Supplementary Material

Repressor CopG prevents access of RNA polymerase to promoter and actively dissociates open complexes

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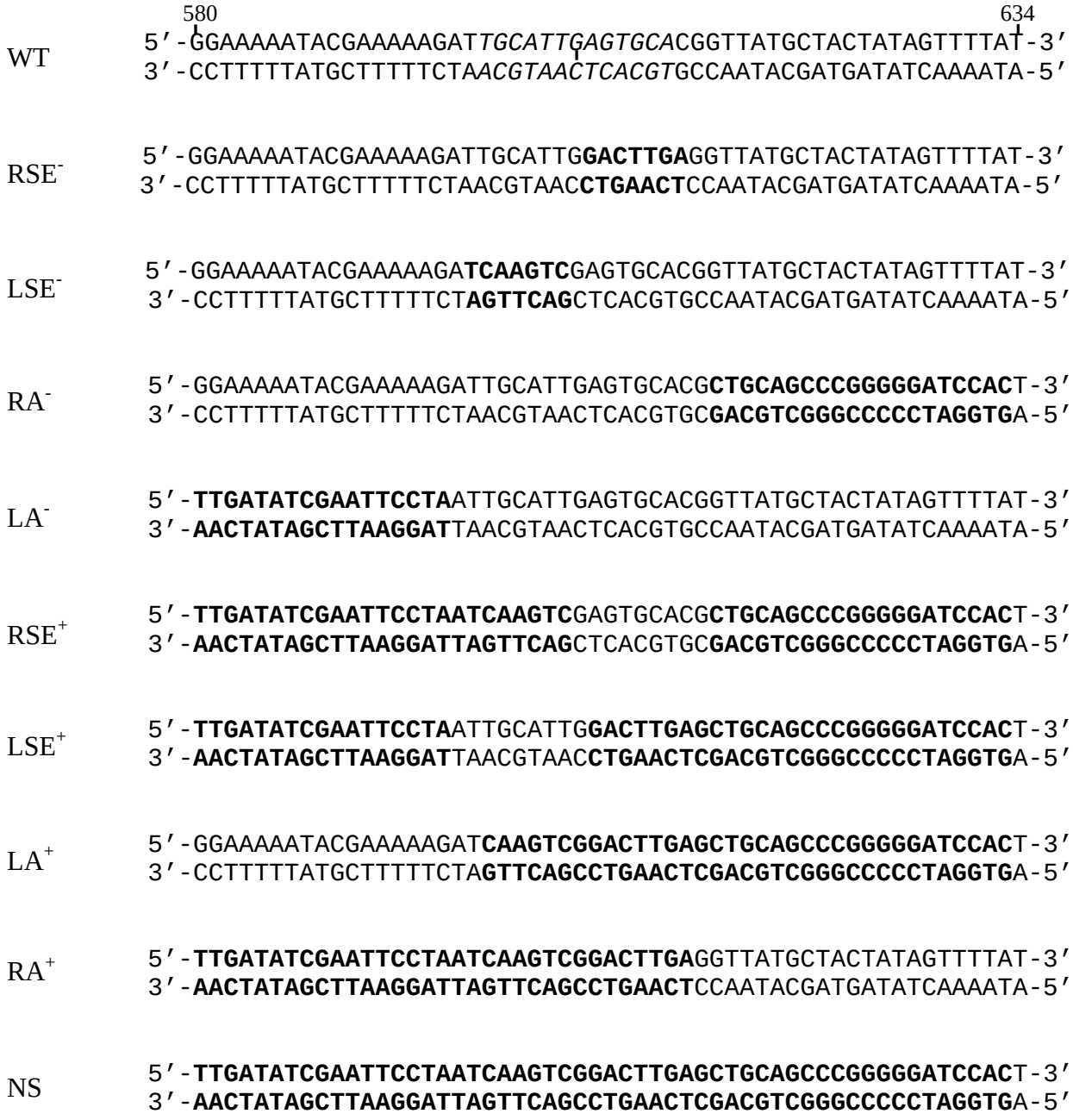
SUPPLEMENTARY MATERIALS AND METHODS

Construction of the operator variants

The following 55-bp operator variants (with the names indicated on the left) were used in the affinity assays:



The numbers above the ends of the wild-type (WT) operator correspond to the coordinates on the pMV158 sequence. The 13-bp pseudo-symmetric element (SE) is displayed in italics on the sequence of the WT operator, with its twofold axis denoted by a small line. Sequence substitutions in the various mutant operators are shown in boldface. The same mutant sequence

(5'-**GACTTGA**-3' from the centre of the SE) was employed to substitute the RSE and the LSE in the corresponding mutant operators lacking these binding sites. The RA and LA were replaced with the sequence at either side of the EcoRI site of the pBluescript SK+ plasmid vector (STRATAGENE), where the SE of the CopG operator had been cloned (unpublished results). In the non-specific (NS) oligonucleotide, the sequence of all four binding sites was substituted accordingly.

The top and bottom strands of each of the above oligonucleotides were synthesized separately and purified by electrophoresis in 12% PAA sequencing gels. The top strand was 5'-end labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase and subsequently annealed to a 3-fold molar excess of the corresponding unlabelled bottom strand.

Relative affinity assays

The relative affinity of binding of CopG to the different operator mutants was determined by competitive electrophoretic mobility shift assays (EMSA), in a way similar to that previously described (1). Basically, CopG was incubated in 6 µl BB buffer with a mixture of the 5'-end labelled 239-bp DNA fragment containing the wild-type promoter/operator sequence and one of the 55-bp operator variants (also 5'-end labelled). Free and complexed DNAs from both fragments were separated by electrophoresis on 8% native PAA gels. Labelled DNA bands were detected and quantified with the storage phosphor technology, with the aid of a FLA-3000 (FUJIFILM) imaging system and the Quantity One software (Bio-Rad). The affinity of CopG for DNA D_1 relative to DNA D_2 [$K_{rel}(D_1/D_2)$] was determined as follows:

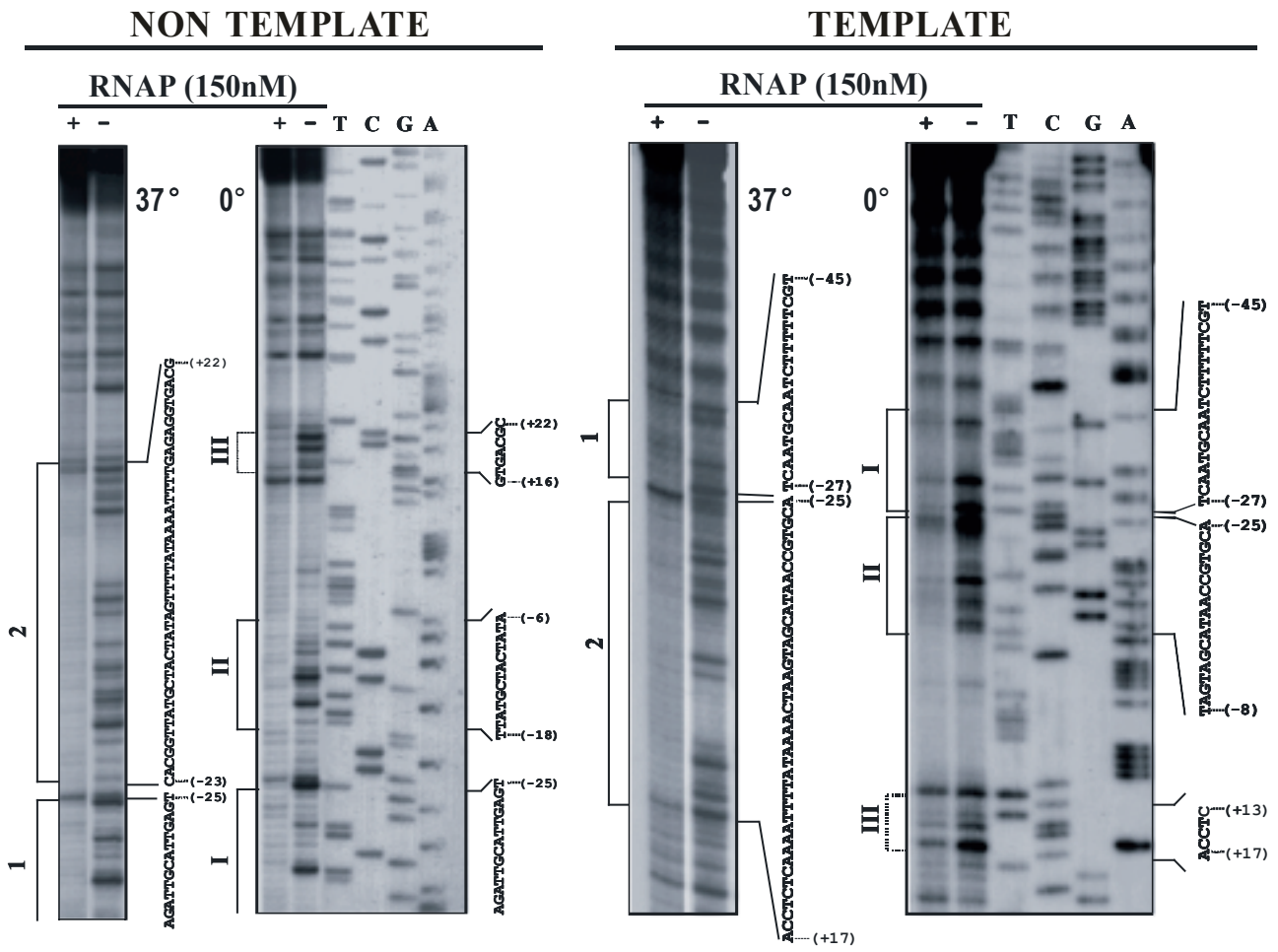
$$K_{rel}(D_1/D_2) = [CopG\text{-}D_1] \cdot [D_2] / [CopG\text{-}D_2] \cdot [D_1]$$

where $[D_1]$ and $[D_2]$ are the concentrations of free DNA D_1 and DNA D_2, respectively. $[CopG\text{-}D_1]$ and $[CopG\text{-}D_2]$ are the sum of the concentrations of the complexes generated by binding of CopG to D_1 and D_2, respectively.

To normalize the data for potential effects due to the different size (239 bp vs. 55 bp) and origin (PCR amplification vs. chemical synthesis) of the DNAs used in these assays, the affinities of CopG for the 55-bp operator mutants relative to the 239-bp wild-type DNA fragment were divided by the ratio between the protein affinities for the 55-bp WT and 239-bp wild-type DNAs.

REFERENCES

1. Ruiz-Maso, J.A., Lurz, R., Espinosa, M. and del Solar, G. (2007) Interactions between the RepB initiator protein of plasmid pMV158 and two distant DNA regions within the origin of replication. *Nucl. Acids Res.*, **35**, 1230-1244.


NON TEMPLATE
TEMPLATE
RNAP (150nM)
RNAP (150nM)
+ − + − T C G A
+ − + − T C G A
37°
0°
37°
0°
1
2
I
II
III
(+22)
(+16)
(−6)
(−18)
(−25)
(−23)
(−25)
(−45)
(−27)
(−25)
(+17)
(−8)
(+13)
AGATTGCATTGAGT CACGGTTATGCTACTATAGTTTTTATAAAATTTTGAGAGGTGACG
GTGACGC
TTATGCTACTATA
AGATTGCATTGAGT
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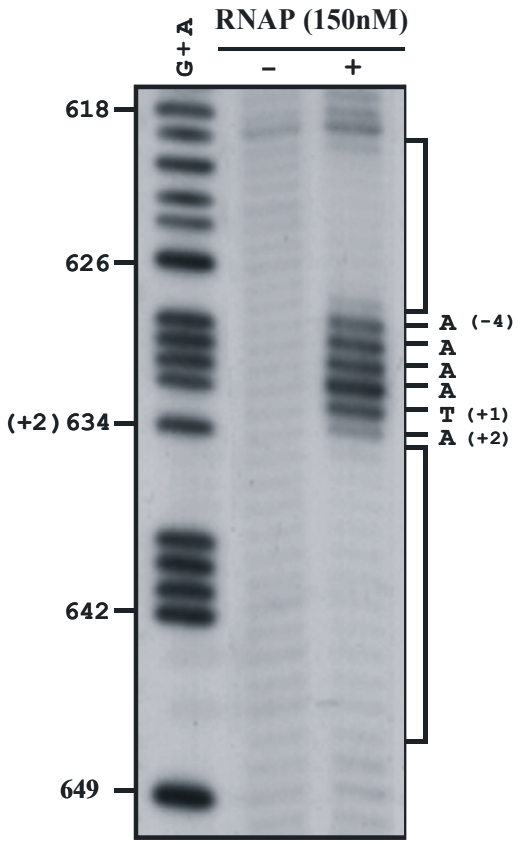
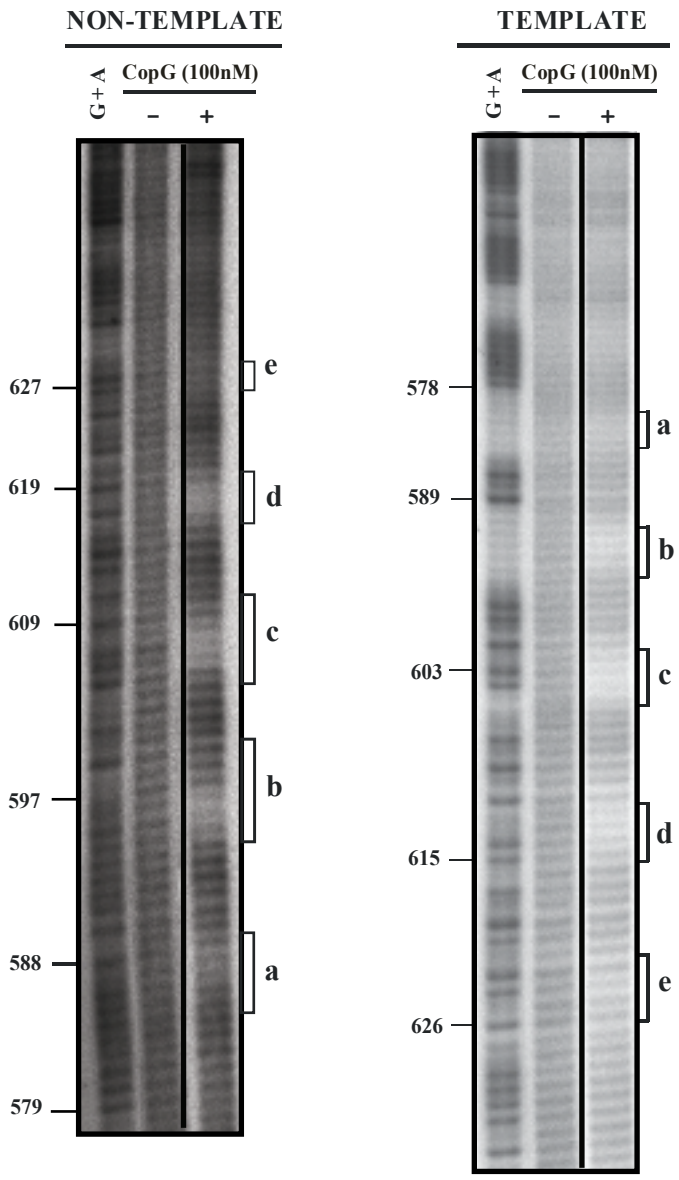
TAGTAGCATAACCGTGCA
ACCTC

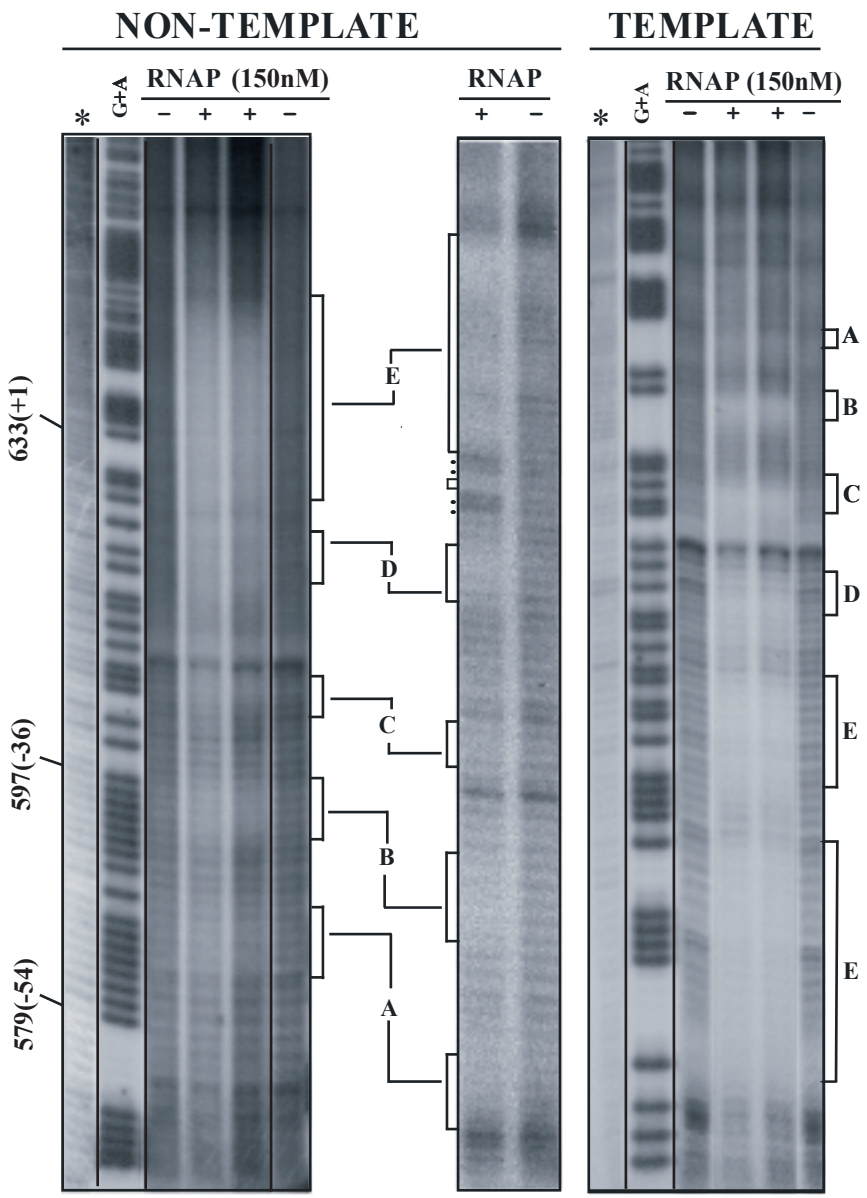
Supplementary Fig 2.



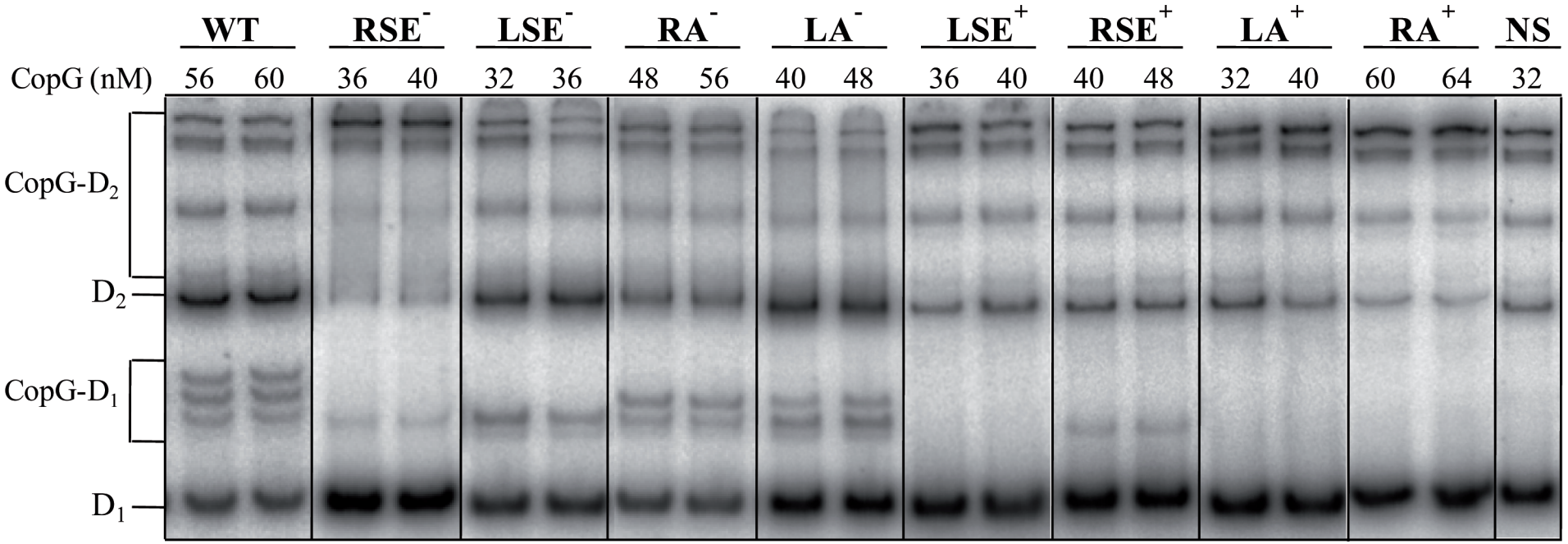
Supplementary Fig 3.



Supplementary Fig 4.



Supplementary Fig 5.



SUPPLEMENTARY FIGURE LEGENDS

Figure S1. DNase I footprinting analysis of RNAP-P_{cr} complexes. Representative footprints on both DNA strands of the RNAP-P_{cr} complexes generated at 0ºC and 37ºC. DNA labelled on either strand (2 nM) was incubated at the indicated temperature in the presence (+) or absence (-) of RNAP, prior to cleavage with DNase I. Protections are shown by numbered brackets that include the sequence of the protected region. (T, G, C, A), dideoxy sequencing reactions performed on the same DNA.

Figure S2. Enhanced HO• reactivity at the +1 region of the template strand of the RNAP-P_{cr} complexes generated at 37ºC. DNA labelled on the template strand (2 nM) was incubated at 37ºC in the presence (+) or in the absence (-) of RNAP, prior to addition of heparin and subsequent cleavage with HO• following method 1 (see Materials and methods). DNA regions protected by RNAP from HO• attack are indicated by brackets. The sequence of the hypersensitive positions at the transcription start site (-4 to +2) is shown. G+A, Maxam and Gilbert sequencing products of the same DNA. Numbers on the left indicate the base coordinates in the pMV158 DNA sequence**.** Coordinate 634 corresponds to promoter position +2.

Figure S3. HO• probing of the contacts of CopG with the DNA backbone of the P_{cr} promoter region at 37ºC. DNA labelled on either strand (2 nM) was incubated in the presence (+) or absence (-) of CopG prior to HO• cleavage following method 1 (see Materials and methods). Five regions protected by the repressor were observed in each strand (a, b, c, d and e). Protections denoted by the same letter in both strands lie across the minor groove of the DNA. G+A, Maxam and Gilbert sequencing products of the same DNA. Numbers on the left indicate the base coordinates in the pMV158 DNA sequence. All the lanes displayed came from the same gel.

Figure S4. HO• probing of the contacts of RNAP with the DNA backbone of the P_{cr} promoter region at 37ºC. DNA labelled on either strand (2 nM) was incubated in the presence (+) or absence (-) of RNAP prior to addition of heparin and subsequent cleavage with HO• following method 2 (see Materials and methods). RNAP protected five regions in each strand (A, B, C, D and E). Protections denoted by the same letter in both strands lie across the minor groove of the DNA. Sites of enhanced HO• reactivity are indicated with dots. No enhancement of the backbone cleavage was observed at the +1 region of the template strand, in contrast to the results obtained in the experiment of Supplementary Figure 2. This difference may be owing to the different method used to generate OH• in each assay. A sample of DNA not treated with OH• is included (*). G+A, Maxam and Gilbert sequencing products of the same DNA. Numbers on the left indicate the base coordinates in the pMV158 DNA sequence. All the lanes displayed came from the same gel.

Figure S5. Competitive EMSA to determine the relative affinity of CopG binding to the different operator variants. CopG (at the stated concentrations) was incubated with a mixture of the indicated 55-bp operator variant (D1 DNA) and the 239-bp wild-type operator DNA fragment (D2 DNA). Concentrations used were: 8 nM (~20000 cpm) for the 239-bp DNA fragment and for the 55-bp WT oligonucleotide; 16 nM (~40000 cpm) for the 55-bp oligonucleotides LSE^-, RA^- and LA^-; and 32 nM (~80000 cpm) for the 55-bp oligonucleotides RSE^-, RSE^+, LSE^+, LA^+, RA^+ and NS.

Table 1. Percentage of free DNA and RNAP-DNA or CopG-DNA complexes obtained by quantification of the free and complexed DNA in EMSA from Fig. 6A.

1			10			30			60			Time(min)
0	40	250	0	40	250	0	40	250	0	40	250	CopG(nM)
93	75	64	89	79	73	87	84	68	84	82	71	%RNAP-DNA complex
0	25	36	0	21	27	0	16	32	0	18	29	%CopG-DNA complex
7	0	0	11	0	0	13	0	0	16	0	0	%fDNA

Table 2. Percentage of free DNA and RNAP-DNA or CopG-DNA complexes obtained by quantification of the free and complexed DNA in EMSA from Fig. 6B.

1			5			10			15			30			Time (min)
0	500	1000	0	500	1000	0	500	1000	0	500	1000	0	500	1000	CopG (nM)
55	17	12	-	13	10	-	15	10	-	19	10	50	23	9	%RNAP-DNA complex
0	83	88	-	87	90	-	85	90	-	81	90	0	77	91	%CopG-DNA complex
45	0	0	-	0	0	-	0	0	-	0	0	50	0	0	%fDNA

Table 3. Percentage of free DNA and RNAP-DNA or CopG-DNA complexes obtained by quantification of the free and complexed DNA in EMSA from Fig. 6C.

1			5			10			15			Time(min)
0	300	600	0	300	600	0	300	600	0	300	600	CopG(nM)
30	19	4	32	11	5	26	16	7	25	16	6	%RNAP-DNA complex
0	81	96	0	89	95	0	84	93	0	84	94	%CopG-DNA complex
70	0	0	68	0	0	74	0	0	75	0	0	%fDNA

Table 4. Percentage of free DNA and RNAP-DNA or CopG-DNA complexes obtained by quantification of the free and complexed DNA in EMSA from Fig. 6D.

1				10				30				Time(min)
150		500		150		500		150		500		Heparin (µg/ml)
0	500	0	500	0	500	0	500	0	500	0	500	CopG(nM)
62	32	57	29	60	26	45	23	55	26	48	25	%RNAP-DNA complex
0	68	0	71	0	74	0	77	0	74	0	75	%CopG-DNA complex
38	0	43	0	40	0	55	0	45	0	52	0	%fDNA