group making essentially no contribution to the total. On the basis of our current understanding, it appears highly likely that the ODPs for the HFCs considered here are all well below 1×10^{-3}. For the key substitute HFC-134a, the best estimate of the ODP is only 1×10^{-5} to 2×10^{-5}.

Note added in proof: Since the submission of this manuscript, several studies on reaction 1 (*14–16*), reaction 2 (*14, 15*), reaction 3 (*17*), and reaction 13 (*17*) have been published. All of these measurements are in agreement with the results reported here.

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Target of the Transcriptional Activation Function of Phage λ cI Protein

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Activation of transcription initiation by the cI protein of phage λ is thought to be mediated by a direct interaction between cI and RNA polymerase at the P_{RM} promoter. Two negatively charged amino acid residues in the DNA binding domain of cI play a key role in activation, suggesting that these residues contact RNA polymerase. The subunit of RNA polymerase involved was identified by selecting polymerase mutants that restored the activation function of a mutant form of cI protein. Although previous studies suggest that several activators interact with the α subunit of RNA polymerase, the results here suggest that cI interacts with the σ subunit. An arginine to histidine change near the carboxyl terminus of σ specifically suppresses an aspartic acid to asparagine change in the activation region of cI. This finding supports the direct-contact model and suggests that a cluster of positively charged residues near the carboxyl terminus of σ is the target of the negatively charged activation region of cI.

Gene expression is frequently regulated by activator proteins that stimulate the rate of transcription initiation at specific promoters. In many cases, activation is thought to involve direct contact between the activator and RNA polymerase on the promoter DNA. Strong evidence for this model has been presented for the *cI* gene product of phage λ, which acts as both a repressor and activator of transcription (*1*). In λ lysogens, cI protein binds to sites in the O_R and O_L operators, thereby turning off two major promoters, P_R and P_L. At the same time, the cI dimer bound to O_R2 turns on transcription of the *cI* gene from the P_{RM} promoter (Fig. 1). The cI dimer bound to O_R2 is thought to interact directly with RNA polymerase bound to P_{RM}, thereby stimulating isomerization of closed polymerase-promoter complexes to open complexes (*2–6*). Part of the evidence for this model was the isolation of a special class of *cI* mutants, called *pc* for positive control, that bind to O_R and repress P_R normally, but cannot activate P_{RM} (*3–6*). The *pc* mutations change amino acids in the first α helix or turn of the helix-turn-helix DNA binding motif of cI. These residues are on the surface of the cI DNA binding domain when it is bound to operator DNA (*7*) and are appropriately positioned to contact RNA polymerase (*4*).

The region of RNA polymerase that contacts cI protein was unknown. The major form of *Escherichia coli* RNA polymerase is composed of a core enzyme ($\alpha_2\beta\beta'$) plus the dissociable σ^{70} subunit, which confers promoter specificity. The σ^{70} subunit is predicted to be close to cI, because σ^{70} recognizes the −35 region of the promoter (*8, 9*), which overlaps O_R2. The α subunit is another likely candidate to interact with cI, because α apparently interacts with several other activators (*10*). To identify which subunit is the target for activation by λ cI protein, we generated a mutant form of RNA polymerase that allows a cI-pc mutant to activate P_{RM}. To obtain this polymerase mutant, we constructed a strain carrying two P22 prophages integrated in the bacterial chromosome (Fig. 2). The first prophage (*11*) carries the *kan* (kanamycin resistance) gene under control of wild-type λ P_{RM}. The second

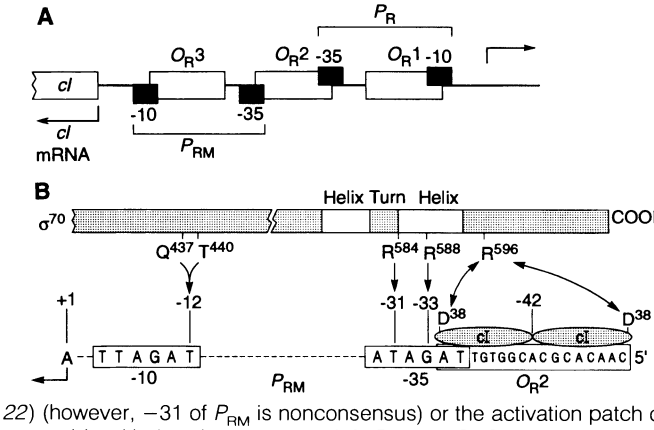

Fig. 1. (**A**) Organization of cI binding sites (open boxes) and promoter elements (filled boxes) in λ O_R region. In λ lysogens cI binds O_R1 and O_R2 (but not O_R3), represses P_R, and activates P_{RM}. (**B**) Proposed interactions between σ^{70} and the promoter or activator. The subunit σ^{70} is aligned with the leftward P_{RM} promoter with a cI dimer bound to O_R2. Arrows represent interactions between σ^{70} residues and consensus base pairs (*8, 9, 22*) (however, −31 of P_{RM} is nonconsensus) or the activation patch of cI. The latter interaction is presumably with the cI monomer at left. D, Asp; Q, Gln; R, Arg; T, Thr.

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prophage (*12*) expresses the λ *cI* gene at a low level from a weak version of the *E. coli lac* (lactose operon) promoter. These fusions are located in a dispensible region of the phage genome (Fig. 3); λ cI protein does not control maintenance of lysogeny by these prophages. Because the *cI* gene carries the *pc2* mutation (Asp^{38} to Asn) (*4*, *5*), activation of the P_{RM}-*kan* fusion is extremely weak and the cells are sensitive to kanamycin. Plasmids containing the *E. coli rpoA* or *rpoD* gene (encoding the α or σ^{70} subunit, respectively) were mutagenized by polymerase chain reaction (PCR) and introduced into the selection strain (*13*). Cells able to grow on plates containing kanamycin were selected. Mutant plasmids that increase kanamycin resistance were obtained with the use of the σ-encoding plasmid, but not the α-encoding plasmid.

Eight independent mutant plasmids were analyzed. Restriction fragment exchange localized the mutations to a 150–base pair (bp) segment of *rpoD* downstream of a Cla I site (Fig. 2). Sequencing of this region revealed that all eight isolates have the same G to A transition, changing codon 596 from CGC (Arg) to CAC (His). We call this mutation *rpoD-RH596* and the mutant protein σ^{70}-RH596.

The effect of the mutant σ was measured with the use of strains that express the *lacZ* (β-galactosidase) gene from λ P_{RM} (Table 1 and Fig. 2) (*14*). In the absence of λ cI protein, σ^{70}-RH596 has no effect on the basal level of transcription from P_{RM}. The mutant σ fully restores the ability of cI-pc2 protein to activate P_{RM}; the mutant polymerase responds to the mutant activator slightly better than wild-type polymerase responds to the wild-type activator. In contrast, the mutant σ does not respond to the cI-pc1 (*3*) or cI-pc3 (*4*) mutant activators and is somewhat defective in responding to wild-type cI. Thus, the Arg^{596} to His change in σ^{70} specifically suppresses the activation defect caused by the Asp^{38} to Asn change in λ cI. This result strongly suggests that cI protein activates P_{RM} by interacting with the COOH-terminus of the σ^{70} subunit of RNA polymerase.

The Arg^{596} residue is just downstream of a putative helix-turn-helix motif of σ^{70} that recognizes base pairs in the −35 region of promoters (*8*, *9*) (Fig. 1B). The operator O_R2, centered at −42 with respect to P_{RM}, overlaps the −35 region. Consequently, when RNA polymerase binds to the P_{RM} promoter, Arg^{596} of σ^{70} is expected to be close to the cI dimer bound to O_R2, which mediates positive control. Thus, although the suppressor mutation was not directed to this (or any) particular region of *rpoD*, the location of the mutant residue agrees with the independently derived alignment of σ^{70} with respect to the promoter. Because Ptashne and co-workers predicted that a basic patch on RNA polymerase contacts the acidic activation patch of cI (*4*, *6*), it is also fitting that Arg^{596} of σ^{70} is basic and lies in a region rich in basic residues.

The *rpoD-RH596* allele was previously isolated as a mutation affecting regulation of the *E. coli araBAD* (arabinose) operon (*15*, *16*). Maximal expression from the *araB* promoter normally requires two activators, CAP (catabolite activator protein) and AraC. Because CAP is active only when bound to adenosine 3′,5′-monophosphate (cAMP), strains defective in adenylate cyclase (*cya* gene product) are deficient in *ara* gene expression. The *rpoD-RH596* mutation was obtained as a suppressor that reverses the Ara^- phenotype of cya^- mutants. This effect requires an intact *araC* gene, suggesting that the mutant σ enhances the ability of AraC protein to activate the *araB* promoter without assistance from CAP-cAMP (*16*).

Here we report a polymerase mutant that suppresses the activation defect of a mutant activator. Previously, most genetic studies of polymerase-activator interactions involved the isolation of polymerase mutants that fail to respond to one or more wild-type activators. In most cases, such mutants have amino acid substitutions or deletions in the COOH-terminal half of the α subunit (*10*). With the exception of the *ara* studies, the only published evidence implicating σ^{70} in positive control is the

Table 1. Allele-specific suppression of λ *cI-pc2* by σ^{70}-RH596. Strains are derivatives of MS1868 (*20*) carrying a P_{RM}-*lac8* prophage (*14*) plus either no second prophage or one of four P_{lac}-*cIA* prophages (*12*) and an *rpoD* plasmid. The activity of β-galactosidase was measured (*21*) and normalized to that of the strain carrying the wild-type plasmid and wild-type cI (set at 100; corresponds to 255 Miller units). The mean and standard deviation of three to six measurements are given.

Activator protein	Relative activity of P_{RM}-*lacZ* fusion	
	Wild-type *rpoD* plasmid*	Mutant *rpoD* plasmid†
None	16.4 ± 2.6	16.5 ± 1.9
cI (wild-type)	(100)	53.4 ± 3.4
cI-pc1 (Gly^{43} → Arg)	6.3 ± 0.2	6.9 ± 0.4
cI-pc2 (Asp^{38} → Asn)	18.5 ± 0.2	120 ± 2.9
cI-pc3 (Glu^{34} → Lys)	0.9 ± 0.5	1.0 ± 0.3

*pMS1297 (*13*). †pMS1366 [identical to pMS1297, except the small Cla I fragment (Fig. 2) is from one of the original *rpoD-RH596* plasmids].

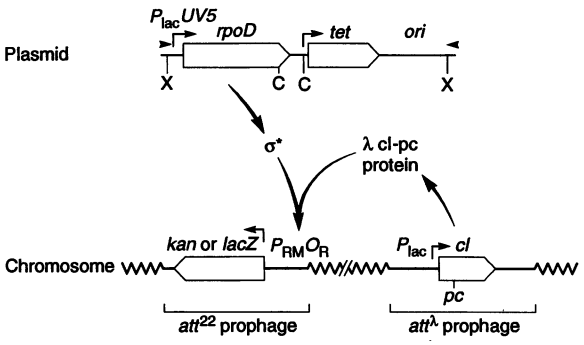

Fig. 2. Selection and analysis of σ^{70} mutants. A P22 prophage integrated at the λ attachment site produces low levels of λ cI-pc2 protein from a weak version of the *lac* promoter. Mutant σ^{70} (σ*), produced by a derivative of plasmid pMS1297 (drawn to scale) (*13*), allows the mutant activator to activate the λ P_{RM} promoter, which controls the *kan* or *lacZ* gene on a second prophage integrated at the P22 attachment site. The *kan* fusion is used to select mutants by demanding increased kanamycin resistance. The *lacZ* fusion is used to analyze mutants by measuring β-galactosidase amounts. No Lac repressor is present. Bent arrows represent promoters; arrowheads represent primers used for PCR mutagenesis (*13*). C, Cla I; X, Xba I.

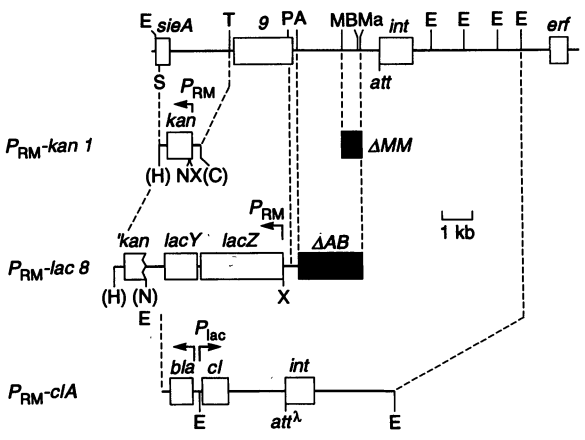

Fig. 3. Map of P22 substitutions. All Eco RI sites are shown; otherwise, only relevant sites are shown: A, Acc I; B, Bbs I; C, Cla I; E, Eco RI; H, Bam HI; M, Mlu I; Ma, Mam I; N, Nru I; P, Hpa I; S, Sau 3AI; T, Taq I; and X, Xho I. Sites in parentheses were destroyed in the final constructs.

recent demonstration that two *rpoD* mutations (Glu^{575} to Lys and Asp^{570} to Gly) prevent activation of several *E. coli* operons by wild-type PhoB (*17*). However, those *rpoD* mutations can also affect promoter recognition directly, because they enhance recognition of the wild-type *lac* promoter in the absence of CAP-cAMP (*18*). The Glu^{575} to Lys mutant also raises the activities of several mutants in the *lac* promoter (±CAP-cAMP) and P22 *ant* promoter (which has no activator) (*8*). In contrast, the Arg^{596} to His mutant has no effect on the activities of 20 P_{lac} alleles (±CAP-cAMP) and 18 P_{ant} alleles (*8*). Thus, all known properties of σ^{70}-RH596 indicate that it does not affect promoter recognition directly but affects positive control by λ cI and AraC. It is likely that further evidence for the role of σ^{70} in positive control will be forthcoming (*19*).

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11. The phage P22-3957 carries P_{RM}-*kan1*, a substitution replacing DNA between a Taq I site near gene *9* and a Sau 3AI site in *sieA* with a fusion of λ P_{RM} to the *kan* gene from pUC71K. From 5′ to 3′ (right to left in Fig. 3), one strand of P_{RM}-*kan1* contains a 26-nucleotide (nt) Cla I–Eco RI segment of pBR322 (nts 23 to 4359, with the Cla I site joined to the compatible Taq I site of P22); wild-type λ P_{RM} and O_R (nts 38,150 to 38,041); and *kan* DNA from the start codon to a downstream Bam HI site, with the Bam HI site joined to the compatible Sau 3AI site of P22. P22-3957 also carries Δ*MM*, an Mlu I–Mam I deletion.
12. The substitution P_{lac}-*clA* replaces DNA between a point in *sieA* and an Eco RI site near *erf* with a fusion of the *E. coli lacZ* promoter and ribosome binding site to λ *cI*. The promoter lacks part of the CAP-cAMP binding site and part of the operator. From 5′ to 3′ (left to right in Fig. 3), one strand of P_{lac}-*clA* contains *bla* of pBR322 (nts 3146 to 2); P_{lac} (nts −59 to −7 fused to nts +15 to +36); λ *cI* (nts 37,943 to 36,967); nts 27 to 369 and 652 to 2066 of pBR322; CTAGAG; and Hind III–Eco RI *att-int* segment of λ (nts 27,480 to 31,752), with the Eco RI site in λ *exo* joined to the Eco RI site near P22 *erf*. Phages P22-3997 through −4000 carry pc^+, *pc1*, *pc2*, and *pc3* versions of λ *cI*, respectively. They also carry four *cI* mutations that do not change the amino acid sequence but create restriction sites.
13. The *rpoD* plasmid pMS1297 (Tet^R, 5.0 kb) (Fig. 2) contains CTCTAGAG; Mun I(filled in)–Stu I fragment of P22 *sieA*; CTCTAGAGAATT; P_{lac}*UV5*, −59 to +36; CCCATGGG; Hpa I–Apa LI(filled in) fragment containing wild-type *E. coli rpoD*, followed by a G residue; and nts 4359 to 1429, 2297 to 3232, and 3428 to 3540 of pBR322. The *rpoA* plasmid pMS1250 (Tet^R, 4.3 kb) is identical, except it has wild-type *E. coli rpoA* (Hpa I–Apa I, made blunt-ended by T4 DNA polymerase, followed by a C residue) instead of *rpoD*. In both plasmids, the 208-bp *sieA* segment is dispensible, contains a unique Ssp I site, and is flanked by Xba I sites. For PCR mutagenesis, we linearized plasmid DNA with Ssp I and amplified by 30 cycles of standard PCR using Taq DNA polymerase and *sieA*-derived primers (Fig. 2). Products were cleaved with Xba I, producing sticky-ended plasmid DNA, which was circularized with T4 DNA ligase, precipitated with ethanol, dissolved in water, and electroporated into *Salmonella typhimurium* JR501 [S. P. Tsai *et al.*, *J. Gen. Microbiol.* **135**, 2561 (1989)]. Cells carrying pMS1297 were always propagated at ≤30°C. Electroporated cells (~40 μl) were diluted with 0.9 ml of SOC medium (Bio-Rad), incubated for 2 hours (at which time cultures contained ≥10^4 Tet^R transformants), diluted with 5 ml of LB broth + tetracycline (50 μg/ml), and incubated overnight. Plasmid DNA was extracted and electroporated into MS4020, which is JR501 carrying P22-3957 (P_{RM}-*kan1*) (*11*) and P22-3999 (P_{lac}-*clA-pc2*) (*12*). Outgrowth was repeated as above. Overnight cultures were plated on LB broth + tetracycline (20 μg/ml) + kanamycin (6 μg/ml) plates. About 0.05% of Tet^R transformants grew on this concentration of kanamycin, but most did not have a mutant plasmid. For each of eight PCR reactions, ~500 Kan^R colonies were pooled. Plasmid DNA extracted from each pool was electroporated into MS4020, and outgrowth and plating were repeated. If mutant plasmids were present, the Kan^R frequency was now at least 100-fold that in the previous step. One plasmid from each pool was analyzed.
14. The phage P22-4229 carries P_{RM}-*lac8*, a substitution replacing DNA between a Hpa I site in gene *9* and a Sau 3AI site in *sieA* with a fusion of λ P_{RM} to *E. coli lacZ*. We derived P_{RM}-*lac8* from P_{RM}-*kan1* by replacing an Xho I–Nru I *kan* fragment with *lac* sequences. From 5′ to 3′ (right to left in Fig. 3), one strand of P_{RM}-*lac8* contains GTT of the Hpa I site joined to GACTCTAGAG; wild-type λ P_{RM} and O_R (nts 38,107 to 37,941); nts 1 to 35 of *kan* coding sequence, followed by TTAA, which creates a stop codon for *kan*; wild-type *lacZ* and *lacY* (nts 1268 to 6274 of *lac*), except the Eco RI site in *lacZ* is mutated and *lacZ* codons 7 to 9 are replaced with CCC; AATT; and Nru I–Bam HI fragment carrying the 3′ end of *kan*, with the Bam HI site joined to the compatible Sau 3AI site in *sieA*. P22-4229 also carries Δ*AB*, an Acc I–Bbs I deletion.
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Catalytic Activity of an RNA Domain Derived from the U6-U4 RNA Complex

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U6 RNA contains two regions that are essential for proper splicing of nuclear precursor messenger RNA (pre-mRNA). A comparison of putative secondary structures of the U6-U4 RNA complexes from different phyla revealed a conserved domain that is similar to the catalytic hammerhead RNA motif. Although no catalytic activity was detected in the mammalian U6-U4 RNA complexes, two nucleotide changes in U6 RNA and one in U4 RNA conferred cleavage activity to the complex. Furthermore, the highly conserved domain of the wild-type complex, without the accompanying flanking regions, cleaved an RNA substrate and exhibited other characteristics of the hammerhead ribozyme. The possible involvement of this structure in pre-mRNA splicing is also discussed.

The splicing of nuclear pre-mRNA is a complex process involving both proteins and small nuclear RNAs (snRNAs) in a multimolecular structure called the spliceosome. The requirement for RNAs with highly conserved sequences, higher order structures, or both, has led to speculation that pre-mRNA splicing may have originated from, or may still rely on, catalytic properties of RNA. The lariat intermediates in pre-mRNA splicing and in the self-splicing group II introns have fostered these notions because if these intermediates denote a common evolutionary origin, then pre-mRNA splicing should be based on RNA chemistry despite the requirement for protein factors (*1*, *2*).

To clarify this possible relation, atten-

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