal in the context of very weak promoters. Conversely, as discussed below, the up-effect of the up-mutations is minimal or even negative in the context of very strong promoters. Second, the effect of context is greatest when the down-mutation is at position −12, within a few base pairs of the up-mutation. This finding seems intuitively reasonable, since one might expect that a perturbation in the protein-DNA contacts at one position would be most likely to influence other contacts nearby. A related idea is that suppression may be most effective if the down- and up-mutations affect the same step in the multistep process of promoter recognition and transcription initiation. Biochemical analysis of the effects of these mutations on polymerase-promoter interactions is needed to address this idea.

It is doubtful whether these context effects are related to the fact that two alternative −10 hexamers overlap the "true" −10 hexamer of P_{ant} (see Figure 1). That the middle hexamer is the active one in wild-type P_{ant} is demonstrated by the analysis of down-mutations: of 13 substitutions in this region, none is in the base pairs unique to the alternative hexamers, and two of these *down*-mutations *improve* one or the other alternative hexamer (YOUDERIAN, BOUVIER and SUSSKIND 1982). The P_{ant}↑*R8* and P_{ant}↑*RU4* mutations not only strengthen the homology with consensus of the "true" −10 sequence, but also weaken the homology of the alternative hexamers to the left and right, respectively. The fact that the up-effect of these mutations is greatest in the presence of down-mutations at position −12 suggests that in this context, part of the effect of the up-mutations may be to reduce interference by the alternative hexamers. However, this idea predicts that P_{ant}↓*RU523* (−12A), which improves the alternative hexamer to the left, should be suppressed better by P_{ant}↑*R8* than by P_{ant}↑*RU4*, since *R8* weakens the same hexamer that *RU523* improves.

Since this is not the case, the relevance of the alternative −10 hexamers is uncertain.

It is also unclear whether the context effects in P_{ant} are related to the fact that the P_{ant} region also contains P_{mnt}, a divergent, overlapping promoter for leftward transcription of the *mnt* repressor gene. Thus, the −35 and −10 hexamers of P_{mnt} are at positions −2 to −7 and −25 to −30 of P_{ant}. Transcription experiments *in vitro* show that the two promoters compete for RNA polymerase (VERSHON *et al.* 1987; D. HAWLEY and W. R. MCCLURE, unpublished results). On the wild-type template, P_{ant} is by far the dominant promoter; in fact, P_{mnt} activity is virtually undetectable unless P_{ant} is damaged by mutation or repressed by Mnt protein. Although it is clear that competition from P_{ant} limits P_{mnt} activity, it is not known whether P_{mnt} ever limits P_{ant} activity. None of the mutations used in this study affects any of the base pairs predicted to be important for the "intrinsic" activity of P_{mnt}. Hence, it is not obvious how P_{mnt} might account for the context effects shown here.

A surprising context effect: up-mutations can have a down-effect: Perhaps the most remarkable result of this study is that the up-mutations do not increase P_{ant} activity in the absence of a down-mutation. When the promoter sequence is otherwise wild-type, P_{ant}↑*R8* has essentially no effect, and P_{ant}↑*RU4* has a slight down-effect on promoter strength. Furthermore, when the two up-mutations are combined in an otherwise wild-type promoter, activity decreases more than twofold. Thus, each up-mutation has a substantial down-effect in the presence of the other (compare P_{ant}↑*R8*↑*RU4* with P_{ant}↑*RU4*, and compare P_{ant}↑*R8*↑*RU4* with P_{ant}↑*R8*). In contrast, in the presence of P_{ant}↓*RE167*, the single up-mutations increase promoter strength fivefold (*R8*) or fourfold (*RU4*), while both up-mutations together increase activity tenfold (Table 3). Thus, as the sequence of P_{ant} approaches conformity with consensus, the up-mutations decrease promoter strength, even though the same mutations increase promoter strength in the presence of a down-mutation. Therefore, the direction as well as magnitude of the effects of these mutations depend on context.

Previous studies provide precedence for the idea that changes toward consensus can impair the activity of a strong promoter. INOUYE and INOUYE (1985) and MANDECKI *et al.* (1985) altered the *E. coli lpp* and *lac* promoters, respectively, by the sequential addition of changes toward consensus. In both cases, the final change to complete conformity with consensus in both hexamers caused a slight decrease in promoter activity *in vivo*. Similarly, BUJARD *et al.* (1987) showed that the λ P_L promoter becomes less active when two base pairs are simultaneously changed toward consensus, which makes the resulting "P_L-*con*" promoter identical with consensus in both hexamers.

Since there must be an upper limit to promoter strength, it is reasonable that the sequential addition of up-mutations would eventually stop increasing promoter activity. Why adding up-mutations would decrease promoter strength is a more difficult question. Since initiation of transcription is a multistep process, it is possible that a surfeit of consensus base pairs makes one of the intermediates too stable to proceed to the next. For example, contacts between RNA polymerase and the promoter in the initial closed complex might be too strong to permit rapid isomerization to an open complex. Alternatively, strong contacts might inhibit promoter clearance by the initiating complex. In the case of the λ P_L-*con* promoter, the latter hypothesis is supported by evidence that P_L-*con* forms open complexes faster than P_L^+ *in vitro*, yet is less active than P_L^+ *in vivo* (BUJARD *et al.* 1987). In addition, Bujard and coworkers have shown that P_{con}, a synthetic consensus promoter, becomes more productive, both *in vivo* and *in vitro*, if the sequences between −4 and +20 are changed (KAMMERER *et al.* 1986), even though these changes do not alter the rate of open complex formation (BUJARD *et al.* 1987). These observations suggest that P_L-*con* and P_{con} are defective in the promoter clearance step of transcription initiation. The same is probably true of the consensus version of the *lac* promoter. Even P_{lac}*UV5*, which differs from consensus at one position in the −35 hexamer and in the length of the spacer region, shows a promoter clearance defect *in vitro*: initiating complexes tend to "stall" and release short RNA products while maintaining the contacts characteristic of the open complex (CARPOUSIS and GRALLA 1985; STRANEY and CROTHERS 1987).

SZOKE, ALLEN and DEHASETH (1987) found that the λ P_{RM} promoter becomes increasingly more active, both *in vivo* and *in vitro*, as four nonconsensus base pairs are changed toward consensus, ultimately making P_{RM} identical with consensus in both hexamers and at position −14. In general, P_{RM} promoter strength is inversely proportional to the number of nonconsensus base pairs in both hexamers. Why this is the case for P_{RM} but not for P_{ant}, P_{lac}, P_{lpp}, and P_L is unclear. We note, however, that the results of SZOKE, ALLEN and DEHASETH (1987) do show some evidence of context effects. For example, changing the −10 hexamer of P_{RM} from 5′-TAGA*T*T-3′ to 5′-TAGA*A*T-3′ increases promoter strength *in vivo* 12-fold if the −35 hexamer is 5′-TAGATA-3′, but has no effect if the −35 hexamer is 5′-TTGACA-3′.

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