Transcription Activation by cAMP Receptor Protein (CRP) at the *Escherichia coli gal* P1 Promoter. Crucial Role for the Spacing between the CRP Binding Site and the −10 Region†

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ABSTRACT: The cAMP–CRP complex activates the initiation of transcription at the *Escherichia coli gal* P1 promoter, and the activation efficiency is highly sensitive to the location of the complex on this promoter region. Moving the CRP binding site by one base pair toward the start of transcription significantly decreases the extent of activation in vivo and actually turns the cAMP–CRP complex into an inhibitor in in vitro experiments. A structural analysis of open complexes formed on the two promoter fragments at 37 °C has revealed three elements crucial for an optimal activation process: a strong upstream anchorage of RNA polymerase, a cooperative binding of CRP and RNA polymerase, and an accurate orientation of the two promoter regions located upstream and downstream of the CRP binding site. Furthermore, structural analysis of polymerase promoter complexes at lower temperatures suggests that RNA polymerase initially recognizes the upstream region of the *gal* P1 promoter and subsequently interacts with sequences from the −10 to +20 region to yield the final open complex structure. The involvement of CRP in these sequential events has been examined.

The cAMP–CRP[1] complex modulates transcription of several operons in *Escherichia coli*. It was evident, many years ago, that the functional DNA binding sites for this complex are located at different distances from the −10 region of the promoters (deCrombrugghe et al., 1984). The compilation of the locations found in natural CRP-dependent promoters, as well as the study of synthetic promoters constructed with CRP binding sites located at various distances from the −10 region, has permitted the determination of the spacings between CRP and the promoter which result in activation (Aiba, 1989; Gaston, et al., 1990; Ushida & Aiba, 1990). The three most efficient positions of the CRP binding site are in phase on the DNA sequence and located at positions −41.5, −61.5, and −70.5 (+1 refers to the transcription start point). These distances correspond respectively to the natural CRP binding sites at the *gal*, *lac*, and *MalT* promoters. Kinetic assays performed in vitro (Buc et al., 1987; Gaston et al., 1990) as well as results from CRP mutagenesis (Bell et al., 1990) or extensive deletions in the *rpoA* gene coding for the α subunit of RNA polymerase (Igarashi & Ishihama, 1991; Igarashi et al., 1991) indicate that the crucial interactions between the three components, promoter, RNA polymerase, and cAMP–CRP complex, are not the same when the distance between the CRP binding site and the −10 region is changed.

We wondered how sensitive is the system to small displacements of the CRP binding site from these optimal positions? It is expected on the basis of simple biophysical considerations that the shorter the distance, the more severe this spacing condition is, since the free energy for twisting or bending the DNA increases markedly when the distance is reduced [cf. Wang and Giaever (1988)]. It is interesting to note that the binding site of most prokaryotic activators which bind in the vicinity of the start site of transcription is around position −40 [see Collado-Vides et al. (1991) for a recent compilation and analysis of the location of activator binding sites]. The CRP binding site of the *gal* P1 promoter is located at the shortest optimal distance of the CRP activating sites. In 1982, Busby et al. observed that, when the CRP binding site was moved only 1 bp closer to the Pribnow box, open complex formation on this promoter was repressed by CRP (Busby et al., 1982). More recently, on variants of the *deo*P2 and *tsx*P2 promoters, where the spacing between the CRP binding site and the upstream boundary of the Pribnow box is around 30 bp, a 1–2 bp increase or decrease in the distance around the optimal position was shown to be sufficient to abolish activation in vivo (Valentin-Hansen et al., 1991).

In order to study more precisely the effect of spacing between the CRP binding site and the −10 region, a double mutant of the *gal* control region was first constructed which eliminated RNA polymerase recognition of the *gal* P2 promoter so that only the *gal* P1 promoter is active, in the absence and in the presence of CRP. Using this modified *gal* P1 promoter, we observed that the deletion of 1 bp at position −30 resulted in a significant loss in activation by CRP in vivo and to repression in vitro. To understand the molecular basis of this effect, the synergy of binding of CRP and RNA polymerase was investigated by footprinting techniques at various temperatures.

MATERIALS AND METHODS

Materials

Chemicals. ATP, CTP, GTP, UTP, and CpA were purchased from Pharmacia, (α-^{32}P)dATP and (γ-^{32}P)ATP were from Amersham, and (α-^{32}P)UTP was from N.E.N.

Proteins. Restriction enzymes were from New England Biolabs, calf intestinal alkaline phosphatase and T4 polynu-

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[1] Abbreviations: bp, base pair; cAMP, cyclic AMP; CRP, cAMP receptor protein; DNase I, deoxyribonuclease I; $(OP)_2Cu^+$, 1,10-phenanthroline–cuprous ion complex; PCR, polymerase chain reaction; RNP, RNA polymerase.

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Table I: Functional Parameters of Three Mutants of the *gal* P1 Promoter[a]

	−CRP				+CRP							
	in vitro				in vitro				activation factor			
	K_B	k_f	K_Bk_f	in vivo	K_B	k_f	K_Bk_f	in vivo	in vitro			in vivo
mutant	($M^{-1} \times 10^{-6}$)	($s^{-1} \times 10^{2}$)	($M^{-1}\,s^{-1} \times 10^{-5}$)	$U_{\beta gal}$	($M^{-1} \times 10^{-6}$)	($s^{-1} \times 10^{2}$)	($M^{-1}\,s^{-1} \times 10^{5}$)	$U_{\beta gal}$	K_B	k_f	K_Bk_f	$U_{\beta gal}$
gal 16C	3.7 ± 1.1	0.68 ± 0.16	0.25 ± 0.02	110 ± 30	3.2 ± 1	4.8 ± 1	1.6 ± 0.9	1190 ± 80	0.9	7.1	6.4	10.8
gal 9A16C(0)	48.1 ± 27	2.1 ± 0.4	10 ± 3.1	380 ± 25	19.4 ± 5.1	7.9 ± 1.2	15.3 ± 1.8	2390 ± 200	0.4	3.8	1.5	6.3
gal 9A16C(−1)	40.4 ± 10	2.4 ± 0.2	9.5 ± 1.9	380 ± 25	39.7 ± 8.5	0.64 ± 0.1	2.5 ± 0.2	660 ± 40	1	0.3	0.3	1.7

[a] This table presents the in vivo and in vitro parameters of transcription initiation on three mutants of the *gal* P1 promoter. In vivo parameters are given as the β-galactosidase levels ($U_{\beta gal}$) measured in *E. coli* strains M182 Δ*crp* (−CRP) and M182 *crp*$^+$ (+CRP) transformed with a pAA187 vector containing the promoter fragment upstream of the *lacZ* gene. These β-galactosidase activities are corrected for β-lactamase activities which are an indicator of the plasmid copy number. In vitro parameters were obtained by abortive initiation assays on linear *Eco*RI–*Hin*dIII promoter fragments. According to the classical two-step model of transcription initiation (McClure, 1985), K_B and k_f constants, respectively, refer to the initial binding constant and the isomerization rate. Their product is representative of the overall strength of the promoter. Details about the measurements of the in vivo and in vitro constants are given in Materials and Methods.

cleotide kinase were from Boehringer Mannheim, and DNase I was from Worthington. *E. coli* RNA polymerase was purified according to the procedure of Burgess and Jendrisak (1975) as modified by Lowe et al. (1979). The concentrations of RNA polymerase were calculated from protein concentrations determined by absorption measurements ($E^{1\%}_{280nm}$ = 6.2), and titration experiments have shown that the enzyme was about 50% active. *E. coli* CRP was prepared as described in Ghosaini et al. (1988).

Methods

Promoter Construction and in Vivo Assays. A variation of the PCR techniques of Gyllenstein et al. (1988) and Higuchi et al. (1988) was used to mutagenize the *gal* control region. The *Eco*RI–*Hin*dIII fragment obtained was cloned into a *lacZ* expression vector, pAA 187 (Busby et al., 1984), and the resulting plasmid was used to transform M182 crp$^+$ and M182 Δ*crp* strains. β-Galactosidase activities were performed during exponential growth phase (Miller, 1972). These activities were corrected for β-lactamase activity (O'Callaghan et al., 1972), which is an indicator of the plasmid copy number.

In Vitro Transcription Assays. Run off transcription and abortive initiation assays were performed as described by Spassky et al. (1984) and McClure et al. (1978), respectively. According to the simple two-step model of open complex formation, the kinetic constants (K_B and k_f) were calculated from a plot of lag versus the reciprocal of RNA polymerase concentration using the program "Enzfitter" (R. J. Leatherbarrow, Elsevier Biosoft, Cambridge, U.K., 1987) on an IMB-PC computer.

Footprinting. The two 9A16C fragments were labeled on both strands at their *Eco*RI ends. The complexes formed at 37 °C with RNA polymerase (150 nM) and/or CRP (100 nM), were treated at the same temperature with DNase I (Spassky et al., 1984), hydroxyl radicals (Tullius & Dombroski, 1986), $(OP)_2Cu^+$ (Sigman et al., 1985), and $KMnO_4$ (Sasse-Dwight & Gralla, 1989). When the complexes were analyzed at lower temperatures, DNase I concentrations were increased and, in order not to change the pH at which RNA polymerase complexes were formed, Tris was replaced by Hepes as buffer.

HinfI Protection Experiments. Labeled fragments were incubated 30 min at 37 °C, in the presence of cAMP (200 μM) and various combinations of RNA polymerase (150 nM) and CRP (100 nM). Heparin was added to a final concentration of 40 μg/mL; 1 min later 18 units of *Hinf*I were added. Digestion was stopped at different times and then analyzed on a 7.5% acrylamide native gel.

RESULTS

(1) Gal Control Region Mutagenesis. The *gal* control region has been transferred to a 144 bp fragment (*Eco*RI–*Hin*dIII). This region contains the two overlapping promoters, P1 (CRP activated) and P2. Two mutations were introduced by PCR mutagenesis into this region, in order to eliminate the *gal* P2 promoter and enhance the activity of the *gal* P1 promoter. First, an A → C change at position −16 was created (called p16C mutant). This mutation is known to completely inactivate the *gal* P2 promoter, both in vivo and in vitro (Kuhnke et al., 1986; Lavigne et al., 1992). However, this p16C mutation also weakens the *gal* P1 promoter, so that inhibitory effects by CRP cannot be easily studied. Consequently, a second mutation was introduced at position −9 (G → A) so as to change the sequence of the *gal* P1 Pribnow box from TATGGT to TATAGT, which is closer to the consensus sequence for a Pribnow box. The strength of this promoter was then measured in vivo and in vitro in the presence and absence of the cAMP–CRP complex and compared with that of the parent construct p16C.

The 144 bp fragment carrying these two mutations, called 9A16C(0), was cloned into a *lacZ* expression vector, pAA187 (Busby et al., 1984). The resulting plasmid was used to transform M182 *crp*$^+$ and M182 Δ*crp* strains. The strength of the modified promoter could then be compared in these two strains, using a reference the *gal* P16C single mutant. β-Galactosidase activities measured during exponential growth phase are given in Table I. First, as expected, the change in the *gal* P1 Pribnow box enhances the overall strength of the promoter both in the presence and in the absence of a functional cAMP–CRP complex. Second, the promoter can still be activated in vivo by the presence of CRP; however, the activation factor is less than that measured on the p16C mutant.

Transcription initiation on the linear 9A16C fragment was then analyzed in vitro. By "run off" assays, we verified that the *gal* P1 promoter was the only active promoter on the 9A16C(0) fragment, both in the absence and in the presence of CRP (data not shown). The kinetics of the initiation reaction were analyzed by abortive initiation assays (McClure et al., 1978). When RNA polymerase was added to a mixture of promoter and substrates allowing the initiation of transcription, a latency characterized by a lag time τ occurred before the production of the expected short transcripts at a constant rate. The dependence of this lag time on the enzyme concentration was interpreted according to the two-step irreversible model where the first binary complex is inactive and in fast equilibrium with the free species. Within the framework of this model, the slope of a plot of τ versus 1/(RNA polymerase) yields the reciprocal of the product K_Bk_f and

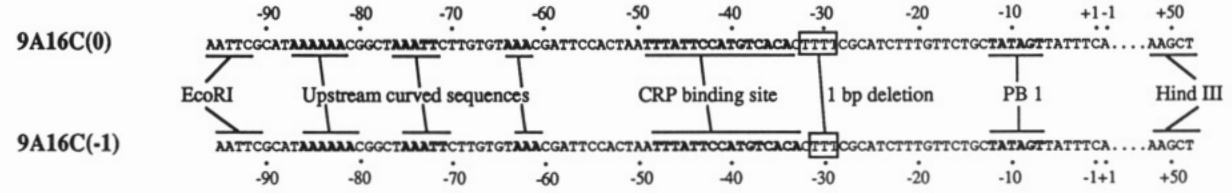


FIGURE 1: Sequence and organization of the two 9A16C mutants of the *gal* P1 promoter. The sequence of the upper strand of the two *gal* P1 mutants is given between the transcriptional start point (noted +1) and the *Eco*RI site (introduced at –94). These two fragments differ from the wild type *gal* sequence by the presence of two point mutations (–9 G → A and –16 A → C). The main elements of the *gal* P1 promoter, i.e., the Pribnow box (PB1) (also called –10 region in the text), the CRP binding site, and the upstream curved sequences, are indicated. The 9A16C(–1) mutant is derived from the 9A16C(0) mutant by a 1 bp deletion at position –30.

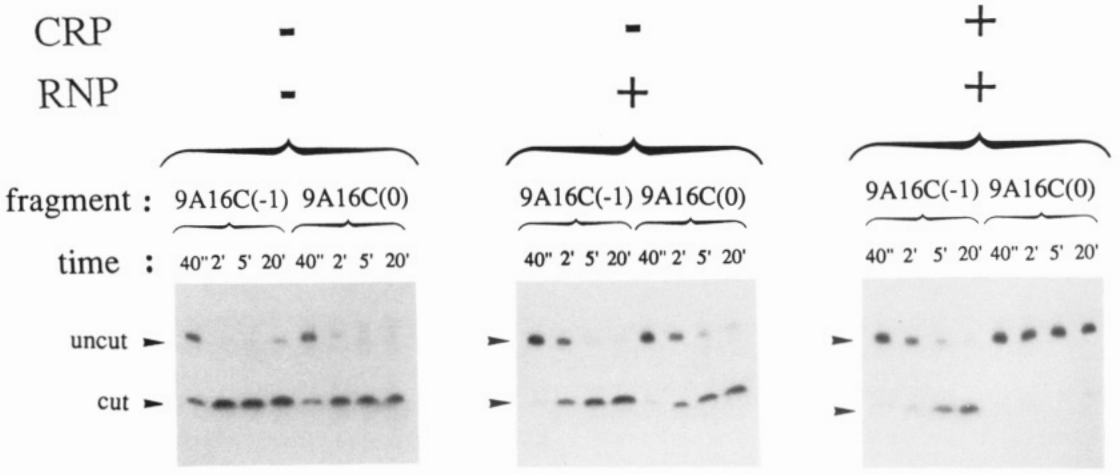


FIGURE 2: Protection by RNP and RNP + CRP of the –58 *Hin*fI site of the two 9A16C fragments. The two 9A16C fragments contain an *Hin*fI site around position –60. These fragments were labeled at the *Eco*RI site (upper strand) and incubated 30 min at 37 °C, in the presence of cAMP (200 μM) and various combinations of RNP and CRP as indicated by "–" and "+" symbols. Heparin was added to a final concentration of 40 μg/mL, 1 min later *Hin*fI digestion was performed as described in Materials and Methods, and digestion products were separated on a 7.5% acrylamide gels. An autoradiogram of the dried gels is presented in this figure. Arrows indicate the position of the fragments before and after cutting.

reflects the overall promoter strength. The intercept with the ordinate corresponds to k_f^{-1}, the characteristic time required for open complex formation from the inactive closed intermediate, while the intercept with the abscissa yields K_B, the constant for the association of the free species in the closed complex. Values obtained by abortive initiation assays in our system are given in Table I. The value of the $K_B k_f$ product shows that the –9 G → A mutation, in a p16C background, makes the *gal* P1 promoter stronger but less activatable by CRP. For both fragments prior addition of CRP essentially increases the first-order rate constant for open complex formation k_f.

In summary, we have constructed a mutant of the *gal* promoter region on which, in both the presence and the absence of CRP, RNA polymerase only makes an open complex at the *gal* P1 promoter. This mutant promoter is fairly strong but is still activatable by CRP. It is therefore suitable to study open complex formation at the *gal* P1 promoter and the activation by CRP of this enzymatic process.

(*2*) *In Vivo and in Vitro, the Distance between the CRP Binding Site and the –10 Region Is Crucial for Transcription Activation by CRP at gal P1.* By PCR mutagenesis, we obtained a fragment which is missing 1 bp at position –30. As a result, the CRP binding site and the upstream curved sequences located between positions –90 and –60 are moved 1 bp nearer to the Pribnow box (see Figure 1). Otherwise, this construct called 9A16C(–1) is identical to its parent 9A16C(0). The assays performed on the 9A16C(0) mutant were repeated on this new construct.

In vivo, in the Δcrp strain, the deletion of 1 bp in –30 region has no effect on promoter strength (cf. column 5 in Table I). However, comparison of the results obtained in the crp^+ and the Δcrp strains indicates that the activation effect due to CRP is reduced by a factor 3.6 (last column).

In vitro, "run off" assays showed that this deletion does not affect the transcriptional start point. In the absence of the cAMP–CRP complex, the K_B and k_f parameters deduced from the abortive initiation assay parameters are similar for both fragments (columns 2–4). However, when the cAMP–CRP complex was bound prior to RNA polymerase addition, the kinetics of open complex formation were markedly affected by the deletion. As shown in Table I, CRP no longer functions as an activator but actually inhibits the promoter, the overall isomerization rate constant k_f being reduced by 75%.

Consistent results were therefore obtained in vivo and in vitro. The loss of 1 bp in the interval between the CRP binding site and the –10 region abolishes the activating effect of CRP. The effect is however more marked in vitro where a significant inhibition is observed; i.e., CRP is behaving now as a repressor.

(*3*) *Stability of Open Complexes.* In order to know whether the differential effect of CRP on the overall rate for open complex formation at the two promoters could also be detected at equilibrium, we first compared the stabilities of open complexes formed on the two fragments. As described in section 5, all footprints of open complexes (performed at 100 nM RNA polymerase concentration, and when indicated at 100 nM CRP) extend upstream beyond the –55 to –60 region where a site for the restriction enzyme *Hin*fI is located. The rate of attach of this site by *Hin*fI has already been used by Shanblatt and Revzin to probe the stability of ternary complexes formed on a wild-type *gal* promoter fragment (Shanblatt & Revzin, 1983). Stability of the open complexes

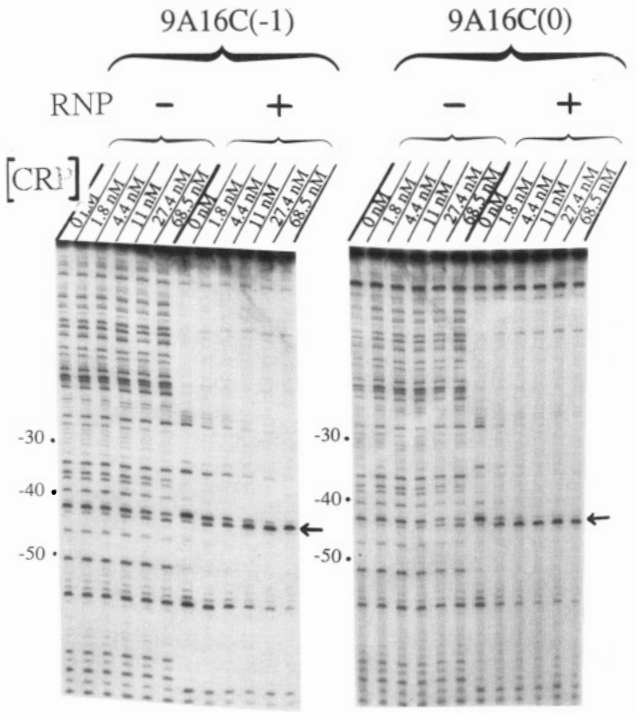


FIGURE 3: Positive interaction between CRP and RNA polymerase revealed by DNase I footprinting on the two 9A16C fragments. The 9A16C(0) and 9A16C(–) fragments were labeled on their lower strand at the *Eco*RI site and incubated at 37 °C, in the presence of cAMP (200 μM) and various concentrations of CRP and in the presence or in the absence of RNP (as indicated by the "–" and "+" symbols). The complexes were then treated with DNase I (Spassky et al., 1984), and the digestion products were separated on an 8% sequencing gel and then visualized by autoradiography. The arrows indicate phosphodiester bonds (–37.5) on the 9A16C(0) fragment and (–36.5) on the 9A16C(–1) fragment which hyperreact to DNase I specifically in the presence of CRP on the DNA binding site.

formed on the 9A16C(0) and 9A16C(–1) fragments, in the presence and in the absence of CRP, was therefore monitored by this technique (see Figure 2). The reactions always go to completion, but the rates of attack depend on the *Hin*fI concentrations. Formation of an open complex in the absence of CRP slows down equally *Hin*fI cutting at both promoters. CRP enhances markedly the local stability of the open complex formed on the 9A16C(0) fragment but had no effect on the complex formed on 9A16C(–1). Therefore, the location of the CRP binding site on 9a16C(–1) leads to the loss of a stabilizing interaction around position –60 within the open complex.

(*4*) *Positive Interaction between CRP and RNA Polymerase Bound to the Two Fragments.* A more quantitative determination of the synergy existing during the binding of CRP and RNA polymerase on a given promoter fragment can be obtained by measuring the change in affinity of one of the partners when the other is added [as previously reported in Spassky et al. (1984), Ponnambalam et al. (1987) and Li and Krakow (1988) and more extensively in Ren et al. (1988)]. DNase I quantitative footprinting was used for this purpose (see Figure 3). In the absence of RNA polymerase, the affinity of CRP for its DNA site is identical on the two fragments ($K_d \approx 5.5 \times 10^{-8}$ M). In the presence of RNA polymerase, this affinity is increased on the two fragments but not equally. CRP has at least a 3-fold higher affinity for its DNA site in the presence of RNA polymerase on the 9A16C(0) fragment ($K_d \leq 10^{-9}$ M) than on the 9A16C(–1) fragment ($K_d \approx 3.5 \times 10^{-9}$ M). Therefore, the presence of RNA polymerase on the modified *gal* P1 promoter stabilizes CRP binding by a factor higher than 55 when the CRP to (–10) distance is 29 bp as in wild type but by only a factor of 16 when this distance is 1 bp shortened. Consequently, the decrease in synergy, already detected in the kinetic experiments, is also apparent in this equilibrium determination.

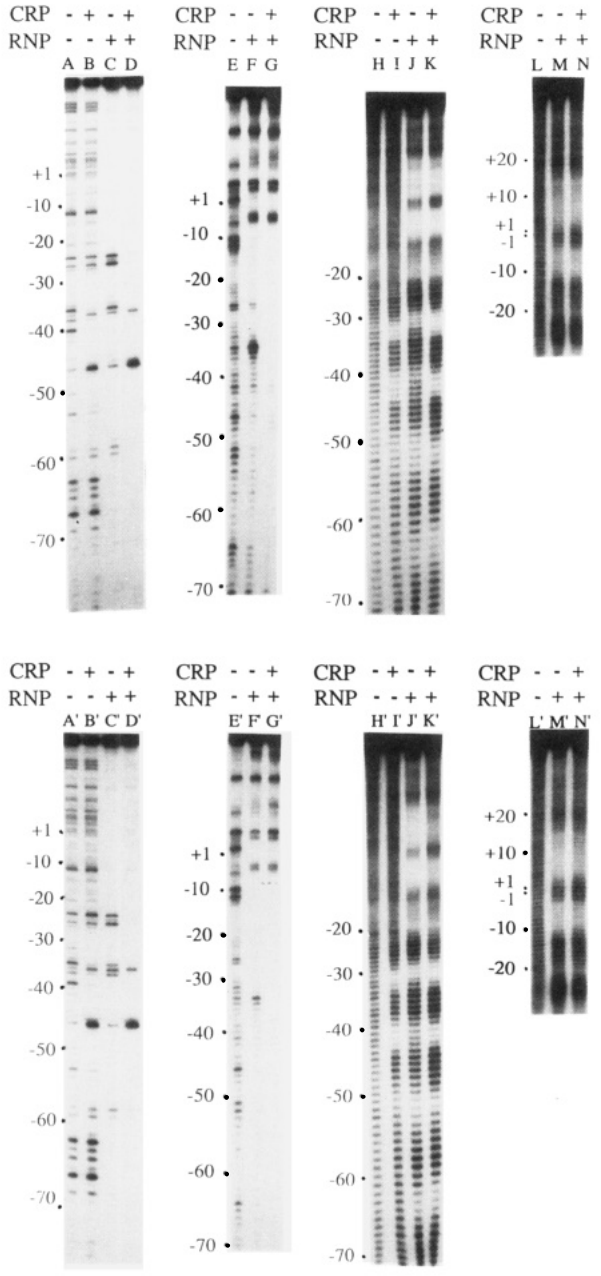
In the kinetic experiments, the overall differential effect exerted by the deletion on CRP activation corresponded to a reduction by a factor of 5 (an activation factor of 1.5 in one case versus 0.3 in the other case). In the equilibrium determinations, the decrease in synergy is higher than 3. We conclude that although CRP still stabilizes the ternary complex on 9A16C(–1), the overall changes in synergy observed in the two types of experiments are compatible.

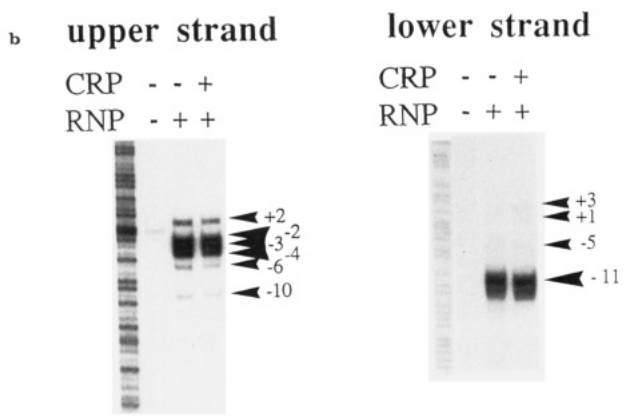
(*5*) *Comparison of the Open Complexes Formed on the Two Fragments.* Different DNA footprinting techniques were employed to characterize the RNA polymerase complexes formed on the two fragments. Figure 4 shows some of the results obtained from DNase I, $(OP)_2Cu^+$, and hydroxyl radical footprints performed on the lower strand (main results summarized in Figure 5). Protections and hyperreactivities are indicated by boxes and arrows, respectively. Open symbols correspond to the normally spaced promoter while shaded symbols refer to the –1 deletion located at position –30. Consequently, the sequences are aligned downstream of the deletion and shifted by 1 bp upstream of this point.

(*a*) *Footprints of Binary Complexes.* Experiments performed with DNase I, hydroxyl radicals, and $(OP)_2Cu^+$ clearly demonstrate that the binary complex formed by RNA polymerase on both 9A16C fragments extends up to position –65 (Figure 5). In order to rule out the hypothesis of nonspecific binding of a second RNA polymerase at the *Eco*RI end, we also performed DNase I and hydroxyl radical analysis on the equivalent *Eco*RI–*Hin*dIII *gal* fragment, where the only functional promoter is *gal* P2, due to a mutation at position –14 of the *gal* P1 promoter (Busby et al., 1984, 1987). In this case, the upstream boundary of the RNA polymerase protection is located around position –45 (data not shown). Protection at –65 is therefore a result of RNA polymerase binding at *gal* P1.

Downstream of the 1 bp deletion, the patterns due to DNase I, hydroxyl radical, $(OP)_2Cu^+$, and $KMnO_4$ attacks are identical on the two fragments. As an example, $KMnO_4$, which reacts with single-stranded regions of DNA, results in a similar set of reactive bands for complexes formed on both fragments (results summarized in Figure 5). Upstream of the 1 bp deletion, we have focused our attention on results obtained by hydroxyl radical footprinting, a technique largely independent of the sequence, which can therefore be used to study the consequence of the deletion on RNA polymerase positioning at the *gal* P1 promoter. From position –30 to position –55, the bases protected or enhanced against hydroxyl radical attacks are located at the same distance from the transcriptional start site. On the other hand, above position –55, the hydroxyl radical footprint is shifted by 1 bp, implying that RNA polymerase recognizes the same bases of the two fragments in this upstream region.

In summary, the shortening of the fragment by 1 bp at position –30 has no effect on the structure of the binary open complex in the region downstream of the deletion including the transcriptional bubble. Furthermore, the open complexes formed by RNA polymerase in the absence or CRP recognize the CRP binding site region in a spacing-dependent manner (relative to the transcriptional start site and therefore the –10 region) and the upstream region site in a sequence-dependent manner.





(*b*) *Footprints of CRP.* CRP binding by itself produces identical changes in the reactivities of both fragments: the sites of maximal protection against hydroxyl radicals and the positions of enhanced DNase I bands are out of phase by 5 bp [as already observed by Shanblatt and Revzin (1987)]. This observation is consistent with the idea that DNase I recognizes preferentially minor grooves located on the outside of the nucleoprotein complex while the narrower minor grooves on the inside are less accessible to hydroxyl radical attack.

Furthermore, the DNase I and hydroxyl radical attacks clearly show that, around the CRP binding site, the CRP and RNP footprints on DNA are overlapping. Interestingly, this overlap is not identical on the two 9A16C fragments. On the 9A16C(0) fragment, with a wild-type CRP to (–10) spacing, the minima of hydroxyl radical reactivity occur 1 bp higher in the CRP footprint than in the RNA polymerase footprint. Otherwise, on the 9A16C(–1) fragment, the CRP binding site is 1 bp closer to position +1 and the minima in the footprints due to either CRP or RNA polymerase are now located at identical positions.

(*c*) *Footprints of Ternary Complexes.* On the two 9A16C fragments, CRP does not modify the patterns observed in the binary RNA polymerase–promoter complex downstream of position –20 (in particular, the pattern due to $KMnO_4$ is identical in all cases).

Upstream of position –20, the pattern of chemical and nucleolytic reactivities reveals interesting features when ternary complexes are compared to the corresponding binary complexes. On both fragments, we observed at least three changes due to the presence of CRP in the open complexes: a loss of hyperreactivity to $(OP)_2Cu^+$ in the –35 region, a change in the DNase I hypersensitivity to the pattern expected for CRP binding, and finally, a 1 bp shift in the downstream direction of the protection due to RNA polymerase in the far upstream region. Furthermore, it is always the footprint due to CRP which is observed in the region corresponding to its binding site. As noted above, the hydroxyl radical footprints due to CRP or RNA polymerase alone, in this region, are in phase on the 9A16C(–1) fragment and out of phase by 1 bp on the 9A16C(0) fragment. Hydroxyl radical footprints of ternary complexes are consistent with this observation. Protection and enhanced cleavages due to CRP and RNA polymerase are in phase and reinforce each other on the 9A16C(–1) fragment whereas the footprint of the open complex in the CRP binding site region is displaced by 1 bp upstream when CRP is present on the 9A16C(0) fragment.

Furthermore, a subtle difference appears in the –30 region. On the upper strand, polymerase alone protects three deoxyriboses centered at –28 on both fragments. CRP also protects

FIGURE 4: Footprint analysis of the open complexes formed at 37 °C by CRP, RNP, and RNP + CRP, on the two 9A16C fragments. (a) The 9A16C(0) and 9A16C(–1) fragments were labeled on their lower strand at the *Eco*RI site and incubated at 37 °C, in the presence of cAMP (200 μM) and various combinations of CRP (100 nM) and RNP (100 nM), as indicated by the "–" and "+" symbols. The complexes were then treated with DNase I [A → D for 9A16C(0) and A′ → D′ for 9A16C(–1)] (Spassky et al., 1984), $(OP)_2Cu^+$ [E → G for (A16C(0) and E′ → G′ for 9A16C(–1)] (Sigman et al., 1985), and hydroxyl radicals [H → N for 9A16C(0) and H′ → N′ for 9A16C(–1)] (Tullius & Dombroski, 1986. Finally, the digestion products were separated on 8% sequencing gels and visualized by autoradiography. (b) Labeled 9A16C(0) fragments (upper and lower strand at the *Eco*RI site) were incubated with CRP and RNA polymerase as in (a) and attacked with $KMnO_4$ (Sasse-Dwight & Gralla, 1989). The attack products were then treated to piperidine, separated on 8% sequencing gels, and visualized by autoradiography.

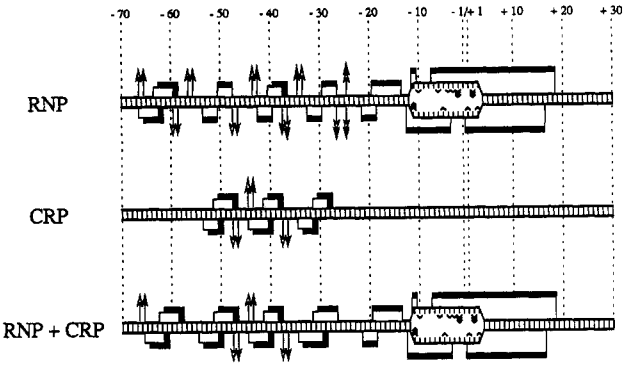


FIGURE 5: Summary of the protection and enhancement patterns seen on the two 9A16C fragments in the presence of CRP, RNP, and RNP + CRP at 37 °C. This figure summarizes data obtained from DNase I, hydroxyl radicals, and $KMnO_4$ probing on the 9A16C(0) and 9A16C(−1) fragments. DNA is represented by a horizontal ladder in which the bars correspond to the base pairs. Thymines modified by $KMnO_4$ are indicated by carets, phosphodiester bonds hyperreactive to DNase I by arrows, and bases protected from hydroxyl radical cleavage by caret boxes. When the arrowheads or the boxes are open, the symbols correspond to reactivities observed on the 9A16C(0) fragment, and when they are shaded, to reactivities observed on the 9A16C(−1) fragment.

three positions centered respectively at −30 for 9A16C(0) and at −29 for 9A16C(−1) so that the enzyme and the activator appear to have their maximal protections displaced by an angle of 72° for 9A16C(0) and only 36° for 9A16C(−1). Some steric hindrance between the binding of CRP and polymerase might result, especially for the (−1) mutant where the footprints of the two proteins overlap over two positions. Indeed, the −29 position of the lower strand of the (−1) mutant, which was not protected by CRP or RNA polymerase alone, is now protected by both proteins, suggesting that CRP has pushed polymerase 1 bp further downstream, in this region.

In summary, the most significant difference observed between the footprints of the complexes formed on the two fragments (plus or minus CRP) remains that CRP shifts the positioning of the open complex only on the promoter having the wild-type spacing and not on the promoter having a 1 bp reduced spacing. This 1 bp shift also results in a more regular periodicity, consistent with a B-DNA structure for the nucleotides involved in the ternary complex in the region of the CRP binding size and above this site.

(6) In Vitro Footprints of Low-Temperature Complexes. In order to try to correlate the kinetic parameters measured previously with differences between DNA binding patterns monitored by footprinting, we repeated the footprints at 4, 16, and 27 °C. A set of them is given in Figure 6, with a summary in Figure 7.

At 4 °C, RNA polymerase footprints could be observed on both fragments, but they were much clearer in the presence of CRP. In all cases, DNA remains double-stranded in the complexes and RNA polymerase protects only an upstream region, from position −11 to position −65 (as judged from the footprints observed on the lower strand). Furthermore, those protections are strikingly similar to the ones observed in the same region by the ternary complexes formed at 37 °C [using $(OP)_2Cu^+$ and DNase I as well as hydroxyl radicals as probing reagents].

At 16 °C, in the absence of CRP, the binary complexes formed between the two fragments and RNA polymerase are stable enough to be probed by footprinting techniques. The patterns are very similar to those observed at 37 °C, although two differences can be pointed out. First, RNA polymerase alone still opens the transcriptional bubble as judged from $KMnO_4$ attack but gives less protection to the region downstream of position −11 against DNase I and hydroxyl radical attack. This difference can be understood if the footprint observed is in fact a mixture of the footprints of open and 4 °C complexes, suggesting a dynamic equilibrium between the two complex forms. Second, base −15 on the lower strand is hyperreactive to $(OP)_2Cu^+$. This observation suggests the existence at this temperature of a stress in the −10 to −20 region of the *gal* P1 promoter, in the absence of CRP.

At 16 °C, prior addition of CRP drastically and specifically affects patterns observed on 9A16C(−1). The ternary complex formed on the 9A16C(0) *gal* fragment at 16 °C is similar to the ones formed at 27 or 37 °C, as judged by the reactivity patterns obtained with the various reagents. By contrast, on the 9A16C(−1) fragment, the complex formed by RNA polymerase and CRP is characterized by the complete absence of a transcriptional bubble, the reactivity to $(OP)_2Cu^+$ or bp −18 on the lower strand, and only a partial protection against DNase I and hydroxyl radical attacks, which extend downstream only as far as position −11 and −20, respectively.

Thus, at 16 °C, RNA polymerase is able to form an open complex both in the presence and in the absence of CRP, on 9A16C(0) where the CRP binding site is located at the wild-type distance from the −10 region of the *gal* P1 promoter. An open complex is also formed if RNA polymerase alone is bound to the fragment shortened by 1 bp. But in this case, prior binding of CRP to a site located 1 bp closer to the −10 region precludes open complex formation and positions RNA polymerase in an intermediate type of complex very similar to the complex characterized at 4 °C but with a DNA distortion at the downstream limit of this protection [as revealed by $(OP)_2Cu^+$ reactivity].

Finally, at 27 °C the complexes are very similar to those obtained at 37 °C; a single-stranded region was created in all four cases examined. Furthermore, it was verified at each temperature by runoff assays that $KMnO_4$ reactivity is associated with the presence of an active complex. Consequently, the binary complexes formed on both fragments and the ternary complex formed on the 9A16C(0) fragment can initiate transcription below 16 °C while the ternary complex formed on the 9A16C(−1) fragment can initiate only above 16 °C.

DISCUSSION

By PCR mutagenesis a double mutant of the *gal* control region has been constructed, making it suitable for accurate kinetic and structural studies of the process of initiation of transcription at *gal* P1 and of its activation by CRP. This mutant promoter is fairly strong in vivo and in vitro, and its activity is still increased by CRP. A similar activation factor was measured in vitro on a linear template and in vivo in β-galactosidase activity of a *gal–lacZ* fusion. With respect to the parent promoter, *gal* 16C, the G to A change at position −9 of the Pribnow box increases both K_B and k_f, the two parameters which define the rate of open complex formation in the classical two-step model. Activation by CRP affects mainly k_f as in the case of *gal* 16C and *gal* wild type [see Lavigne et al. (1992) and for different results, Goodrich and McClure (1992)]. However, the extent of activation, in vivo and in vitro, is smaller, a result which indicates that the efficiency of a positive regulator is strongly dependent of the strength of the regulated promoter [for a more striking example of this rule, see the case of OmpR protein which can either stimulate or repress transcription at *ompF* gene depending on

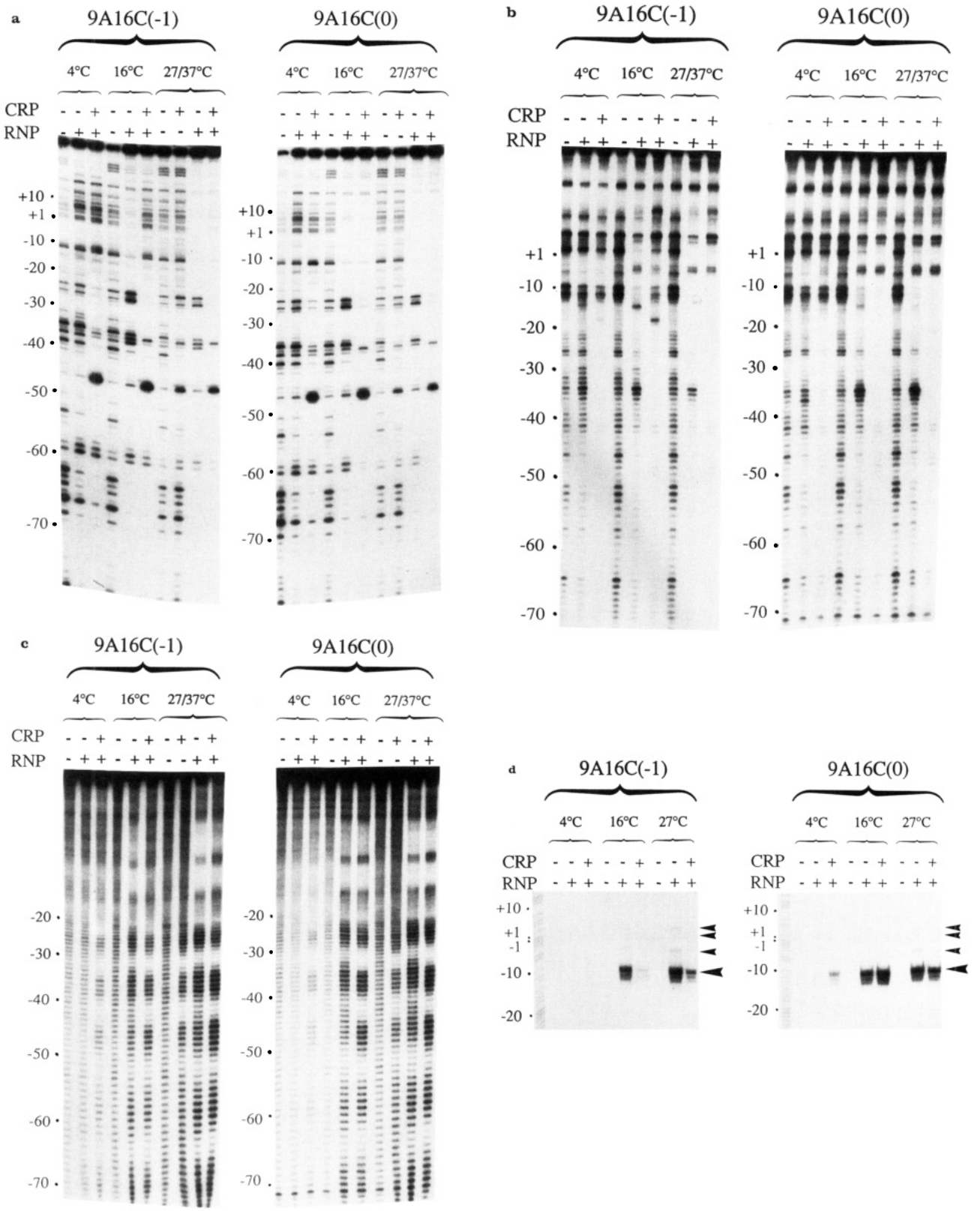


FIGURE 6: Footprint analysis of the complexes formed by RNP at 4, 16, and 27/37 °C in the absence and in the presence of CRP. The 9A16C(0) and 9A16C(–1) fragments were labeled on the lower strand at the *Eco*RI site and incubated 30 min at 27, 16, and 4 °C, in the presence of cAMP (200 μM) and various combinations of CRP (100 nM) and RNP (100 nM), as indicated by the "–" and "+" symbols. The complexes were then treated with DNase I(a), (Spassky et al., 1984), $(OP)_2Cu^+$ (b) (Sigman et al., 1985), hydroxyl radicals (c) (Tullius & Dombroski, 1986), and $KMnO_4$ (d) (Sasse-Dwight & Gralla, 1989). Finally, the digestion products were separated on an 8% sequencing gel and visualized by autoradiography.

the basal level of transcription at this promoter (Tsung et al., 1990)]. With this promoter we have constructed the positioning of RNA polymerase at *gal* P1 can be conveniently studied both in the absence and in the presence of CRP.

Promoters where the CRP site is centered near position –41 lack any consensus –35 hexamer. It was therefore important to delineate the role of the nonconsensus region located upstream of position –35 in the process of promoter recognition.

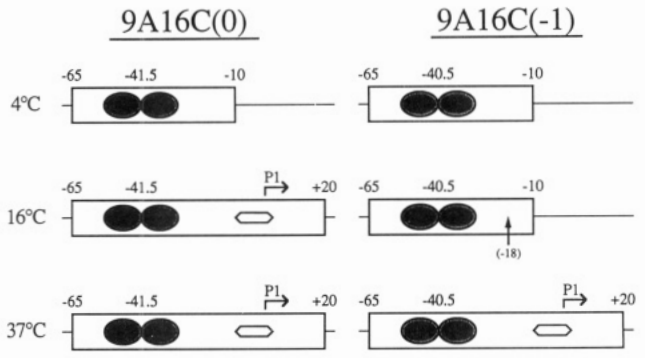


FIGURE 7: Global shape of the ternary complexes formed at 4, 16, and 37 °C on the two 9A16C fragments. The rectangles define the promoter region protected by CRP + RNP, and the two ellipses represent the presence of the CRP dimer on its site. Transcription initiation efficiency at *gal* P1 is represented by a horizontal arrow and the opening of the transcriptional bubble by a hexagon. A vertical arrow indicates the hyperreactivity to $(OP)_2Cu^+$ of the base at position −18 on the lower strand, obtained on the 9A16C fragment at 16 °C.

The comparison of two promoters (0) and (−1) where the sequence was shifted by 1 bp above position −30 was found to be very informative, both in the absence and in the presence of CRP.

In the absence of CRP, the two promoters are of equal strength (same K_B and k_f values). All the other parameters which have been measured (stability of the open complex, rate of dissociation) are also identical. Therefore, the shift in the sequence upstream of position −30 makes no significant difference in all the processes studied, implying that sequence specificity for promoter recognition above this point is negligible. However, this upstream region is the one recognized by the enzyme at low temperature, and many previous studies performed on the *gal* P1 promoter suggested the involvement of this region in the process of initiation of transcription at this promoter (Busby et al., 1983, 1987). In particular, Chan et al. (1990) have recently shown that open complexes formed on promoters with a consensus-like −35 box (like *gal* P2) protect DNA only up to position −45 while protection can extend far upstream on promoters without any recognizable −35 box (like *gal* P1). The footprints of the present open complexes extend up to position −65. Comparison of the footprints obtained with our two promoters indicates a similar positioning of RNA polymerase and a similar distortion of the DNA around the transcriptional start point. Between positions −10 and −55, the hydroxyl radical footprints indicate the same regular protection, with a helical periodicity between the protected regions independent of the nature of the sequence. Only in the far upstream region (above bp −55), we noticed a similar positioning of RNA polymerase with respect to the common sequence of the two promoters (and no longer with respect to the distance to the start site). These observations suggest a rather high flexibility of the DNA above −30 in these open complexes.

These observations (extended and phased footprints) are consistent with the idea that the DNA is partially wrapped around RNA polymerase [as already suggested for the *lac* UV5 promoter by Amouyal and Buc (1987)]. RNA polymerase was also shown to bend the DNA in the open complex formed on another *gal* P1 promoter mutant (Kuhnke et al., 1989). In those promoters devoid of −35 sequences, bending and wrapping would essentially put in register the −50, −60, and −10 regions in a manner reminiscent of the phasing requirement of the −35 and −10 regions in a classical promoter.

In the presence of CRP, the strength of the two 9A16C promoters is now very different. We attribute these significant effects to the change in spacing between the center of the CRP site and the promoter start because, in the absence of CRP, the shift of the same sequence elements by 1 bp downward had a negligible effect. CRP turns out to be a moderate activator on the promoter having a normal CRP to −10 spacing and a slight inhibitor on its (−1) variant. By themselves the footprints of the corresponding ternary complexes on the two promoters do not yield any clue about this difference. The presence of CRP does not extensively change the borders of the footprint nor the overall periodicity of the hydroxyl radical footprints, although it does make them more regular. In the ternary complex, the footprint made by RNA polymerase in the −60 region is moved 1 bp downstream. After this relocation by CRP of the upstream contacts, the regions protected by the activator and by RNA polymerase around −60 are now separated by an integral number of turns of B-DNA helix.

However, the energetic *cost* of establishing these similar structures on the two promoters is clearly different. There is a significant decrease in the synergy of binding of the two proteins when the CRP to −10 spacing is reduced. Also, protection of the restriction site at −60 against cleavage is very much enhanced when the spacing is correct and when CRP and RNA polymerase are present. From the comparison between the footprints of the two ternary complexes, there is no evidence for a local change in the disposition of the two proteins in the vicinity of the restriction site. We assume therefore that it is the *connection* between the upstream and the downstream regions of the promoter which is hampered when the CRP to −10 distance is shortened. Activation by CRP and synergy in CRP–RNA polymerase binding appear to be linked together. It should be remembered that CRP bends the DNA (Wu & Crothers, 1984; Schultz et al., 1991) and its hydroxyl radical footprint shows that it is located on the same face of the DNA as RNA polymerase. Our results therefore suggest that CRP helps open complex formation by correctly orienting the regions located downstream and upstream of its binding site.

The most clearcut effect of the change in CRP to −10 spacing is a 12-fold decrease in the rate of isomerization between the closed to the open complex in the presence of CRP. Open complex formation is a rather fast process at 37 °C precluding, for the time being, kinetic footprinting assays. The structure and activity of the complexes formed at low temperature were therefore investigated, assuming, as done previously by various authors, that low-temperature complexes are analogous to the sequential kinetic intermediates in open complex formation.

At 4 °C, in both the absence and the presence of CRP, RNA polymerase forms an inactive complex on the two 9A16C fragments which protects the DNA from −65 to −11 positions. If this 4 °C complex is representative of a closed complex, then RNA polymerase must recognize the *gal* P1 promoter by its upstream sequences. It has been noticed previously, on other promoters, that, at 4 °C, RNA polymerase protects an upstream part of these promoters (Hofer et al., 1985; Kovacic, 1987; Cowing et al., 1989; Schickor et al., 1990; Mecsas et al., 1991). However, in general, the −10 region was totally included in the 4 °C protected region [called recognition domain in Schickor et al. (1990)]. On the *gal* P1 promoter, the downstream border of the 4 °C complex is at position −11 (lower strand). Furthermore, we observed that an increase in temperature to 12 °C is sufficient to make RNA polymerase alone extend its positioning downstream and open the transcriptional bubble (data not shown). This behavior, already observed on the *gal* p19T mutant (Grimes et al., 1991),

indicates that the single-stranded region forms very easily in the *gal* P1 promoter family.

At 16 °C, in the absence of CRP, RNA polymerase can form on both fragments a complex which is active and produces a footprint similar to the open complex one. However, at the same temperature, RNA polymerase can initiate transcription in the absence of CRP but not in the presence of CRP on the (−1) mutant promoter. This inhibition of the formation of an active complex is correlated with at least two changes in the ternary complex footprint, compared to that characterized on the same fragment at 37 °C. First, the footprint is less extended in its downstream part, reaching position −11. Second, base −18 on the lower strand becomes hyperreactive to $(OP)_2Cu^+$. We think that these clearcut differences observed at 16 °C are a reflection of the repressing effect of CRP which manifests itself at 37 °C. Furthermore, there is a perfect correlation between the capacity to form an open complex at 16 °C and the production of an extended footprint covering the downstream region.

This study of low-temperature complexes leads us to present a speculative model of the formation of an open complex at the *gal* P1 promoter and of its modulation by CRP (cf. Figure 7). We propose that RNA polymerase initially recognizes the *gal* P1 promoter upstream of the −10 region. It becomes capable of initiating transcription at the same time as the protection extends to the −10 to +20 region. The opening of the transcriptional bubble is probably not the limiting step of this process since it always occurs as soon as RNA polymerase protects the downstream region. Interaction of RNA polymerase with the −10 to +20 region, as monitored by the extension of the footprint to this region, is therefore likely to be the limiting step for open complex formation on this promoter and the target of CRP regulation. Activation occurs only if CRP is correctly oriented (i.e., as on the wild-type sequence). With regard to the enzymatic machinery, a 1 bp shift of the CRP binding site, i.e., a 34 °C phase shift, is enough to disturb the protection of the downstream sequences. Finally, although the open ternary complex can accommodate the 1 bp shift of the CRP location, there is a loss of positive interactions between CRP and RNA polymerase as shown by all the experiments performed at 37 °C and discussed above.

The difficult coupling between the upstream and the downstream contacts which has to be made by RNA polymerase on the (−1) fragment in the presence of CRP leads at 16 °C to an inability to create the downstream contact (and hence to open the bubble) and at 37 °C, when the single-stranded region is created, to a weakening of the upstream contact of RNA polymerase around −60. These considerations illustrate the delicate balance of forces existing in the activation process at the *gal* P1 promoter with a normal spacing and the precise fit which should result. Furthermore, thermodynamic values reported in this study show that the events involved in this enzymatic process have small energy barriers and therefore probably require only minor modifications to both DNA and enzyme structures.

It is interesting to compare how important the locations of the CRP binding site and of the −35 hexamer are for the regulation of the initiation of transcription. The length and the structure of the −35 to −10 spacer were shown to be important for an efficient open complex formation (Stefan & Gralla, 1982; Auble et al., 1986; Auble & deHaseth, 1988; Ayers et al., 1989; Martello et al., 1989; Werel et al., 1991). Most of the alterations to the −35 to −10 spacer (gap, deletion, insertion, substitution by particular sequences, or drug binding) affected the initial binding step (K_B constant), and many of these authors concluded that the relative orientation of the −10 and −35 elements is a key element in the initial binding of RNA polymerase which produces the closed complex. More recently, an underwinding of the −35 and −10 spacer by about 33° was shown to be crucial for positive control of transcription by Hg-MerR (Ansari et al., 1992).

If a CRP site at −41.5 replaces a −35 consensus hexamer, we have shown that similar considerations apply with two significant differences only. CRP helps to put in register the domain of RNA polymerase creating the single-stranded region with a domain of interaction located far upstream, and this event, with this type of promoter fragment, occurs during the isomerization step rather than during the formation of the closed complex.

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REFERENCES

Aiba, H., Hanamura, A., & Tobe, T. (1989) *Gene 85*, 91–97.
Amouyal, M., & Buc, H. (1987) *J. Mol. Biol. 1985*, 795–808.
Ansari, A. Z., Chael, M. L., & O'Halloran, T. V. (1992) *Nature 355*, 87–89.
Auble, D. T., & deHaseth, P. L. (1988) *J. Mol. Biol. 202*, 471–482.
Auble, D. T., Allen, T. L., & deHaseth, P. L. (1986) *J. Biol. Chem. 261*, 11202–11206.
Ayers, D. G., Auble, D. T., & deHaseth, P. L. (1989) *J. Mol. Biol. 207*, 749–756.
Bell, A., Gaston, K., Williams, R., Kolb, A., Buc, H., Minchin, S., Williams, J., & Busby, S. (1990) *Nucleic Acids Res. 18*, 7243–7250.
Buc, H., Amouyal, M., Buckle, M., Herbert, M., Kolb, A., Kotlarz, D., Menendez, M., Rimsky, S., Spassky, A., & Yeramian, E. (1987) *RNA polymerase and the regulation of transcription* (Reznikoff, W. S., et al., Eds.) pp 115–125, Elsevier Science Publishing Co., Inc., New York.
Burgess, R. R., & Jendrisak, J. J. (1975) *Biochemistry 14*, 4634–4638.
Busby, S., Aiba, H., & de Crombrugghe, B. (1982) *J. Mol. Biol. 154*, 211–227.
Busby, S., Kotlarz, D., & Buc, H. (1983) *J. Mol. Biol. 167*, 259–274.
Busby, S., Truelle, N., Spassky, A., Dreyfus, M., & Buc, H. (1984) *Gene 28*, 201–209.
Busby, S., Spassky, A., & Chan, B. (1987) *Gene 53*, 145–152.
Chan, B., Spassky, A., & Busby, S. (1990) *Biochem. J. 270*, 141–148.
Collado-Vides, J., Magasanik, B., & Gralla, J. D. (1991) *Microbiol. Rev. 55*, 371–394.
Cowing, D. W., Mecsas, J., Record, M. T., & Gross, C. (1989) *J. Mol. Biol. 210*, 521–530.
deCrombrugghe, B., Busby, S., & Buc, H. (1984) *Science 224*, 831–838.
Gaston, K., Bell, A., Kolb, A., Buc, H., Busby, S. (1990) *Cell 62*, 733–743.
Ghosaini, L. R., Brown, A. M., & Sturtevant, J. M. (1988) *Biochemistry 27*, 5257–5261.
Goodrich, J. A., & McClure, W. R. (1992) *J. Mol. Biol. 224*, 15–29.
Grimes, E., Busby, S., & Minchin, S. (1991) *Nucleic Acids Res. 19*, 6113–6118.
Gyllenstein, U. B., & Erlich, H. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7652–7656.
Higuchi, R., Krummel, B., & Saiki, R. K. (1988) *Nucleic Acids Res. 16*, 7351–7367.

Hofer, B., Müller, D., & Köster, H. (1985) *Nucleic Acids Res. 13*, 5995–6013.
Igarashi, K., & Ishihama, A. (1991) *Cell 65*, 1015–1022.
Igarashi, K., Hanamura, A., Makino, K., Aiba, H., Aiba, H., Mizuno, T., Nakata, A., & Ishihama, A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8958–8962.
Kovacic, R. T. (1987) *J. Biol. Chem. 262*, 13654–13661.
Kuhnke, G., Krause, A., Heibach, C., Gieske, U., Fritz, H., & Ehring, R. (1986) *EMBO J. 5*, 167–173.
Kuhnke, G., Theres, C., Fritz, H., & Ehring, R. (1989) *EMBO J. 8*, 1247–1255.
Lavigne, M., Herbert, M., Kolb, A., & Buc, H. (1992) *J. Mol. Biol. 224*, 293–306.
Li, X., & Krakow, J. S. (1988) *J. Biol. Chem. 263*, 3448–3453.
Lowe, P. A., Hager, D. A., & Burgess, R. R. (1979) *Biochemistry 18*, 1344–1352.
Martello, P. A., Bruzik, J. P., deHaseth, P., Youngquist, R. S., & Dervan, P. B. (1989) *Biochemistry 28*, 4455–4461.
McClure, W. R. (1985) *Annu. Rev. Biochem. 54*, 171–204.
McClure, W. R., Cech, C. L., & Johnston, D. E. (1978) *J. Biol. Chem. 253*, 8941–8948.
Mecsas, J., Cowing, D., & Gross, C. A. (1991) *J. Mol. Biol. 220*, 585–597.
Miller, J. H. (1972) *Experiments in molecular genetics*, pp 352–355, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
O'Callaghan, C. H., Morris, A., Kirby, S. M., & Shingler, A. H. (1972) *Antimicrob. Agents Chemother. 1*, 283–288.
Ponnambalam, S., Spassky, A., & Busby, S. (1987) *FEBS Lett. 129*, 189–196.
Ren, Y. L., Garges, S., Adhya, S., & Krakow, J. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4138–4142.
Sasse-Dwight, S., & Gralla, J. D. (1989) *J. Biol. Chem. 264*, 8074–8081.
Schickor, P., Metzger, W., Werel, W., Lederer, H., & Heumann, H. (1990) *EMBO J. 9*, 2215–2220.
Schultz, S. C., Shields, G. C., & Steitz, T. A. (1991) *Science 253*, 1001–1007.
Shanblatt, S. H., & Revzin, A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1594–1598.
Shanblatt, S. H., & Revzin, A. (1987) *J. Biol. Chem. 24*, 11422–11427.
Sigman, D. S., Spassky, A., Rimsky, S., & Buc, H. (1985) *Biopolymers 24*, 183–197.
Spassky, A., Busby, S., & Buc, H. (1984) *EMBO J. 3*, 43–50.
Stefano, J. E., & Gralla, J. D. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1069–1072.
Tsung, K., Brissette, R. E., & Inouye, M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5940–5944.
Tullius, T. D., & Dombroski, B. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5469–5473.
Ushida, C., & Aiba, H. (1990) *Nucleic Acids Res. 18*, 6325–6330.
Valentin-Hansen, P., Holst, B., Sogaard-Andersen, L., Martinussen, J., Nesvera, J., & Douthwaite, S. R. (1991) *Mol. Biol. 5*, 433–437.
Wang, J. C., & Giaever, G. N. (1988) *Science 240*, 300–304.
Werel, W., Schickor, P., & Heumann, H. (1991) *EMBO J. 10*, 2589–2594.
Wu, H., & Crothers, D. M. (1984) *Nature 308*, 509–513.

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