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Promoter Interference in a Bacteriophage Lambda Control Region: Effects of a Range of Interpromoter Distances

MICHAEL G. STRAINIC, JR.,[1] JENNIFER J. SULLIVAN,[1] JULIO COLLADO-VIDES,[2] AND PIETER L. DEHASETH[1]*

Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106-4935,[1] and Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca A.P. 565-A, Morelos 62100, Mexico[2]

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The p_R and p_{RM} promoters of bacteriophage lambda direct transcription in divergent directions from start sites separated by 83 phosphodiester bonds. We had previously shown that the presence of an RNA polymerase at p_R interfered with open complex formation at p_{RM} and that this effect was alleviated by the deletion of 10 bp between the two promoters. Here we present a detailed characterization of the dependence of the interference on the interpromoter distance. It was found that the reduced interference between the two promoters is unique to the 10-bp deletion. The relief of interference was demonstrated to be due to the facilitation of a step subsequent to RNA polymerase binding to the p_{RM} promoter. A model to explain these observations is proposed. A search of known *Escherichia coli* promoters identified three pairs of divergent promoters with similar separations to those investigated here.

In the rightward control region of bacteriophage lambda, transcription is initiated in divergent directions from two promoters, p_R and p_{RM}, that have start sites separated by 83 phosphodiester bonds (pdb; we are using this designation to avoid ambiguity in the representation of the distance between start sites). These two promoters are among those responsible for implementing the decision as to whether viral development will proceed along the lytic or lysogenic pathways (27). The p_R promoter has greater similarity to the promoter consensus sequence than the p_{RM} promoter (27). As a consequence, open complex formation at p_R is accomplished in seconds but under the same conditions requires tens of minutes at p_{RM} (15, 27, 34). Therefore, for the wild-type control region, in vitro RNA polymerase (RNAP)-p_{RM} interactions occur almost exclusively in the context of another RNAP already bound to p_R. It has been previously shown that this p_R-bound RNAP interferes with open complex formation at p_{RM} (16, 17, 21, 34, 37). The effect is not exerted at the initial binding of RNAP to the promoter but rather at a subsequent step (16, 34) that is likely a conformational change in the RNAP (9). Eventually, open complexes do form at p_{RM} and coexist with those at p_R (16, 25). The converse of the situation described above has also been shown: when p_R has been weakened due to base substitutions, its ability to form open complexes is affected by the presence of p_{RM} on the same DNA fragment (11).

Only 13 pdb separate the start site-distal edges of the −35 regions of the p_R and p_{RM} promoters. Given such a short interpromoter distance, it was suggested that the p_R-bound RNAP was slowing open complex formation at p_{RM} because of steric hindrance. Consistent with this notion, deletion of 1 bp between the −35 regions was found to further reduce the rate of open complex formation at p_{RM} (40). However, it has also been shown that when the distance between the −35 regions of the promoters is shortened by the deletion of 10 bp (one turn of the DNA helix), unexpectedly the inhibition of open complex formation at p_{RM} is greatly diminished (21). In other phages where the interpromoter distance at p_R and p_{RM} is even shorter, such as 434 (66 pdb between start sites) and P22 (52 pdb), concurrent occupancy of the promoters is not observed (8, 41).

To further explore this phenomenon, a series of deletions between the −35 regions of p_R and p_{RM} was generated to examine the length dependence of inhibition at the p_{RM} promoter by the presence of RNAP at the p_R promoter. DNA constructs lacking 3, 5, 6, 7, 8, 9, 10, 11, and 12 bp between the −35 regions of the two promoters were made (Fig. 1). The distance between the −35 regions of the p_{RM} and p_R promoters was deleted, starting from the edge of the −35 region proximal to p_{RM}. The constructs are designated as D*n*, where *n* is the number of base pairs that have been deleted. The promoters were constructed from synthetic oligodeoxyribonucleotides and cloned into the pKK232-8 vector by using *Bam*HI and *Hin*dIII restriction sites as described previously (21) and sequenced. The location of the strand-separated region at both promoters was checked by $KMnO_4$ footprinting and found not to be affected by the deletions (data not shown).

Determination of open complex formation by the electrophoretic mobility shift assay. Open complex formation at the p_{RM} promoter was monitored with an electrophoretic mobility shift assay carried out as described by Mita et al. (21). Approximately 1 to 2 nM ^{32}P-labeled promoter DNA was incubated at 37°C with RNAP (activity, 50% ± 10% [mean ± standard deviation]), at a concentration of active enzyme of 100 nM, in 20 μl of HEPES buffer (30 mM HEPES [pH 7.6], 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol) containing 50 μg of bovine serum albumin per ml. After the addition of 1 μl of a 1-mg/ml solution of heparin to inactivate free RNAP as well as closed complexes and incubation for an additional minute at 37°C, 2 μl of a loading solution (30% glycerol, 0.25% bromophenol blue, 0.25% xylenecyanolphenolfluorine) was added to each reaction mixture prior to loading onto a 4% polyacrylamide gel (29:1 acrylamide-bisacrylamide). The gels were run in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) at 6 V/cm for 1.5 h and then exposed to X-ray film to detect the radioactive bands. Two complex bands were observed. On the basis

* Corresponding author. Mailing address: Department of Biochemistry, School of Medicine, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106-4935. Phone: (216) 368-3684. Fax: (216) 368-4544. E-mail: pld2@po.cwru.edu.

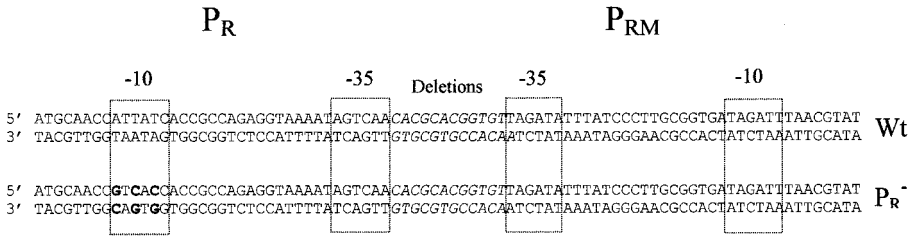

FIG. 1. Constructs used in this study. The sequences shown were cloned into the pKK232-8 plasmid vector for *E. coli* at *Bam*HI and *Hin*dIII restriction sites. The p_R promoter was inactivated by introduction of three base pair substitutions in its −10 region, which are shown in boldface type. The −10 and −35 regions are boxed. The position of the region shortened in the deletion mutants is indicated in italic letters. The deletions start from the upstream edge of the −35 of the p_{RM} promoter and progress towards the p_R promoter. Constructs are designated as D*n*, with *n* indicating the number of base pairs deleted. For example, the sequence between the −35 regions is CACGCACGG (top strand in the figure) for the D3 mutant.

of actual footprinting of the complexes (21), we were able to determine that the faster-moving band represented DNA with an open complex at p_R only and the slower one represented a complex of DNA and RNAP bound in open complexes at both p_R and p_{RM} (see also Results). Open complexes at both promoters are very stable (reference 28 and our unpublished results); thus, no significant dissociation or redistribution of RNAP is expected to occur during electrophoresis of the complexes.

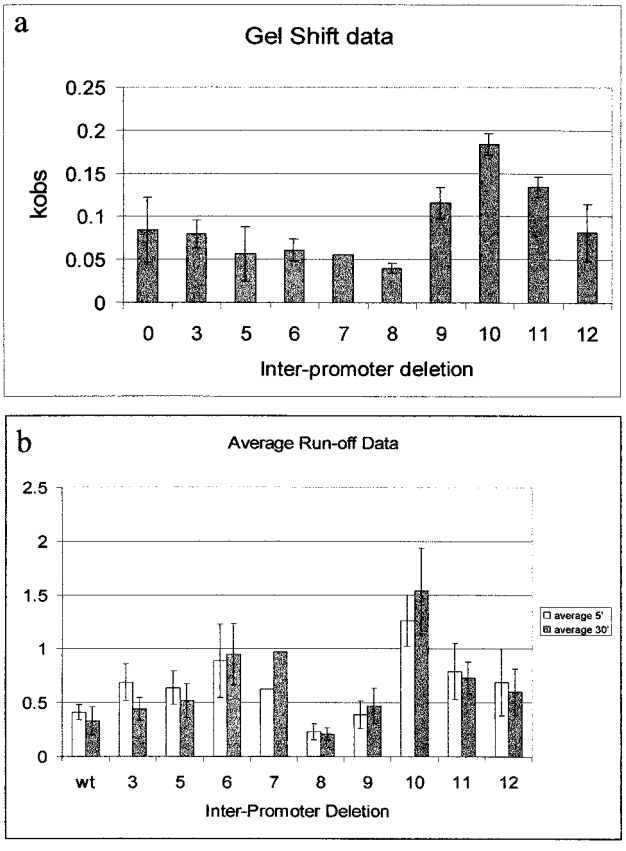

FIG. 2. Activity of the p_{RM} promoter is maximal for a 10-bp deletion in the region separating p_{RM} and p_R in both electrophoretic mobility shift and runoff transcription assays. The *x*-axis represents the number of base pairs deleted between the −35 regions of p_R and p_{RM}. (a) Comparison of the average k_{obs} for open complex formation at p_{RM} for each of the promoter variants. The radioactivity in each band, as a percentage of the total in the lane, was plotted against the time of incubation with the RNAP, and the k_{obs} for each DNA was determined by fitting the data to the equation $y = Y_f \cdot \{1 - \exp[-(t) \cdot k_{obs}]\} + Y_o$, where y = the percent of open complexes formed, t = time after RNAP mixing, and Y_f and Y_o are the limiting values for y. (b) Data from runoff transcription assays. The *y*-axis is the ratio of the band intensity for transcription derived for the p_{RM} promoter compared to the total density of the lane. The empty bars represent the relative amounts of RNA transcribed after incubating the RNAP with promoter for 5 min, while the solid bars represent the relative amounts of RNA synthesized after a 30-min preincubation.

Full saturation of the p_R promoter occurs before our first time point (taken at 2 min) and probably within seconds (21, 28). Next, the much slower process of open complex formation at p_{RM} takes place. Our measurements follow the rate of conversion of DNA with one open complex (at p_R) to that with two open complexes (at p_R and p_{RM}) and thus the rate of open complex formation at p_{RM}. A comparison of the pseudo-first-order rate constants (k_{obs}) for the binding of RNAP to the p_{RM} promoter in the context of the different deletions is graphically shown in Fig. 2a, and the values for k_{obs} for each promoter deletion mutant are given in Table 1. The D10 construct is seen to be unique in the rate with which p_{RM} can form an open complex with RNAP, which was enhanced greater than twofold on this construct. The rate of open complex formation at p_{RM} was slowest for the D8 construct.

Run-off transcription assays. The ability of RNAP to form open complexes at p_{RM} for each of the constructs was also determined with a single-round runoff transcription assay. Approximately 5 nM promoter was incubated with 50 nM RNAP in HEPES buffer for either 5 or 30 min, followed by a 1-min incubation with heparin (50 μg/ml). To allow RNA synthesis, ATP, CTP, and GTP were added to 200 μM and UTP (including [^{32}P]UTP) was added to 2 μM. After 10 min, UTP was added to 500 μM and the reaction mixture was incubated for an additional 5 min to ensure complete elongation of all transcripts. Finally, the products were separated on a denaturing gel. Bands apparent after exposure of the gel to Kodak Biomax film were scanned, and the intensities were normalized to the sum of the intensities of the p_{RM} and p_R bands.

TABLE 1. Average k_{obs} of the promoter deletion mutants

Interpromoter deletion no.	Avg k_{obs}[a] (min^{-1})
0	0.06 ± 0.04
3	0.08 ± 0.02
5	0.06 ± 0.03
6	0.06 ± 0.01
7	0.06[b]
8	0.04 ± 0.01
9	0.12 ± 0.02
10	0.18 ± 0.01
11	0.08 ± 0.01
12	0.09 ± 0.03

[a] The k_{obs} for each DNA was determined by fitting the data as described in the legend to Fig. 2. Values are means ± standard deviations based on averaging the results of three independent determinations except where indicated.
[b] Only one determination.

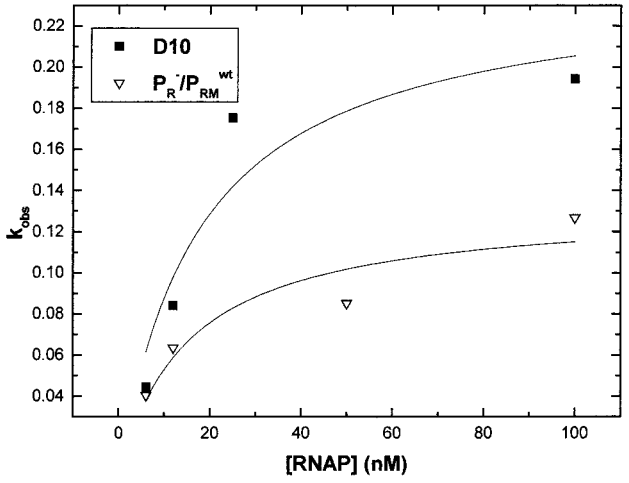

FIG. 3. Dependence of k_{obs} on RNAP concentration for the p_R^-/p_{RM} and D10 constructs. The k_{obs} were determined as a function of [RNAP], and the data were fit to the equation $k_{obs} = (K_B \cdot k_f)$ [RNAP]/(K_B[RNAP] + 1), where [RNAP] is the concentration of enzyme, K_B is the association constant for RNAP binding to the promoter in a closed complex, and k_f is the first-order rate constant for the conversion of the closed to the open promoter complex. The curves are the result of the fits. Symbols: ■, promoter with the 10-bp deletion; ▽, wild-type DNA that has had the p_R promoter inactivated by the base changes indicated in Fig. 1. The DNA concentration was kept constant (approximately 1 to 2 nM) for all concentrations of RNAP.

The amount of runoff product made in this assay is a reflection of the number of open complexes formed during the incubation of RNAP and the promoter, prior to the addition of heparin. The results of these experiments are shown in Fig. 2b. For all constructs, the p_{RM} promoter was found to be competent to initiate RNA synthesis (results not shown). Relative to the other deletion mutants, again a sharp increase is seen in the amount of RNA synthesized from the p_{RM} promoter on the D10 template. In this assay, but not the gel mobility shift experiments, the D6 and D7 constructs also show elevated levels of RNA synthesis, albeit not quite as high as that for D10. We do not understand the underlying cause of this difference between the two assays for these two constructs.

Dependence of k_{obs} on RNAP concentration for the wild type and D10 spacing between p_R and p_{RM}. The results described above, as well as those from our previous studies (21, 37), indicate that utilization of the p_{RM} promoter on the construct with the 10-bp deletion was significantly increased in comparison to that on constructs with the wild-type or other spacings between the p_{RM} and p_R promoters. To better understand the effect of the 10-bp deletion on open complex formation at p_{RM}, we determined the dependence of k_{obs} on RNAP concentration for two promoter mutants, D10 and p_R^-/p_{RM}. The rates of open complex formation were determined for each concentration of RNAP as described above. The dependence of k_{obs} on

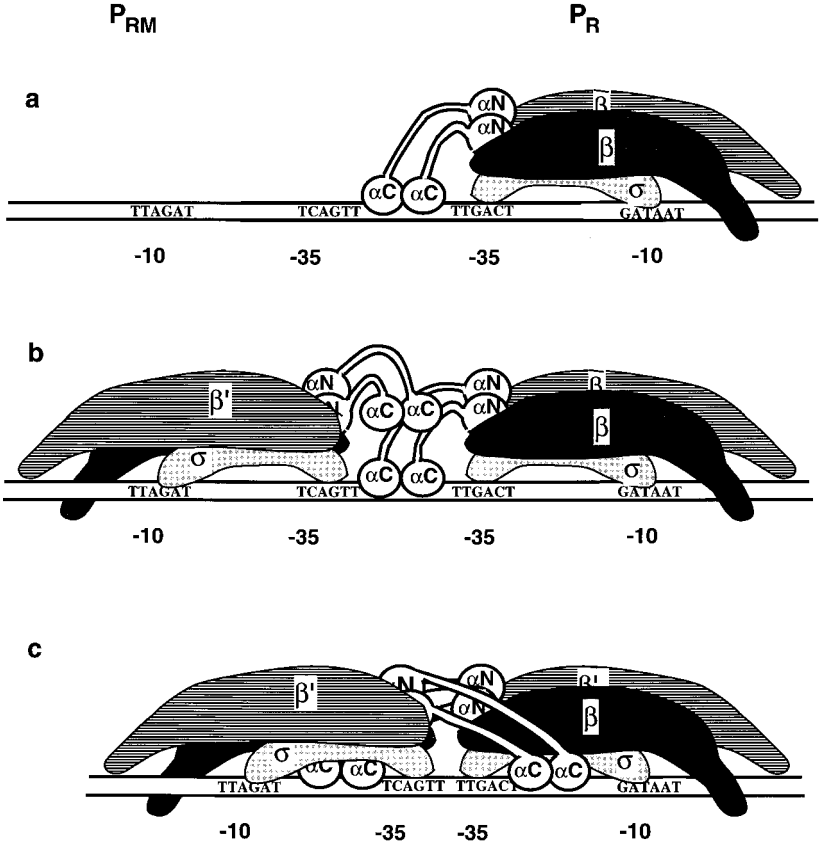

FIG. 4. RNAP at p_R interferes with open complex formation at p_{RM} for the wild-type (a and b) but not the D10 (c) interpromoter distance. The α subunits of RNAP are shown in white, with the N-terminal domains anchored to the β and β′ subunits of the RNAP (gray and striped regions) and the CTDs and the flexible linkers jutting away from RNAP. The p_{RM} promoter is on the left, and p_R is on the right. The sequences of the −10 and −35 regions are indicated for the nontemplate strand of each promoter. The spacer DNAs between the −10 and the −35 regions are shown as devoid of contacts with RNAP. (a) The −35 regions of p_R and p_{RM} are separated by 13 pdb. Within seconds of the addition of RNAP, an open complex forms at the p_R promoter. Proposed upstream contacts of the α-CTDs of the RNAP are shown. (b) Subsequent interaction of RNAP with p_{RM} in the presence of an RNAP at p_R. The RNAP at p_R obstructs upstream access by the α-CTDs of the RNAP at p_{RM}. (c) The −35 regions of p_R and p_{RM} are separated by 2 bp. This closer-in arrangement allows the spacer DNAs of p_R and p_{RM} to be contacted by the α-CTDs of the RNAP at the other promoter, facilitating open complex formation at p_{RM} despite the presence of an RNAP at p_R.

the concentration of RNAP is shown in Fig. 3; the data were fit as described in the figure legend to obtain the values of the association constant for RNAP binding to the promoter in a closed complex (K_B) and the first-order rate constant for the conversion of the closed to the open promoter complex (k_f). The values of K_B ($7 \times 10^7 \pm 3 \times 10^7$ M^{-1}) and k_f (0.13 ± 0.02 min^{-1}) for the p_R^-/p_{RM} construct determined here were similar to those previously reported (16). The fact that mainly k_f is increased when p_R is inactivated (12, 16) indicates that RNAP binding to p_{RM} is not affected but is rather a subsequent step on the pathway to formation of an open complex. For p_{RM} on D10, similar values for K_B ($6 \times 10^7 \pm 3 \times 10^7$ M^{-1}) and k_f (0.24 ± 0.04 min^{-1}) are obtained, indicating that on this template the formation of an open complex at p_{RM} takes place as if the p_R promoter were not occupied. We routinely observe a slightly greater rate of open complex formation at p_{RM} in the D10 than in the p_{R^-} context (reflected here by a twofold-greater k_f) (see also references 21 and 37). However, since the effect is quite small, we have not attempted to characterize it further.

Involvement of the α-CTD in the interference of RNAP at p_R with open complex formation at p_{RM}. In Fig. 4 we present a model, refined from Tang et al. (37), that takes our results into account and also draws upon recent insights into the interaction of the α subunit of RNAP with upstream DNA sequences. As first shown for the *rrnB* P1 promoter, the alpha C-terminal domain (α-CTD) binds sequence specifically to an A+T-rich region located between −40 and −60 (the UP element), thereby greatly activating RNA synthesis in vivo and the rate of open complex formation in vitro (30). However, at other promoters, there is also evidence for interactions of the α-CTD with other DNA sequences in upstream regions at similar locations, both in the presence and absence of activator protein (7, 10, 13, 29, 37). The extent of activation that can result from such interactions has not been systematically studied. Based on results with RNAP deleted for the α-CTD, we estimate that at the p_R and p_{RM} promoters, the interactions with upstream DNA stimulate open complex formation two- to threefold (37). Without the ability of interaction with upstream sequences, RNAP always exhibited a low level of activity at p_{RM}, even when the p_R promoter was inactivated or the template used bore the D10 template (37). These results provide a strong indication that on the D10 template the RNAP at p_{RM} was able to engage in upstream interactions even in the presence of another RNAP at p_R.

In the model presented in Fig. 4, on the template with the wild-type spacing between the two promoters, the interference of p_R-bound RNAP with open complex formation at p_{RM} is exerted via obstruction of interactions between the α-CTD and DNA in the −40 to −60 region of p_{RM}. This obstruction would be relieved for the D10 construct. Here the 10-bp deletion between the −35 regions of p_{RM} and p_R makes the spacer DNA between the −10 and −35 regions of the p_R promoter coincident with bp −44 to −60 with respect to p_{RM}. We envisage that the α-CTD of the RNAP at p_{RM} would be able to reach over the RNAP at p_R and contact this region. Few if any contacts have been demonstrated between promoter-bound RNAP and the spacer DNA (1, 35), so that the α-CTD of the RNAP at p_{RM} may well be able to interact with the spacer DNA of p_R, even when both promoters are occupied. The steep dependence of promoter activity and the rate of open complex formation on the interpromoter distance may reflect several factors. For shorter deletions (longer interpromoter distances), less of the spacer DNA but more of the −10 region of p_R is at −40 to −60 with respect to p_{RM}, leading to obstruction akin to that mentioned above for the wild-type spacer. Conversely, the longer deletions D11 and D12 (with shorter interpromoter distances) would keep the entire spacer DNA within the −40 to −60 region, but steric clashes between the two RNAPs would then become prohibitively severe.

We show that the putative upstream interactions lead to an increase in k_f, which is in agreement not only with the mode of p_{RM} activation obtained when the p_R promoter is inactivated (12, 16) but also with that observed when p_{RM} is provided with a genuine UP element (36, 37). Thus, the model is consistent with the available experimental evidence indicating that upstream interactions of the α-CTD facilitate a step subsequent to the initial binding of RNAP to the promoter.

Divergent promoters of *E. coli*. Divergent promoters are fairly common in *E. coli* as well. In a 1988 review (3), many instances of divergently transcribed promoters in a back-to-back orientation (i.e., directing the synthesis of nonoverlapping transcripts) similar to that of p_R and p_{RM} of phage lambda were recognized. For our current analysis, we focused on pro-

TABLE 2. Back-to-back divergent promoters in *E. coli*

Promoter pair[a]	Transcription[b]	Position[c]	Separation (pdb)[d]	Reference
fepA	Reverse	611892	16	26
fes	Forward	611908		
bioA	Reverse	808515	10	24
bioB	Forward	808525		
fumA	Reverse	1686464	111	20
manA	Forward	1686575		
pdx	Reverse	2435904	28	33
div	Forward	2435932		
udf Px	Reverse	3208209	78	6
rpsUp1	Forward	3208287		
dnaAp1	Reverse	3881590	104	23
rpmHp3	Forward	3881694		
asnC	Reverse	3924656	101	19
asnA	Forward	3924757		
ilvY	Reverse	3955488	45	39
ilvC	Forward	3955533		
metJp1	Reverse	4126138	78	18
metB	Forward	4126216		
trmA	Reverse	4160874	104	14
btuB	Forward	4160978		2
uvrA	Reverse	4271512	78	32
ssb	Forward	4271590		5
IleRp2	Reverse	4445871	108	38
ORF83	Forward	4445979		
smp	Reverse	4622368	64	22
serB	Forward	4622432		

[a] Only pairs with a distance between start sites of 120 pdb or fewer are shown. All are promoters for RNAP holoenzyme containing σ^{70}.
[b] Forward is in a clockwise direction on the genome (in the direction of increasing numbers on the map of Blattner et al. [4]).
[c] Position of start site.
[d] Separation (in total number of pdb) of the start sites between forward and reverse promoters (map number of forward start site − map number of reverse start site).

moter pairs that had start sites separated by 120 pdb or fewer. We chose this distance as an upper limit based on the observed 60-bp upstream extension in DNA interactions at promoters containing upstream elements (30). Thus, it is likely that start site separations beyond this distance will allow unimpeded interactions of RNAP at either promoter. Our search of the database RegulonDB (31) for known *E. coli* promoters satisfying the above criteria identified 13 promoter pairs, five of which were also represented in the earlier compilation (3) (Table 2). Three cases for which the separation between the start sites is in the range of 71 to 83 pdb investigated here were identified. Interestingly, all three have a separation of 78 pdb, similar to that for the D5 deletion (this work and reference 21), where the interference was found to be rather pronounced. The regulatory significance of a separation by this distance has yet to be investigated.

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