The EMBO Journal vol.6 no.13 pp.4227–4234, 1987

A new target for CRP action at the *malT* promoter

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Communicated by H.Buc

In *Escherichia coli*, the transcription of the *malT* gene is activated by the complex formed between cAMP and its receptor protein, CRP. Kinetics of formation of polyribonucleotide products from the corresponding promoter were studied *in vitro* by two sets of techniques, abortive initiation assays and run-off experiments. The first type of assay indicated that open complexes were formed at *malT* with an equivalent efficiency, and at comparable rates, whether CRP–cAMP was present or not. Secondary effects due to the activating complex were observed (increased stability of the open complex, elimination of a weaker binding site for the enzyme, improved Michaelis constants of RNA polymerase for the substrates of the assay, UTP in particular). But, primarily, CRP–cAMP did not exert a significant role in the rate of formation of the initiation complex. In contrast, run-off assays showed that the yield of the full-length transcripts was markedly enhanced by prior incubation of the DNA fragment with CRP– cAMP. Both in the presence and in the absence of activator, the rate-limiting step for this process was markedly slower than the formation of the initial open complex. Short oligonucleotides ($n < 9$), probably arising from a recycling process, were found when the initiation complex was formed in the absence of CRP–cAMP. They were abolished by prior incubation with the activator. Unexpectedly, CRP–cAMP appears to favour the escape of RNA polymerase from the initiation complex at this promoter.

Key words: cAMP–CRP complex/activation of transcription

Introduction

The mechanism by which the complex formed between cAMP and its receptor protein (CRP or CAP) activates initiation of transcription in *Escherichia coli* is still unknown. It is particularly intriguing that the distance between the centre of the main DNA binding site for the cAMP–CRP complex is located at various distances from the initiation start point at different promoters, even in cases where no protein other than the enzyme and the activating complex is known to participate in the nucleic-acid–protein assembly (for a review see de Combrugghe *et al.* 1984). Such is the case for the *lac* control region, the *gal* control region and the *malT* promoter where those distances are 61.5, 41.5 and 70.5 bp, respectively. In the first two cases, extensive kinetic studies have been performed *in vitro* (Malan *et al.*, 1984; Herbert *et al.*, 1986). The most conclusive data emerged from the analysis of promoter mutants, where competing sites for RNA polymerase have been eliminated. In such cases, the kinetics of open complex formation could be analysed according to a simple scheme where a single active complex RP_o formed between RNA polymerase, R and promoter, P, could initiate transcription in the presence as well as in the absence of the CRP–cAMP complex.

$$R + P \underset{K_B}{\rightleftharpoons} RP_c \overset{k_f}{\underset{k_r}{\rightleftharpoons}} RP_o$$

In both cases, it was shown that prior addition of the activating complex increased the rate of open complex formation at low but not at high RNA polymerase concentration, implying that it was the formation of the closed intermediate RP_c which increased (Malan *et al.*, 1984; Buc *et al.*, 1987; M.Herbert *et al.*, in preparation). At the *malT* promoter, it is known that the binding of CRP favours the subsequent specific binding of RNA polymerase. In addition, single-round transcription experiments, performed at 100 mM KCl concentration, indicated that synthesis of full-length transcripts displayed an absolute requirement for CRP at all RNA polymerase concentrations tested (Chapon and Kolb, 1983). We therefore decided to investigate the kinetics of these events by abortive initiation assays and by run-off experiments, under the conditions previously used to study the *lac* and the *gal* promoters.

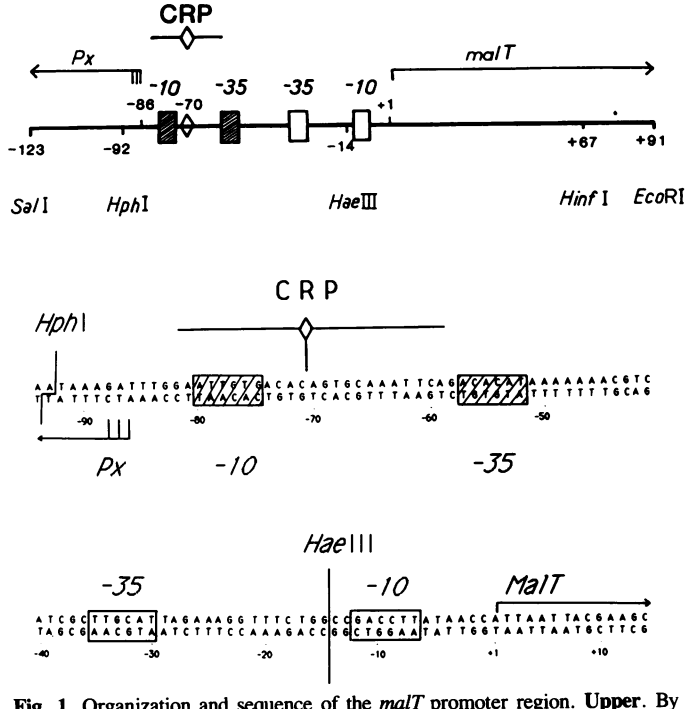

Fig. 1. Organization and sequence of the *malT* promoter region. **Upper.** By convention, position +1 refers to the initiation of the *malT* mRNA. Transcripts from the *malT* and *Px* promoters are indicated by arrows. Bare or hatched boxes show the positions of the '−10' and '−35' regions of the two promoters. The CRP site whose centre is indicated by a diamond is overlined. **Lower.** The sequence of the *malT* promoter from −94 to +14 as determined by Débarbouillé *et al.* (1982) is shown.

Table I. Activities of the *malT* and *Px* promoters

	MalT		*Px*			
	In the presence of *Px*	Alone	In the presence of *malT*		Alone	
Template	214 bp	214 bp cut by *Hph*I	214 bp		214 bp cut by *Hae*III	
Product	ApUpU	ApUpU	CpUpUpU	ApUpC	CpUpUpU	ApUpC
−CRP	38	49	16 ± 7	12 ± 6	19 ± 9	12 ± 6
+CRP	71	74	2 ± 1	1 ± 1	1 ± 1	1 ± 1

The activities or turn-over numbers of the two promoters are expressed as the amount of mols of UMP incorporated/mol of template/min with SD ±20% unless otherwise indicated. The selective synthesis of ApUpU on one hand, and CpUpUpU or ApUpC on the other, were respectively initiated from the *malT* and *Px* promoters. The dinucleotide and UTP concentrations were 500 μM and 50 μM. Polymerase was at 150 nM, DNA template at 4 nM, cAMP at 200 μM and CRP, when present, at 100 nM.

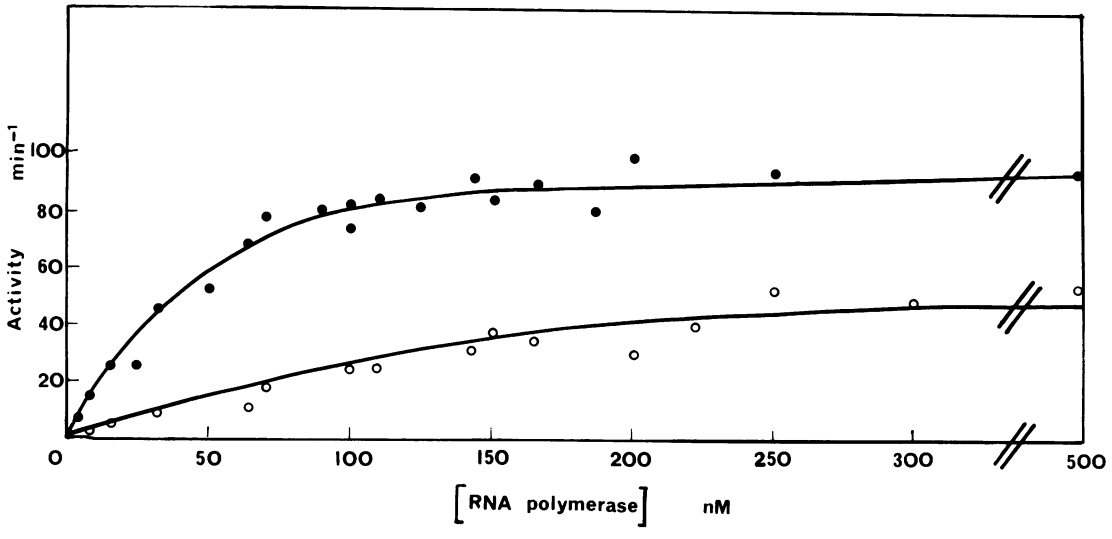

Fig. 2. RNA polymerase titrations of the *malT* promoter in the presence of 50 nM CRP and 200 μM cAMP (●) or in its absence (○). The activities in the abortive initiation assay are plotted versus the RNA polymerase concentration. The abortive initiation reactions, performed in standard buffer at 37°C were initiated by the addition of ApU (0.5 mM) and [α-^{32}P]UTP (0.05 mM), following a 1-h pre-incubation of RNA polymerase and template (2−6 nM) at 37°C. After 15- and 30-min reactions, the incorporation of UTP into ApUpU was determined and the activity calculated as mol UMP incorporated/mol promoter/min. When present, CRP and cAMP were incubated at least 10 min with the DNA template prior to the addition of polymerase.

Results

Organization of the DNA region upstream the RNA start at malT

As already shown in Chapon and Kolb (1983), the *malT* promoter is extremely weak in the absence of added CRP−cAMP complex. Run-off transcription assays, performed under their conditions on the 190-bp *Sal*I−*Hin*fI fragment depicted in Figure 1, yielded several faint sets of long transcripts ($n > 10$) (Figure 6). The most abundant, 69−71 bp in length, was abolished after restriction cleavage of the fragment by *Hae*III, but preserved after cutting with *Hph*I. A 5-fold higher yield of transcripts of the same length was obtained when the 190-bp fragment was pre-incubated with CRP−cAMP. A second set of transcripts, 39−43 bp in length, remained unaffected when the template was cleaved with *Hae*III, and disappeared after restriction with *Hph*I. Also pre-addition of CRP−cAMP completely inhibited its synthesis.

Two divergent sets of transcripts originating around −87 and +1 could therefore be observed (+1 refers to the origin of the CRP activated transcript corresponding to the *malT* gene). The major one arose from the *malT* promoter, since it was markedly stimulated by prior addition of CRP−cAMP, and since it was abolished after truncating the corresponding promoter. The other promoter *Px* was turned off by addition of the activating complex, probably because of a direct interference between CRP binding at −70 and polymerase binding at *Px* (cf. Figure 1).

Abortive initiation assays; choice of the substrates; CRP requirement

The most efficient primers possible were chosen to further characterize these promoters using abortive initiation assays. At the *malT* promoter, the 5′ end of the predominant message is ApUpUpA (cf. Chapon and Kolb, 1983). Open complex formation at *malT* was followed through the abortive synthesis of the trinucleotide ApUpU from ApU and [α-^{32}P]UTP. At *Px*, multiple starts may be observed from positions −86 to −88; we either used ApU and [α-^{32}P]CTP to form ApUpC initiated at position −86 or CpU and [α-^{32}P]UTP to synthesize CpUpUpU, starting at position −88. As shown in Table I, the steady-state rates of formation of these products were affected by the restriction of the 214-bp fragment, as well as by the presence of the CRP−cAMP complex in the expected manner. In particular, removal of the *malT* promoter did not increase the specific activity of transcription at the *Px* promoter and *vice versa*, indicating that, in the absence of CRP, the two sites were not appreciably competing for enzyme binding.

Titration with increasing concentrations of RNA polymerase were performed at the two promoters, using the relevant

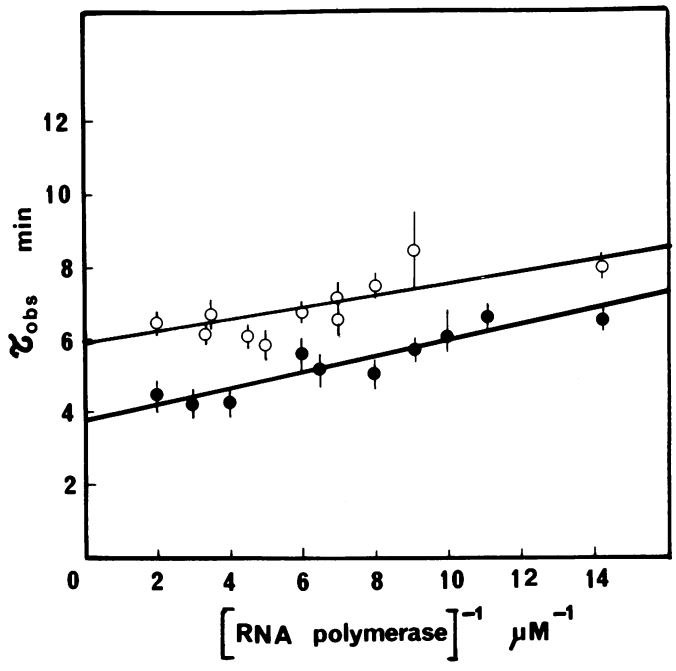

Fig. 3. Influence of CRP–cAMP on the kinetics of open complex formation at the *malT* promoter. τ_{obs}, measured in the presence of 50 nM CRP and 200 μM cAMP (●) and in its absence (○) is plotted versus the reciprocal of the RNA polymerase concentration. Reactions were initiated with the addition of the enzyme, following a 10-min pre-incubation of DNA and nucleotide substrates and eventually CRP and cAMP. Portions of the reaction were removed at various times and the incorporation of UTP determined at each time. Lag curves were constructed and τ_{obs} determined by the graphical method (Buc and McClure, 1985). Error estimates in τ_{obs} are shown as vertical bars.

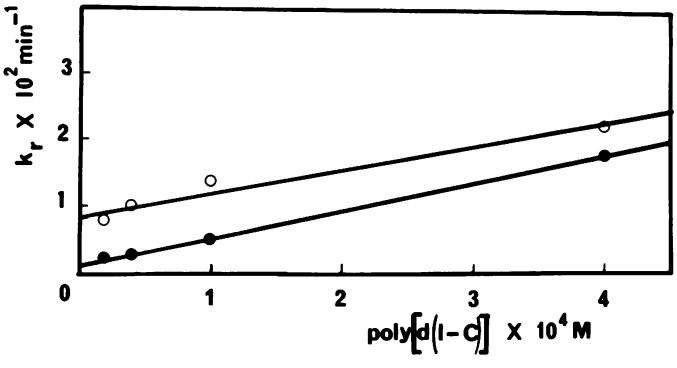

Fig. 4. The effects of various amounts of poly[d(I–C)] on reverse rate constants k_r of open complexes formation at the *malT* in the presence (●) of CRP (80 nM) and cAMP (200 μM) or in their absence (○). Following a 1-h pre-incubation of polymerase and template at 37°C with or without the cAMP–CRP complex, poly[d(I–C)] was added. At selected times thereafter, portions were withdrawn and added to nucleotide substrates. The fraction of open complexes remaining was analysed by measuring the abortive initiation rate in 5- and 7-min reactions. Final concentrations in the assay are given: DNA, 6 nM; polymerase, 175 nM or 100 nM; ApU, 500 μM; UTP, 50 μM; CRP and cAMP as indicated above. k_r was calculated from a semilogarithmic plot of the fraction of open complex remaining versus time.

substrates in separate assays. In the absence of CRP–cAMP, RNA polymerase displayed a poor affinity for both sites (cf. Figure 2 for the *malT* promoter). When the fragment was incubated with 50 nM of CRP and 200 μM of cAMP prior to enzyme and substrate addition, abortive synthesis of ApUpC or CpUpUpU was completely abolished, while the specific activity in the steady-state release of ApUpU was markedly increased. At the plateau there was a 2-fold stimulation, and the response with respect to RNA polymerase concentration was steeper (cf. Figure 2).

The marked effect of CRP–cAMP on the steady-state activity of RNA polymerase in this assay could then be used for titration purposes. The CRP preparation used was partially inactive, as judged from its ability to remain bound to a *lac* binding site in a gel retardation assay (Kolb *et al.*, 1983). We therefore titrated simultaneously the stimulation of the rate of initiation of transcription at two CRP-dependent promoters, *lac*P_1 WT and *malT*. The same quantity of protein, one active dimer per fragment, was required in both cases.

Analysis of forward kinetics at malT

In the abortive initiation assay specific for the *malT* promoter, RNA polymerase was added last, at concentrations ranging from 60 to 500 nM. A latency period was observed before the establishment of the steady-state release of the radioactive product, and from the analysis of this process, times required for open complex formation, τ_{obs}, were measured as indicated in Malan *et al.* (1984) and in Materials and methods. The values were not affected by a 2-fold change in the UTP concentration or by the excision of the *Px* site by *Hph*I. Data obtained in the presence as in the absence of CRP–cAMP could be fitted by the equation:

$$(\tau_{obs})^{-1} = k_r + [k_f K_B (R)/1 + K_B (R)] \sim \frac{k_f K_B (R)}{1 + K_B (R)}$$

This expression holds true whenever the scheme given in the Introduction is valid. Here (R) is the concentration of RNA polymerase, K_B the association constant for the formation of the closed complex, and k_f and k_r the isomerization rate constants for the second step. k_r was independently measured and shown to be negligible (cf. next section). A plot of τ_{obs} versus $(R)^{-1}$ yielded then K_B and k_f (cf. Figure 3). The values were not very different in the absence ($K_B = 2.9 \times 10^7$/M; $k_f = 0.003$/s) or in the presence of activator ($K_B = 1.8 \times 10^7$/M; $k_f = 0.004$/s). It is obvious that contrary to the *lac* and *gal* cases, CRP–cAMP does not drastically affect the rate of open complex formation at this promoter.

Analysis of the reverse kinetics

Preformed open complexes were challenged with poly(deoxyinosinate–deoxycytidylate), poly[d(I–C)], and their decay followed by abortive initiation assays performed after various incubation times (closed complexes are irreversibly dissociated during this challenge) (Cech and McClure, 1980). A single exponential decay of the initial enzymatic activity was observed on > 80% of the process. Contrary to that found for strong promoters, this rate appeared to depend on the competitor concentration (cf. Figure 4). The data were therefore extrapolated to zero competitor concentration, to yield residence times of 125 ± 20 min in the absence, and 1350 ± 300 min in the presence of CRP–cAMP. Addition of substrates did not significantly affect those values. Therefore the stabilization of the enzyme–promoter complex due to CRP (Chapon and Kolb, 1983) arises from a decrease in the dissociation rate constant of the final complex (from 1.3×10^{-4}/s to 1.2×10^{-5}/s), and not by a positive effect on the forward rate process.

Influence of CRP on substrate binding

However, CRP was found to affect the steady-state rate of formation of the abortive products **a** (see above). This quantity was measured at increasing concentrations of ApU (substrate A) and UTP (substrate B), to know whether the main effect exerted by

Table II. Effects of CRP–cAMP on kinetic constants for substrates at the *malT* promoter

	Φ_0	Φ_A	Φ_B	Φ_{AB}	$a_{max/min}$	K_A μM	K_B μM	K_{ia} μM
	× 10^2 min	× 10^6 min	× 10^7 min M	× 10^{11} min M^2	$1/\Phi_0$	Φ_A/Φ_0	Φ_B/Φ_0	Φ_{AB}/Φ_B
−CRP	1.27 ± 0.23	2 ± 0.17	2 ± 0.17	5.7 ± 0.7	78 ± 14	160 ± 50	16 ± 5	280 ± 80
+CRP	1.04 ± 0.06	0.53 ± 0.1	0.23 ± 0.07	0.97 ± 0.13	96 ± 6	52 ± 10	2.2 ± 0.7	420 ± 100

Assay conditions are described in the legend of Figure 5. Kinetic constants Φ_0, Φ_A, Φ_B and Φ_{AB} were determined from double reciprocal plots of the activity data (see Materials and methods). Assuming a steady-state ordered model with ApU binding first, followed by UTP (McClure *et al.*, 1978), the Michaelis constants for ApU (K_A) and UTP (K_B), the dissociation constant of ApU (K_{ia}) and the maximal velocity a_{max} of the ApUpU synthesis were determined as indicated.

CRP was an increase in the maximal velocity of the reaction or an improvement of the Michaelis constants.

One of the substrate concentrations was fixed and the other varied. Data were plotted according to Lineweaver–Burk; Dalziel's methodology and formalism (1957) were followed to fit the measurements of $\mathbf{a} = f[(A),(B)]$ with the equation:

$$\mathbf{a}^{-1} = \Phi_0 + \frac{\Phi_A}{(A)} + \frac{\Phi_B}{(B)} + \frac{\Phi_{AB}}{(A)(B)}$$

The results are summarized in Table II and in Figure 5. At high concentrations of both substrates CRP did not exert any significant influence on the maximal velocity of the reaction (Φ_0 is equal to 1.04 × 10^{-2} min in the presence and 1.27 × 10^{-2} min in the absence of the activator). The most significant effects were observed in the response of the specific rate **a** with respect to [B], the concentration of UTP. The parameter Φ_B decreased by a factor of 10 when CRP–cAMP was initially present. In a similar fashion, the 6-fold change in the value of Φ_{AB} reflected an increased affinity of the enzyme for UTP at low ApU concentration; Φ_A was less affected by the presence of CRP.

Hence, at the *malT* promoter, CRP had no significant effect on the rate of open complex formation or on the maximal velocity of the abortive initiation assay. These findings are in sharp contrast with the observations made on the lactose and galactose promoters. Three positive effects were detected, however; at *malT*, CRP increases the residence time and therefore the stability of the open complex. It also increases the affinity of the enzyme for UTP and eliminates a secondary independent binding site at *Px*. None of these phenomena are expected, however, to contribute appreciably to promoter strength *in vivo* (cf. Discussion).

Run-off assays

The assays reported above indicated that similar amounts of competent complex were formed at *malT* in the absence and presence of CRP. These results, obtained by monitoring the formation of a trinucleotide, were at striking variance with the single-round transcription experiments reported by Chapon and Kolb (1983), who found a significant increase in the amount of full-length transcripts synthesized when CRP was added. Run-off experiments were therefore performed under conditions analogous to the abortive initiation assays. Both the amount of transcripts and their rate of formation were monitored.

Prior addition of CRP to the incubation mixture increased the amount of full length transcripts by a factor of five. Even in the presence of CRP and cAMP, the yield was still significantly lower than when the CRP independent *malT* P_1 promoter mutant was used. This 5-fold increase was maintained when the UTP concentration was varied from 3 to 50 μM, or when the single-round transcription assay was primed with ApU. This was not due to the inactivation of a significant fraction of the open complexes

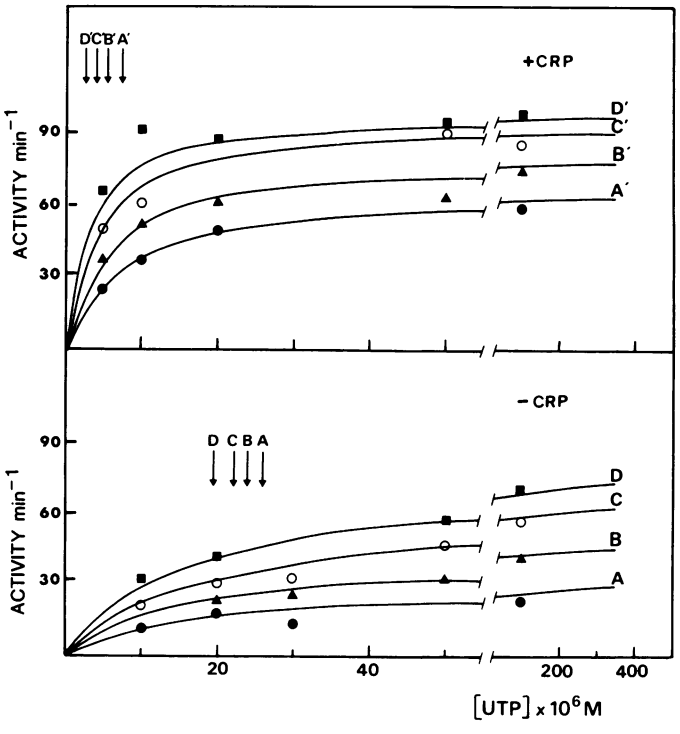

Fig. 5. Activities of RNA polymerase on the *malT* promoter as a function of substrate concentrations. DNA template (3 nM) and polymerase (150 nM) with or without the cAMP–CRP complex were pre-incubated for 1 h and various amounts of UTP (as indicated above) and ApU (●, 100 μM, curves A′ and A; ▲, 200 μM, curves B′ and B; ○, 500 μM, curves C′ and C; ■, 1000 μM, curves D′ and D) added. The arrow under each letter points out the UTP concentration required for half-maximal activity at each ApU concentration. The abortive initiation reaction was followed for 5 and 10 min and the activities determined as described in the legend of Figure 2. Final concentrations of CRP and cAMP were 50 nM and 200 μM respectively.

in one case and not in the other. The sole change was the presence of heparin at a concentration of 50 μg/ml in the run-off assay. This point was checked by performing reverse kinetic experiments similar to the ones described above, replacing poly[d(I–C)] with heparin (final concentration 80 μg/ml). In such experiments, the decay of active species still proceeded via a slow first-order process extrapolating to 100% at zero time (k_r values: 2.8 × 10^{-4}/s in the absence of CRP).

As shown in Figure 7, the latency time required for the completion of the message was significantly longer than the time necessary for open complex formation, as monitored by abortive initiation assays. This was true whether CRP was present or not. Also this time appeared to be dependent on the UTP concentration; it was further increased, at least in the absence of

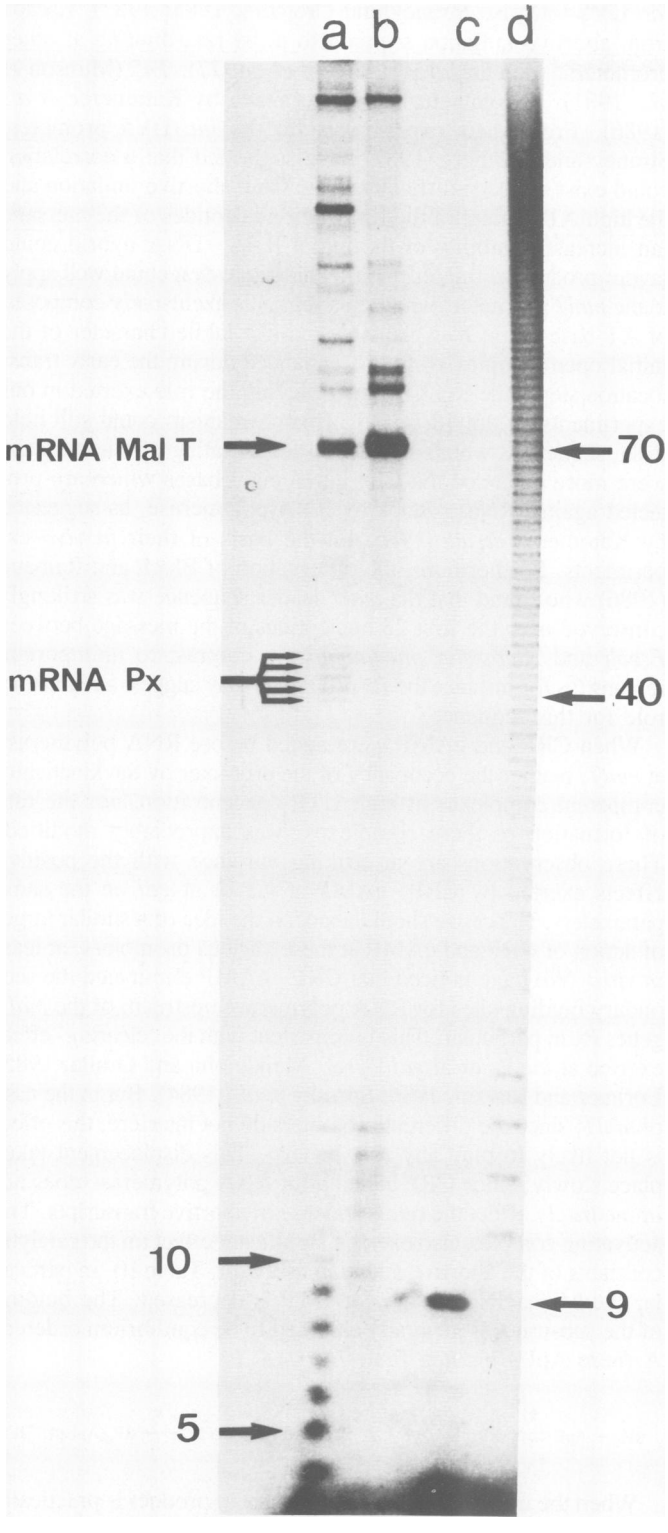

Fig. 6. *In vitro* transcription from *malT* promoter in the absence (**lane a**) and presence (**lane b**) of the cAMP–CRP complex. The 190-bp *HinfI–SalI* DNA fragment at 15 nM was pre-incubated with RNA polymerase (150 nM) for 1 h at 37°C before addition of XTPs. CRP, when added, was at 150 nM. **Lanes c** and **d** are RNA markers, respectively a 9-mer synthesized in the presence of ATP, UTP and 3'OMeCTP from the *malT* promoter and a poly[r(A–U)] ladder. Note that the *Px* transcript (**lane a**) appears only faintly on the autoradiogram, due to its multiple start sites and its low U content.

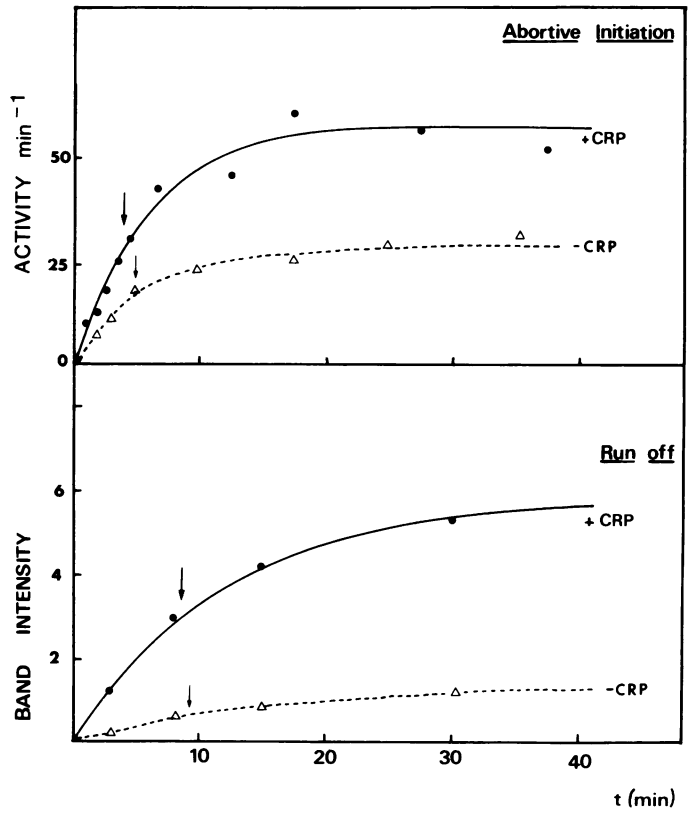

Fig. 7. Comparison of the effect of the cAMP–CRP complex on the *malT* promoter activity by the abortive initiation reaction and the run-off assay. Activities of RNA polymerase on the *malT* promoter in the presence (●) or in the absence (△) of the cAMP–CRP complex was monitored versus time following addition of RNA polymerase either by measuring ApUpU synthesis in a 5- or 7-min assay or by quantifying the *malT* transcript after 5 min. Run-off assays in the presence of ApU as described in Materials and methods. The arrow shows the time following addition of the enzyme when half-maximal activity was obtained.

CRP, when the UTP concentration was lowered from 30 to 3 μM, contrary to that found previously for the trinucleotide synthesis. Therefore, under our conditions, the rate-limiting step for messenger synthesis of *malT* is not the formation of a competent open complex but a later stage, where the nucleotide concentration appears to play a role. The yield of full-length transcript is also CRP dependent.

The observations are best explained if one postulates that RNA polymerase paused at specific elongation steps, slowing down the overall rate of synthesis of the *malT* transcript. In the absence of CRP, RNA polymerase has a larger chance of terminating at these crucial steps. We looked therefore for the presence of shorter transcripts in run-off experiments performed in the absence of the activating protein. Figure 6, lane a, indicates that tetra to nonanucleotides were indeed present in great quantity, suggesting that RNA polymerase was able to recycle at these early pauses. Prior addition of CRP abolished such synthesis (Figure 6, lane b).

Discussion

As expected from its very poor fit with the consensus sequence, the *malT* promoter has a weak affinity for RNA polymerase in the absence of added activator. Several other transcription starts were found on the 214-bp fragment studied, the predominant one

originating from a divergent and 86-bp distant promoter, called here *Px*. This secondary site acted, however, as an independent 'sink' for the enzyme. Contrary to what was found at the lactose and galactose regulatory regions (Musso *et al.*, 1977; Maquat and Reznikoff, 1978; Malan *et al.*, 1984; Spassky *et al.*, 1984) where the P_1 and P_2 loci are overlapping, binding at *malT* and at *Px* are not exclusive, and removal of the second sequence did not substantially affect either the specific activity, monitored by the synthesis of the relevant abortive product, ApUpU, or the rate of open complex formation at *malT*.

With respect to the strong or medium promoters most frequently studied by abortive initiation assays (McClure, 1985), the *malT* promoter behaves rather atypically. The weak specific activity reported in Figure 2 in the absence of CRP−cAMP is not due to a weak probability of forming a complex with RNA polymerase at this site. Rather, as shown in Table II, this is due to a poor Michaelis constant of the enzyme for one of its substrates, UTP. Increasing the UTP concentration brought the specific activity measured in the abortive initiation assay to a level comparable to that observed in the presence of CRP. Certainly, the formation of an open complex does not require the presence of substrates: it was found that a sudden increase in the concentration of UTP resulted in an immediate enhancement of the turnover number of the reaction, while a latency time of > 6 min would be expected if the maximal number of competent species were not already formed under such conditions. Moreover, neither the kinetic constants for the forward process, nor the stability of the final complex, were found to be significantly affected by the UTP concentration. We conclude therefore that the final binary complexes correspond to a pool of species which were competent in the abortive initiation assay.

The low affinity of RNA polymerase for its site results from an unfavourable 'on' rate constant and from a large 'off' rate constant. Values found for the association constant K_B and the isomerizaton rate constant k_f are comparable to the *lac* promoter in the absence of the activator (Malan and McClure, 1984). The backward rate constant is markedly poor (1.3×10^{-4}/s). Hence the final complex is active but labile. Furthermore, and contrary to the other cases studied, the apparent first-order dissociation rate constant appears to depend on the concentration of the added competitor, poly[d(I−C)]. Most likely, the final complex can bind the challenging molecule and RNA polymerase can be dissociated from the promoter by a mechanism involving a direct displacement [for an analysis of a similar case see Cech and McClure (1980) and Busby *et al.* (1981)]. Hence the final fit between the enzyme and the DNA is probably rather loose. This deduction is strengthened by an analysis of the pattern of attack of this complex by DNase I or by orthophenanthroline-Cu^+; protections and enhancements are weak (cf. Chapon and Kolb, 1983; A.Kolb, M.Menendez and H.Buc, unpublished).

This rather labile complex, active when monitored by abortive initiation assays, appeared quite inefficient, however, in completing the subsequent elongation steps leading to escape from the promoter. First, as discussed above, the yield of full-length transcripts in single-round transcription experiments was only 4% of that found for the parent *malT* P_1 promoter. Second, completion of full-length transcripts occurred more slowly than expected if the overall limiting step were the formation of the open complex. Third, the number of short transcripts synthesized during such experiments indicated that RNA polymerase pauses and recycles many times between +3 and +9 before being able to overcome a crucial elongation step. The situation is quite comparable to the one described by Carpousis and Gralla (1980) at *lac* UV5 (cf. also Straney and Crothers, 1985; 1987). Escape from abortive initiation seems also to be rate-limiting at other promoters, such as *gal* P_2 (Aiba *et al.*, 1977), *Tn5* (Munson *et al.*, 1981), on synthetic constructs made by Kammerer *et al.* (1986). From their experiments on the *lac* UV5 promoter, Straney and Crothers (1987) have suggested that a correlation could exist between difficult escape from abortive initiation and the high AT content of the first eight nucleotides of the message (an increased stability of the initial RNA−DNA hybrid could favour productive transcription). This suggestion could well apply to the *malT* promoter, where this region is exclusively composed of AT base pairs. It is plausible that the labile character of the initial open complex would be enhanced during the early translocation steps, the RNA product playing the role exerted in our experiments by poly[d(I−C)]. This mechanism could still hold if the sequences which are crucial for dictating efficient escape were more extended than the initial eight bases which are protected against RNase attack by RNA polymerase, as suggested by Kammerer *et al.* (1986) on the basis of their *in vivo* experiments. Furthermore, the observation of Bloch and Raibaud (1986) who found that the *malT* leader sequence was strikingly conserved over the first 23 nucleotides of the message between *E.coli* and *Klebsiella pneumoniae* in contrast to unimportant regions (as for instance the *Px* promoter) may suggest a regulatory role for this sequence.

When CRP and cAMP were added before RNA polymerase at *malT*, neither the occupancy of the promoter by the kinetically competent complexes at high UTP concentration, nor the rate of formation of these complexes, was appreciably modified. These observations are at striking variance with the positive effects exerted by CRP−cAMP at *lac* or at *gal* on the same parameters. Hence we should abandon the idea of a similar target of action of CRP and cAMP at these various promoters, at least *in vitro*. We have noticed that CRP−cAMP eliminated the secondary binding sites for RNA polymerase upstream of the *malT* gene, *Px* in particular. This is consistent with the 'clearing' effect exerted at *lac* P_2 or at *gal* P_2 (cf. Meiklejohn and Gralla, 1985; Lorimer and Revzin, 1986; Spassky *et al.*, 1984). But in the case of *malT*, since the different promoters do not interfere, this effect is not likely to play any role *in vivo*. This displacement takes place slowly, since CRP added after RNA polymerase does not immediately affect the rate of release of abortive transcripts. The activating complex also exerts a significant effect on the catalytic constants of the abortive initiation assay (cf. Table II), in particular the Michaelis constant for UTP is decreased. The binding of the substrates is generally assumed to be equilibrium ordered, A (here ApU) binding first:

$$RP_o + ApU \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (RP_o,ApU) + UTP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (RP_o,ApU,UTP) \xrightarrow{k} RP_o(ApUpU/PPi)$$

When the transformation of substrates to product is practically irreversible, Φ_B can be written as $(k + k_{-2})/kk_2$ (Ricard, 1973) and, in simple cases, k is equal to Φ_0^{-1}. As Φ_0 is practically unchanged by the addition of CRP−cAMP, this model would imply an increased affinity of UTP for its site when the activating complex is present. However, a strictly ordered pathway has only been documented for the abortive synthesis of pppApU at the λP_R promoter (McClure *et al.*, 1978). It is possible that the scheme would be more complex when translocation is required as in the present case; the interpretation would then be less straightforward. In any case, a distal effect is exerted by the activator complex on the catalytic site of the enzyme; it could be

a long range allosteric effect if CRP were still contacting the enzyme in the open complex. Alternatively CRP could simply commit RNA polymerase towards a different final complex where the catalytic site would be more properly positioned. The absolute values of the Michaelis constants are, however, rather low, compared with the concentration of the pool of UTP within the cell (Danchin *et al.*, 1984), and it is therefore unlikely that the rate of formation of the first phosphodiester bonds would be affected *in vivo* when cAMP is present.

The most significant effect exerted by CRP−cAMP in the abortive assays is the reduction of the backward rate constant. Here again this factor cannot directly affect promoter strength as indicated above, but a labile complex with a rather poor affinity for its substrate could experience considerable difficulty in translocating at specific pausing sites. We have seen that CRP prevents the recycling of RNA polymerase. It could do so by increasing also, at this pausing site, both the residence time of the enzyme and the affinity for the incoming substrate. Similar mechanisms have already been proposed by others studying elongation of the message; during *in vitro* elongation of transcripts initiated at the T_7A_1 promoter, Kassavetis and Chamberlin (1981) have found abnormally high apparent Michaelis constants for ribonucleotides at some of the pausing sites explaining why pausing is so markedly enhanced at low triphosphate concentrations. We have observed a similar trend for the dependence of the overall rate of the *malT* transcript on UTP concentration. The fluctuation in the pool of UTP could then affect the efficiency of transcription *in vivo* as demonstrated for the *pyrB* and *E* genes of *Salmonella typhimurium* (Jensen *et al.*, 1986). As shown previously CRP improves the Michaelis constant for UTP in the abortive initiation assay; it could possibly play a similar role later on in the pathway favouring translocation of the enzyme over termination.

We do not know yet whether such a mechanism is operating *in vivo* though, as pointed out earlier, the conservation of the sequences in the first nucleotides of the message is an indication for their functionality. Genetic work is in progress on that issue. Spontaneous point mutants have been previously isolated, close to the *malT* promoter, which express *malT* in the absence of CRP (Chapon, 1982). In such a class, one should find mutants affecting the pausing sites detected in this study, though a rather extensive modification of the region is likely to be required to observe a clear effect.

The molecular nature of the positive effect observed at *malT* could be either direct (CRP moving along with RNA polymerase) or indirect (CRP engaging RNA polymerase in a better open complex, from which it is easier to escape). In this second hypothesis, one could postulate a mechanism similar to that proposed by Straney and Crothers (1985) to account for their results at *lac* UV5. In the first one, the mode of action would be very similar to the mechanism suggested by Roberts and collaborators to account for the action of the Q protein favouring the escape of RNA polymerase from a pausing site located 16 bp downstream of the phage late gene promoter and then allowing the enzyme to pass the downstream terminator (Grayhack *et al.*, 1985). This is also in agreement with the data of Ullmann *et al.* (1979) and Guidi-Rontani *et al.* (1984) who have repeatedly stressed the role of the CRP−cAMP complex as an antiterminator in some polycistronic transcriptional units including the *lac* and *gal* operons.

Materials and methods

DNA fragments

A 214-bp *SalI*−*Eco*RI fragment (Chapon and Kolb, 1983) includes the *malT* promoter sequence from −117 to +91. It was purified from plasmid pOM50 as described in Maniatis *et al.* (1982), and its concentration was determined from UV spectra, employing an absorption coefficient at 260 nM of 6.5/mM/cm/DNA phosphate. The 214-bp *malT* fragment cut by *Hin*fI restriction enzyme was also used as a template in run-off assays. To study the *malT* or *Px* promoter alone, the 214-bp fragment was cut respectively with either *Hph*I or *Hae*III restriction enzymes.

Proteins

E.coli RNA polymerase was isolated by the procedure of Burgess and Jendrisak (1975). Holoenzyme was separated from the core according to Lowe *et al.* (1979). The preparations used in this study were characterized for their activity on the *lac* UV5 promoter as described (Malan *et al.*, 1984), and estimated to be 60% active. *E.coli* CRP was the kind gift of D.Kotlarz. The protein was homogeneous on polyacrylamide gel electrophoresis; its concentration was determined spectrophotometrically using $\epsilon_{278} = 4.1 \times 10^4$ M/cm; its activity was judged to be 30% by titration with the *Hha*I−*Bst*NI 41-bp *lac* fragment containing the wild-type *lac* CRP site in gel retardation assays (Kolb *et al.*, 1983).

Abortive initiations assays

These were performed as described by McClure *et al.* (1978). The standard buffer used was 40 mM Tris, pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, 1 mM DTT and 0.1 mg/ml bovine serum albumin (BSA). Standard reactions included 0.5 mM dinucleotide and 0.05 mM [α-^{32}P]UTP or CTP (8−40 Bq/pmol). The abortive initiation reactions products synthesized at 37°C were assayed by spotting 20 μl-portions of each reaction mixture onto Whatman 3 MM paper which had been prespotted with 100 mM EDTA, and chromatograms developed as described (McClure *et al.*, 1978). The ApUpU product synthesized at the *malT* promoter had an R_f of 0.07. The activity of RNA polymerase on the promoter was expressed in each case in mol of ApUpU synthesized/mol of promoter/min.

Final steady-state synthesis and values of τ_{obs} were estimated at each RNA polymerase concentration by the graphical method given in Buc and McClure (1985).

Determination of open complex lifetimes were performed according to Cech and McClure (1980). The quantity of open complexes remaining after challenge by various amounts of poly[d(I−C)] or heparin (80 μg/ml) was followed for 2 h, using 5- and 7-min enzymatic assays. The k_r values, measured by challenge with heparin (80 μg/ml) were $(1.51 \pm 0.06) \times 10^{-4}$/s in the presence of CRP (80 nM) and cAMP (200 μM) and $(2.77 \pm 0.1) \times 10^{-4}$/s in their absence.

Determination of the kinetic constants

Abortive initiation assays allowed the determination of activities of polymerase on the *malT* promoter as a function of substrate concentrations. First reciprocal activities were plotted versus reciprocal ApU concentration (substrate A) at fixed concentrations of UTP (substrate B) indicated. The slopes and intercepts of these straight lines were replotted versus the reciprocal UTP concentration. The intercepts on the ordinates of these plots gave Φ_0 and Φ_A, while the slopes yielded Φ_B and Φ_{AB}. Second, the activities were plotted again in the same way, but with UTP as the variable substrate and ApU held constant. The slope and intercept replot versus reciprocal ApU concentrations allowed the determinations of Φ_0 and Φ_{AB}.

Transcription reactions

Transcription assays were performed with 15 nM DNA fragment at 37°C in the standard buffer, except that we used acetylated BSA. Transcription was allowed to proceed for 5 min after simultaneously adding 200 μM each of ATP, CTP and GTP and 50 μM UTP (70 Bq/pmol) and 50 μg/ml of heparin. The reaction was stopped by adding 2 vol of a solution containing 96% formamide (v/v), 20 mM EDTA, 0.01% bromophenol blue and xylenecyanol blue dyes and loaded directly on a 10% urea−polyacrylamide sequencing gel. By using only 200 μM ATP and 50 μM UTP in the presence of 3′-*O*-methyl cytidine triphosphate (3′OMeCTP) in the transcription assay, the *malT* transcript was shortened to a 9-mer oligonucleotide. In some experiments ApU (200 μM) was included to the reaction mixture, thereafter supplemented by addition of XTP at 50 μM each and heparin as above.

The poly(deoxyadenylate−thymidylate) (poly[d(A−T)] transcription mixture included 20 μM poly[d(A−T)], expressed in phosphate concentration, 150 nM polymerase, 50 μg/ml heparin, 200 μM ATP and 50 μM [α-^{32}P]UTP; the reaction was stopped after 30 s incubation with XTPs to get an RNA ladder from 9 to 150 nucleotides long. RNA bands were calibrated versus the poly(adenylate−uridylate) ladder and quantified after autoradiography on Kodak-XR5 film using a Biorad 620 densitometer equipped with a Shimadzu C-R3A integrator.

Acknowledgements

We are very grateful to Pascal Roux for expert technical assistance. We thank Denise Kotlarz for abundant supplies of purified CRP. We are indebted to Olivier Raibaud for helpful suggestions. We are particularly grateful to Evelyne Richet, who pointed out the existence of the *Px* promoter. This work was supported by

grants from Centre National de la Recherche Scientifique (U.A. 1149) and Institut National de la Santé et de la Recherche Médicale (no. 83 1004). M.M. is a postdoctoral fellow from the C.S.I.C.

References

Aiba,H., Adhya,S. and de Crombrugghe,B. (1981) *J. Biol. Chem.*, **256**, 11905–11910.
Bloch,M.A. and Raibaud,O. (1986) *J. Bacteriol.*, **168**, 1220–1227.
Buc,H. and McClure,W.R. (1985) *Biochemistry*, **24**, 2712–2723.
Buc,H., Amouyal,M., Buckle,M. Herbert,M., Kolb,A., Menendez,M., Rimsky,S., Spassky,A. and Yéramian,E. (1987) In Reznikoff,W.S. *et al.* (eds), *RNA Polymerase and the Regulation of Transcription. A Steenbock Symposium*. Elsevier, New York, pp. 115–125.
Burgess,R.R. and Jendrisak,J.J. (1975) *Biochemistry*, **14**, 4634–4638.
Busby,S., Spassky,A. and Buc,H. (1981) *Eur. J. Biochem.*, **118**, 443–451.
Carpousis,A.J. and Gralla,J.D. (1980) *Biochemistry*, **19**, 3245–3253.
Cech,C.L. and McClure,W.R. (1980) *Biochemistry*, **19**, 2440–2447.
Chapon,C. (1982) *J. Bacteriol.*, **150**, 722–729.
Chapon,C. and Kolb,A (1983) *J. Bacteriol.*, **156**, 1135–1143.
Dalziel,K. (1957) *Acta Chem. Scand.*, **11**, 1706.
Danchin,A., Dondon,L. and Daniel,J. (1984) *Mol. Gen. Genet.*, **193**, 473–478.
Débarbouillé,M., Cossart,P. and Raibaud,O. (1982) *Mol. Gen. Genet.*, **185**, 88–92.
de Crombrugghe,B., Busby,S. and Buc,H. (1984) In Yamamoto,K. (ed.), *Biological Regulation and Development*. Plenum Press, New York, Vol. 3B, pp. 129–167.
Grayhack,E.J., Yang,X., Lau,L.F. and Roberts,J.W. (1985) *Cell*, **42**, 259–269.
Guidi-Rontani,C., Danchin,A. and Ullmann,A. (1984) *Mol. Gen. Genet.*, **195**, 96–100.
Herbert,M., Kolb,A. and Buc,H. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2807–2811.
Jensen,K.F., Fast,R., Karlström,O. and Larsen,J.N. (1986) *J. Bacteriol.*, **166**, 857–865.
Kammerer,W., Deuschle,U., Gentz,R. and Bujard,H. (1986) *EMBO J.*, **11**, 2995–3000.
Kassavetis,G.A. and Chamberlin,M.J. (1981) *J. Biol. Chem.*, **256**, 2777–2786.
Kolb,A., Spassky,A., Chapon,C., Blazy,B. and Buc,H. (1983) *Nucleic Acids Res.*, **11**, 7833–7852.
Lorimer,D.D. and Revzin,A. (1986) *Nucleic Acids Res.*, **14**, 2921–2938.
Lowe,P.A., Hager,D.A. and Burgess,R.R. (1979) *Biochemistry*, **18**, 1344–1352.
Malan,T.P. and McClure,W.R. (1984) *Cell*, **39**, 173–180.
Malan,T.P., Kolb,A., Buc,H. and McClure,W. (1984) *J. Mol. Biol.*, **180**, 881–909.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY.
Maquat,L. and Reznikoff,W.S. (1978) *J. Mol. Biol.*, **125**, 467–490.
McClure,W.R. (1985) *Annu. Rev. Biochem.*, **54**, 171–204.
McClure,W.R., Cech,C.L. and Johnston,D.E. (1978) *J. Biol. Chem.*, **253**, 8941–8948.
Meiklejohn,A.L. and Gralla,J.D. (1985) *Cell*, **43**, 769–776.
Munson,L.M. and Reznikoff,R.W. (1981) *Biochemistry*, **20**, 2081–2085.
Musso,R.E., Di Lauro,R., Adhya,S. and de Crombrugghe,B. (1977) *Cell*, **12**, 847–854.
Ricard,J. (1973) *Cinétiques et Mécanismes d'Action des Enzymes (cinétique enzymatique phénoménologique)*. Doin, Paris.
Spassky,A., Busby,S. and Buc,H. (1984) *EMBO J.*, **3**, 43–50.
Straney,D.C. and Crothers,D.M .(1985) *Cell*, **43**, 449–459.
Straney,D.C. and Crothers,D.M. (1987) *J. Mol. Biol.*, **193**, 267–278.
Ullmann,A., Joseph,E. and Danchin,A. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3194–3197.

Received on July 31, 1987; revised on October 10, 1987