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Action of CAP on the *malT* Promoter In Vitro

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DNase I footprinting experiments demonstrated that CAP, the cyclic AMP receptor protein of *Escherichia coli*, binds around position −70 at the promoter of *malT*, the positive regulator gene of the maltose regulon. The binding of CAP in the presence of cyclic AMP favored the subsequent specific binding of RNA polymerase. Initiation of *malT* transcription in vitro displayed an absolute requirement for CAP at all tested RNA polymerase concentrations. However this was not the case with a mutant promoter (*malTp1*), which leads to CAP-independent *malT* expression in vivo. In that case an effect of CAP was seen only at the lower concentrations of RNA polymerase. These results, which suggest that CAP stimulates *malT* expression by promoting the binding of polymerase to the promoter, are compared with those obtained in other systems.

CAP, the catabolite activator protein of *Escherichia coli*, exerts a positive control on the expression of many genes, principally on those that are involved in the catabolism of substrates. In the presence of its effector, cyclic AMP (cAMP), it stimulates transcription initiation at the promoter of these genes (14, 33, 45a, 48; de Crombrugghe et al., Biol. Reg. Dev., in press). The primary sequence of CAP is known (1, 2, 8), its three-dimensional structure has been established at 0.29-nm resolution (29), and the sequence of its binding site at several promoters has been determined. In spite of these and numerous other studies the exact mechanism whereby CAP stimulates the initiation of transcription is still a matter of debate (19, 32, 39, 43). The most extensive studies performed so far have concerned the *lac*, *gal*, and *ara* operons (3, 15, 17, 20, 22, 25, 31, 40–42, 45, 47), and the results obtained in these three systems are quite different (see below). To gain more insight into the mechanism of CAP action we undertook a study of a fourth promoter, namely, the one which controls the expression of *malT*, the positive regulator gene of the maltose regulon (7, 10, 11, 36). This system seemed relatively simple since the activation by CAP and cAMP is the only regulation known to be exerted on *malT* expression (6, 10). We determined the site at which CAP binds and demonstrated that it enhances the binding of RNA polymerase at the *malT* promoter and that it stimulates the initiation of transcription at this promoter, presumably by enhancing RNA polymerase binding.

MATERIALS AND METHODS

Strains and media. The following strains of *E. coli* K12 were used. pop3, which was isolated under the designation MC4100 by M. Casadaban (10), is F^- *araD139 Δlac-169 rpsL relA thiA*. pop3931 derives from pop3 and carries the *malTp1* mutation (6, 35). Strain JM83 (30) was supplied by P. Stragier and is *ara Δ(lac-pro) thi rpsL* [φ80 d*lacZ* Δ(*lacM15*)].

Complete medium (ML) and synthetic medium (M63) were as previously described (6). Ampicillin was used at 50 μg/ml in solid and liquid media.

Materials. Purified CAP was kindly given by B. Blazy. It was more than 98% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Its concentration was determined spectrophotometrically by using $\varepsilon_{278} = 4.1 \times 10^4$ M/cm per CAP dimer (44).

Purified *E. coli* RNA polymerase was kindly given by A. Spassky. It was isolated by the procedure of Lowe et al. (23). Holoenzyme was separated from core by the method of Lowe et al. (23).

Plasmids pUR222 (38) and pUC9 (30) were kindly provided by O. Raibaud and P. Stragier, respectively.

Construction of plasmids. Plasmid pOM50 is a derivative of plasmid pUR222, in which we inserted a 208-base-pair (bp) *mal* DNA fragment. The construction of pOM50 involved two steps. We first purified an 802-bp *Hpa*II-*Hpa*II *mal* DNA fragment from plasmid pOM1 (36). This fragment carries the control region for both the *malT* gene and the *malPQ* operon. We cloned this fragment into the *Eco*RI site of pBR322 after treatment of both the fragment and the *Eco*RI-digested plasmid with DNA polymerase Klenow fragment (26). By this operation we created an *Eco*RI site on each side of the *mal* DNA segment. The 208-bp *Sau*3AI-*Eco*RI subfragment containing the *malT* promoter (Fig. 1A) was then cloned between the *Bam*HI and *Eco*RI sites of pUR222.

Plasmid pOM51 is a derivative of plasmid pUC9, in which we inserted a 206-bp *Hpa*II-*Sau*3AI *malT* DNA fragment carrying the *malTp1* mutation. The *malTp1* DNA fragment, purified from plasmid pOM1 *malTp1* (7), was cloned into the *Acc*I and *Bam*HI sites of plasmid pUC9. Plasmid pUC9 contains the beginning of the *lacZ* gene and is able to render strain JM83 Lac^+

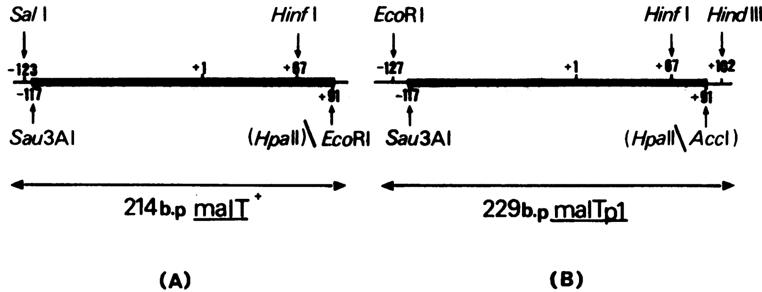


FIG. 1. Schematic representation of the 214-bp *malT*+ DNA fragment and the 229-bp *malTp1* DNA fragment. The *malT* fragment, represented as a heavy line, was sequenced previously (7, 9). The plasmid vectors are represented as a thin line. The position of relevant restriction sites is shown. The numbers are their distance, in bp, from the starting point of transcription (+1). Parenthesis indicate that the restriction site has been lost during the cloning step.

by α-complementation (30). The *mal* insertion at the *Acc*I-*Bam*HI sites led to a frameshift in the amino terminus of the *lacZ* sequence, preventing plasmid pUC9 from α-complementation of strain JM83. The resulting plasmid, pOM51, was then introduced into strain pop3931, which also harbors the *malTp1* mutation on its chromosome.

DNA labeling. The purifications of plasmid DNA and of restriction fragments were as described previously (9). The 214-bp *Eco*RI-*Sal*I *mal* fragment was labeled on the nontranscribed strand at its *Eco*RI site or on the transcribed strand at its *Sal*I site by published procedures with DNA polymerase Klenow fragment (26) and [α-^{32}P]dATP (nontranscribed strand) or [α-^{32}P]dTTP (transcribed strand). Labeled DNA was precipitated by spermine (18). The 194-bp *Hin*fI-*Eco*RI *malTp1* fragment was labeled on the nontranscribed strand at its *Hin*fI site with DNA polymerase Klenow fragment and [α-^{32}P]dATP.

DNA sequencing. DNA sequencing was performed by the technique of Maxam and Gilbert (28).

DNase protection experiments. DNase I "footprinting" was performed as described by Galas and Schmitz (16). DNA fragments (2.5 mM), ^{32}P labeled at one extremity, were incubated in buffer (50 μl, final volume) containing 40 mM Tris-hydrochloride (pH 8.0), 30 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg of bovine serum albumin per ml, and 5% glycerol. When present, 50 nM CAP and 200 μM cAMP were added first. Incubation proceeded for 3 min at 37°C. Incubation in the presence of various RNA polymerase concentrations (see Fig. 3), was then carried out for 20 min at 37°C. DNase I was added at a final concentration of 0.075 μg/ml for 15 s. The digestion was stopped by adding 8 μl of 3 M sodium acetate and 1 mg of tRNA per ml. The mixture was extracted with phenol, precipitated and rinsed with ethanol, and dried. The DNA was redissolved and loaded on a 10% urea-polyacrylamide gel as in DNA sequencing.

Transcription reactions. Transcription assays were performed with 5 nM DNA fragments. Incubation procedures were exactly as described for DNase I protection experiments, except that bovine serum albumin was omitted and the KCl concentration was 100 mM. Transcription was allowed to proceed for 10 min at 37°C after simultaneously adding 300 μM each ATP, CTP, and GTP, 90 μM UTP, 5 μCi of [α-^{32}P]UTP (3,000 mCi/mmol), and 0.15 mg of heparin per ml. The reaction was stopped with 0.35 M sodium acetate and 9 mM sodium EDTA (pH 8.0). DNA was precipitated with ethanol, dissolved, and loaded on a 7% urea-polyacrylamide gel as in DNA sequencing.

In the case of the experiment described in Fig. 4C, [γ-^{32}P]ATP was used as the labeled nucleotide instead of [α-^{32}P]UTP. The procedures were as described above except that we used 50 nM DNA, 300 μM UTP, 90 μM ATP, and 50 μCi of [γ-^{32}P]ATP (3,000 mCi/mmol).

RESULTS

CAP binding site at the *malT* promoter. In glucose-grown cells, *malT* expression is stimulated 5 to 10 times by CAP and cAMP (6, 10). Deletion analysis has shown that all of the DNA sequences required for activity of the *malT* promoter, including those necessary for the action of CAP and cAMP, are located less than 83 bp upstream from the transcription starting point (35). To localize more precisely the site at which the CAP-cAMP complex binds, we employed the DNase I footprinting technique of Galas and Schmitz (16). We used a 214-bp *Eco*RI-*Sal*I DNA fragment containing the *malT* promoter (see above and Fig. 1). Each strand was labeled separately at its 3′ end, and the resulting labeled DNA was incubated with DNase I in the presence or absence of CAP and cAMP. The concentration of CAP, when present, was 50 nM (this concentration was shown to be sufficient for binding at the *malT* promoter; Kolb et al., Nucleic Acids Res., in press). The results (Fig. 2) indicate that CAP protects the region extending from position −57 to −82, except that position −65 on the nontranscribed strand and −67 on the transcribed strand actually exhibited increased sensitivity to DNase I. The effects were not observed when cAMP was omitted from the reaction mixture. The region protected against DNase I presumably constitutes the CAP binding site at the *malT* promoter. It presents strik-

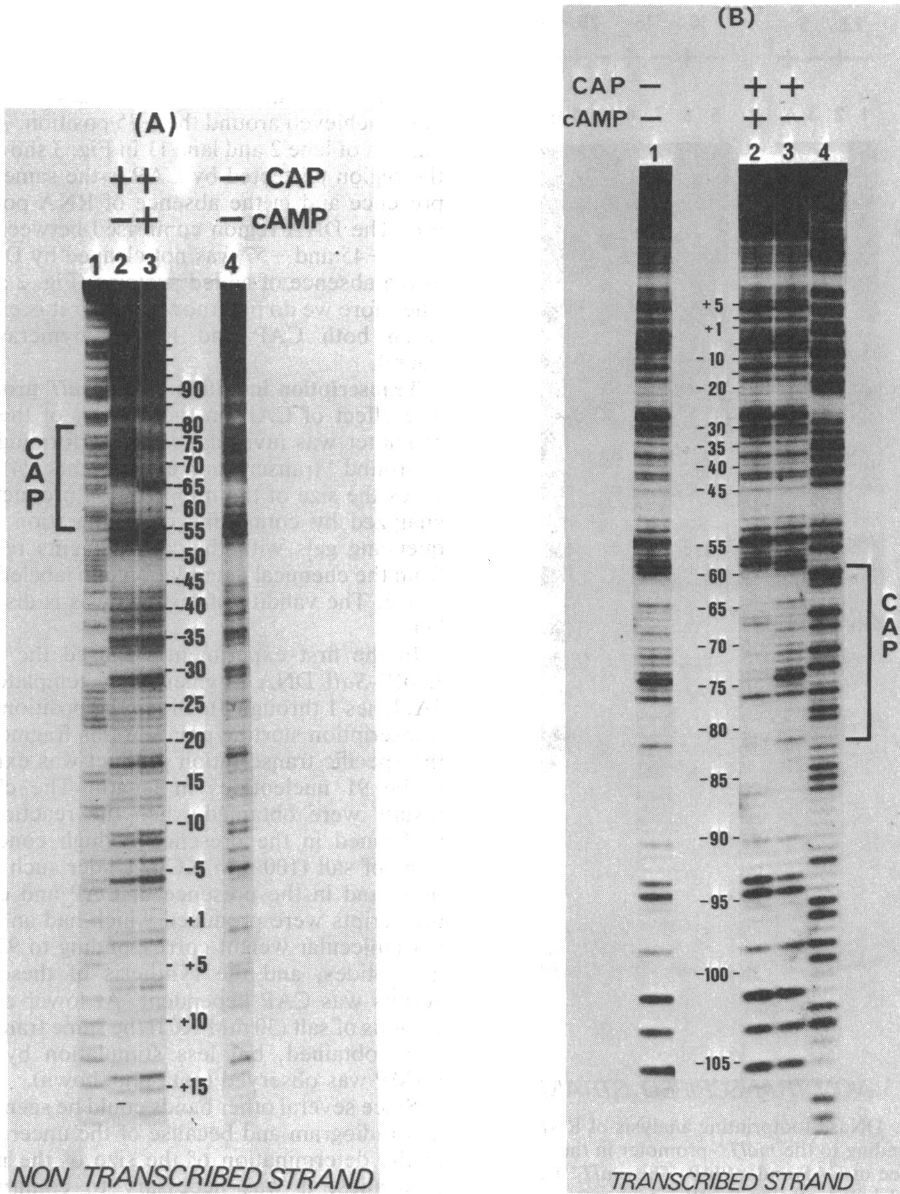


FIG. 2. DNase footprinting analysis of CAP-cAMP protection of *malT*+ DNA. The *malT*+ DNA fragment was end labeled at one or the other 3' end and digested under partial reaction conditions with DNase I as described in the text. The reaction products were fractionated on a 10% polyacrylamide–7 M urea gel. (A) Analysis of the nontranscribed strand. The *mal* fragment was labeled at the *Eco*RI site. Lanes: 1, A+G reaction; 2, CAP (50 nM); 3, CAP (50 nM) and cAMP (200 μM); 4, no CAP or cAMP. (B) Analysis of the transcribed strand. The *mal* fragment was labeled at the *Sal*I site. Lanes: 1, no CAP or cAMP; 2, CAP (50 nM) and cAMP (200 μ); 3, CAP (50 nM); 4, A+G reaction.

ing homologies with the CAP binding sites found in other systems (see below). Attempts to determine the bases that make contacts with CAP by studying the protection exerted by CAP against methylation of the DNA by dimethyl sulfate (45) were unsuccessful; no protection was observed.

CAP and cAMP enhance the binding of RNA polymerase at the *malT* promoter. The binding of RNA polymerase to the nontranscribed strand of the 214-bp *Eco*RI-*Sal*I DNA fragment was analyzed with the DNase I footprinting technique described above (Fig. 3). In the absence of CAP and at a high RNA polymerase concentration (25 nM), there was a general decrease in the

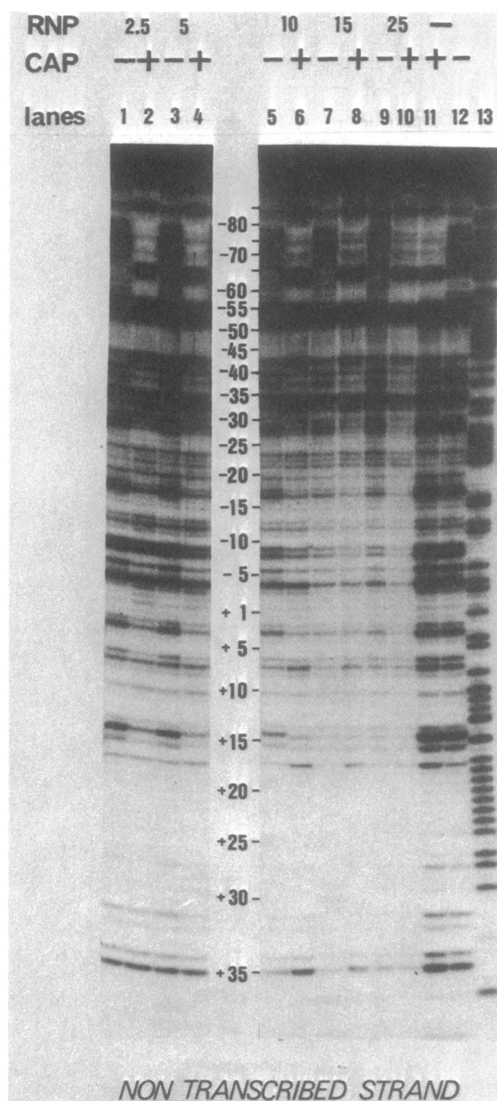


FIG. 3. DNase footprinting analysis of RNA polymerase binding to the *malT*⁺ promoter in the absence or presence of CAP and cAMP. The *malT*⁺ fragment was end labeled at the *Eco*RI site (nontranscribed strand). The partial DNase I digestion was conducted as in Fig. 2. The nanomolar concentrations of RNA polymerase (RNP) are indicated. When present, CAP was at 50 nM. cAMP (200 μM) was added in every sample. Lane 13, A+G reaction.

intensity of the bands corresponding to the different DNase I cleavage products, compatible with a nonspecific binding of the polymerase all along the fragment (Fig. 3, lanes 9 and 10). In the presence of CAP and cAMP (CAP at 50 nM), and at lower concentrations of RNA polymerase (2.5 and 5 nM), specific alterations of the DNase I digestion pattern were observed (Fig. 3, lanes 1 through 4). Even though these alterations were not as clear as those resulting from the binding of CAP between positions −57 and −82, they indicated binding of RNA polymerase between positions −45 and +15, the strongest protection being achieved around the −35 position. A comparison of lane 2 and lane 11 in Fig. 3 shows that the region protected by CAP is the same in the presence and in the absence of RNA polymerase. The DNA region comprised between positions −45 and −57 was not cleaved by DNase I in the absence of added proteins (Fig. 2 and 3). Therefore we do not know whether it is exposed when both CAP and RNA polymerase are bound.

Transcription initiation at the *malT* promoter. The effect of CAP on the activity of the *malT* promoter was investigated by performing "single-round" transcription experiments (24). In all cases the size of the transcription products was analyzed by comparing their migration on sequencing gels with that of fragments resulting from the chemical degradation of a labeled DNA probe. The validity of this analysis is discussed below.

In the first experiment we used the 214-bp *Eco*RI-*Sal*I DNA fragment as a template (Fig. 4A, lanes 1 through 3). Given the position of the transcription starting point on this fragment (7), the specific transcription product was expected to be 91 nucleotides in length. The clearest results were obtained when the reaction was performed in the presence of high concentrations of salt (100 mM KCl). Under such conditions and in the presence of CAP and cAMP, transcripts were produced which had an apparent molecular weight corresponding to 92 to 95 nucleotides, and the synthesis of these transcripts was CAP dependent. At lower concentrations of salt (30 mM KCl) the same transcripts were obtained, but less stimulation by CAP-cAMP was observed (data not shown).

Since several other bands could be seen on the autoradiogram and because of the uncertainties in the determination of the size of the mRNA molecules, it was necessary to confirm that CAP-dependent transcripts were authentic *malT* transcripts. Additional evidence that this was the case was obtained by predigesting the DNA template with *Hin*fI, which cleaves 24 bp upstream from the *Eco*RI end of the fragment (Fig. 1); correspondingly shorter CAP dependent transcripts were obtained (Fig. 4A, lanes 4 and 5; Fig. 4B, lanes 1 and 2).

Previous in vivo mapping of the transcription starting point had shown that *malT* mRNA starts with an A (7). In agreement with this result we found that the in vitro-synthesized, CAP-dependent transcripts were also labeled when we used [γ-^{32}P]ATP instead of [α-^{32}P]UTP in the transcription reaction (data not shown). No labeling

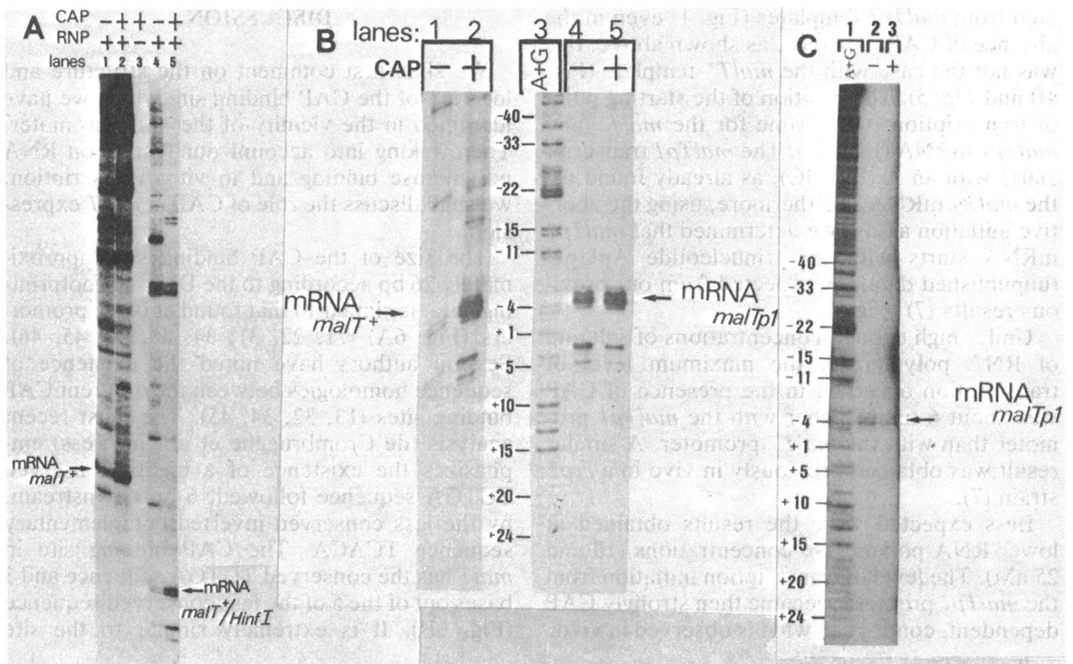


FIG. 4. In vitro transcription from *malT*+ and *malTp1* DNA templates. Reaction conditions are described in the text. cAMP (200 μM) was added in every sample. (A) In lanes 1, 2, and 3, the 214-bp *Eco*RI-*Sal*I *malT*+ DNA fragment (5 nM) was used as a template. In lanes 4 and 5, the 190-bp *Hin*fI-*Sal*I *malT*+ DNA fragment (5 nM) was used as a template (Fig. 1A). The concentration of RNA polymerase (RNP) was 10 nM. When present CAP was at 100 nM. (B) In lanes 1 and 2, the 190-bp *Hin*fI-*Sal*I *malT*+ DNA fragment (5 nM) was used as a template. In lanes 4 and 5, the 194-bp *Hin*fI-*Eco*RI *malTp1* fragment (5 nM) was used as a template (Fig. 1B). The concentration of RNA polymerase was 37.5 nM. The concentration of CAP when present was 100 nM. Lane 3, A+G reaction. (C) Transcription reaction from the *Hin*fI-*Eco*RI *malTp1* DNA fragment (50 nM) with [γ-^{32}P]ATP as the labeling nucleotide. The concentration of RNA polymerase was 100 nM. Lanes: 1, A+G reaction; 2, absence of CAP; 3, 1 μM CAP.

was observed when using another γ-^{32}P-labeled nucleotide.

Although all of the evidence strongly suggests that the above-mentioned CAP-dependent in vitro transcripts were really initiated at the same point as the *malT* transcript obtained in vivo, examination of the data in Fig. 4 deserves further comments.

First, the CAP-dependent transcripts display some heterogeneity in size, consisting mainly in a family of four molecules differing in length from one another by one nucleotide and of two additional molecules of slightly lower size. Since this heterogeneity was also found when labeling with [γ-^{32}P]ATP (data not shown; Fig. 4C), it could not have resulted from the existence of a multiple choice in the initiation starting point (4, 5). Rather, it must have resulted from an imprecision in the termination point (12).

Second, the size of the transcripts seemed in all cases slightly longer than expected (by four to seven nucleotides). The main reason for this discrepancy is probably that RNA molecules do not migrate exactly like DNA fragments. However other factors must also be involved. Chemical degradation of the DNA eliminates the modified base and leaves a fragment with a phosphorylated 3′ end, whereas the transcripts have a 3′-OH end but a triphosphate at the 5′ end. In addition, in Fig. 4B and C the transcribed strand was longer by two nucleotides at its 5′ end than the strand that was labeled before chemical degradation.

Effect of an up-promoter mutation on in vitro transcription. The "Pribnow box" of the *malT* promoter, GACCTT, is rather atypical, and this probably accounts, at least in part, for its inefficiency (7). A previously described mutation, *malTp1*, was found to convert it to the sequence TACCTT, closer to the consensus sequence TATAAT (7, 37). The effect of *malTp1* was to increase 5- to 10-fold the expression of *malT* in a *crp*+ background and to render it almost completely independent of the presence of CAP and cAMP. These effects could be reproduced in vitro, at least when using a high enough concentration of salt (100 mM KCl) and of RNA polymerase (100 to 200 nM). Transcription was initi-

ated from *malTp1* templates (Fig. 1) even in the absence of CAP, whereas, as shown above, this was not the case with the *malT*⁺ template (Fig. 4B and Fig. 5). The position of the starting point of transcription is the same for the *malT*⁺ and *malTp1* mRNA (Fig. 4B). The *malTp1* transcript starts with an A (Fig. 4C), as already found for the *malT*⁺ mRNA. Furthermore, using the abortive initiation assay, we determined that *malTp1* mRNA starts with the trinucleotide ApUpU (unpublished data), as expected from our previous results (7) (Fig. 6).

Under high enough concentrations of salt and of RNA polymerase, the maximum level of transcription observed in the presence of CAP was about 6 times higher with the *malTp1* promoter than with the *malT*⁺ promoter. A similar result was obtained previously in vivo in a *crp*⁺ strain (7).

Less expected were the results obtained at lower RNA polymerase concentrations (10 and 25 nM). The level of transcription initiation from the *malTp1* promoter became then strongly CAP dependent, contrary to what is observed in vivo.

DISCUSSION

We shall first comment on the structure and location of the CAP binding site which we have identified in the vicinity of the *malT* promoter. Then, taking into account our results on RNA polymerase binding and in vitro transcription, we shall discuss the role of CAP in *malT* expression.

The size of the CAP binding site, approximately 26 bp according to the DNase I footprinting data, is similar to that found at other promoters (Fig. 6A) (21, 22, 31, 34, 40, 42, 45, 46). Several authors have noted the existence of sequence homologies between the different CAP binding sites (13, 32, 34, 45). The most recent analysis (de Crombrugghe et al., in press) emphasizes the existence of a highly conserved TGTGA sequence followed, 6 bp downstream, by the less conserved inverted complementary sequence TCACA. The CAP binding site in *malT* has the conserved TGTGA sequence and 3 bases out of the 5 of the less conserved sequence (Fig. 6B). It is extremely similar to the site

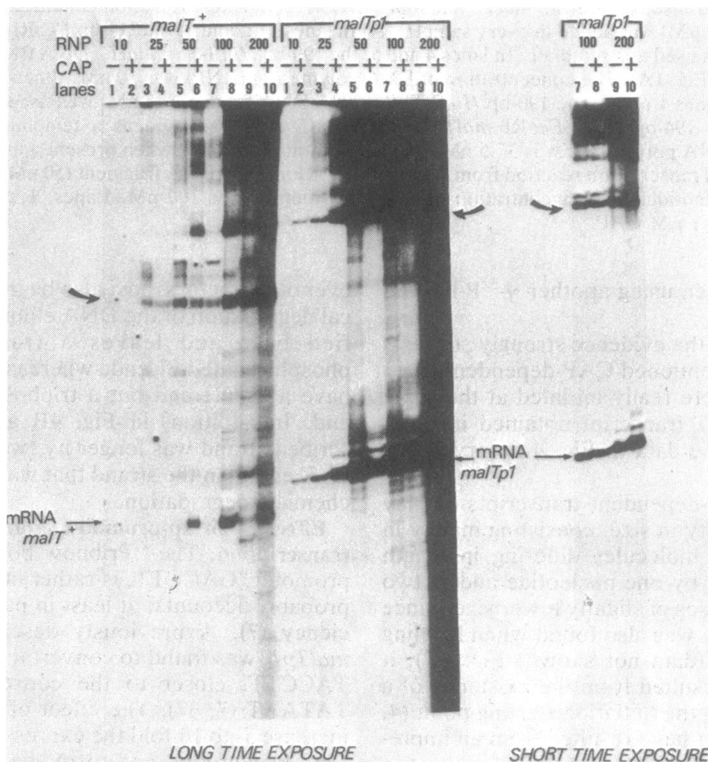


FIG. 5. Effect of RNA polymerase concentration on in vitro transcription from a *malT*⁺ or *malTp1* DNA template. In vitro transcription experiments were conducted as in Fig. 4. Either the 214-bp *Eco*RI-*Sal*I *malT*⁺ DNA fragment (5 nM) or the 229-bp *Hin*dIII-*Eco*RI *malTp1* DNA fragment (5 nM) was used as a template as indicated. Since the *malTp1* DNA template is 11 bp longer than the *malT*⁺ DNA template at its 3' end, the *malTp1* transcript is longer than the corresponding *malT*⁺ transcript. The nanomolar concentrations of RNA polymerase (RNP) are indicated. Other details are as for Fig. 4.

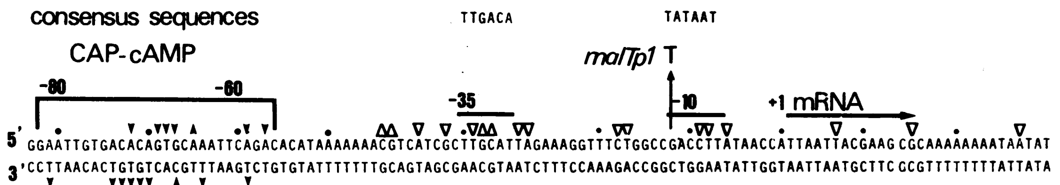
involved in the common control for *araC* and *araBAD* (22, 31).

One point of special interest with respect to the mechanism of CAP action is the position of the CAP binding site relative to the transcription startpoint. Three situations have been described. In the first one, exemplified by the *gal* promoter, the CAP and RNA polymerase binding sites overlap, at least when examined on the linear DNA sequence. In the second, of which the *lac* promoter is the prototype, the sites are juxtaposed. In the third situation, found in the case of the *araBAD* promoter, the two sites are separated by a long stretch of DNA which constitutes a binding site for another activator protein, AraC in that particular example. The *malT* promoter seems to fall in the second class. However in this case the distance between the CAP site—taking as a reference the consensus TGTGA sequence—and the transcription starting point is 9 bp longer than in the *lac* promoter (42). Differences of this kind must be taken into account in models (32, 45a; de Crombrugghe et al., in press) that assume that CAP acts via a direct interaction with RNA polymerase. Essentially, they make it necessary to consider the promoter not as a rigid linear molecule, but as a more complex, three-dimensional structure.

Protection experiments against DNase I have also shown that CAP and cAMP are required for the correct binding of RNA polymerase at the wild-type *malT* promoter. Transcription initiation at this promoter also required the presence of CAP and cAMP at all the RNA polymerase concentrations tested. In contrast a mutant promoter, altered at position −12 and known to allow CAP-independent *malT* expression in vivo, was active in vitro even in the absence of CAP. However this independence from CAP was not absolute since this protein was required, even with the mutant promoter, at low RNA polymerase concentrations.

These observations suggest that the wild-type promoter has a low affinity for RNA polymerase and does only bind it in the presence of the CAP-cAMP complex. The mutant promoter seems to have a higher affinity for RNA polymerase and, provided the enzyme is present at a high enough concentration, can bind it even in the absence of CAP. However in this situation CAP still stimulates the binding of RNA polymerase at lower RNA polymerase concentrations. Taken together these results suggest that, as already proposed in studies dealing with the *lac* promoter (24, 27; P. Malan, Ph.D. thesis, Harvard University, Cambridge, Mass., 1980), CAP facilitates the initial binding of RNA polymerase. In the case of the *lac* promoter and using the abortive

(A)



(B)

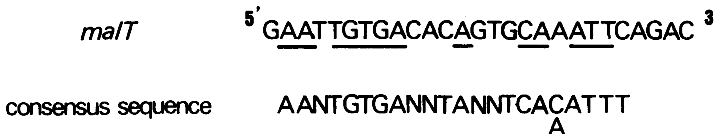


FIG. 6. Summary of DNase I protection data. (A) Presented here are 113 bp of the *malT* gene (sequenced by Débarbouillé et al. (9) with the base pair notation relative to the starting point of transcription. Dark arrows indicate either protection from (▼) or enhancement of (▲) DNase I cleavage at the 3' side of the indicated base after binding of the CAP-cAMP complex. Open arrows indicate either protection from (▽) or enhancement of (△) DNase I cleavage after binding of RNA polymerase in the presence of the CAP-cAMP complex. Heavy lines indicate the position of the so-called −10 (Pribnow box) and −35 regions of the promoter. The horizontal arrow indicates the beginning of the mRNA (7). The CAP-cAMP binding site is represented by parentheses. The base change corresponding to *malTp1* is also shown (7). (B) Comparison of the CAP-cAMP binding site at the *malT* promoter (first line) with the consensus sequence for the CAP-cAMP binding site proposed by de Crombrugghe et al. (in press) (second line). Bases that are homologous in both the *malT* DNA sequence and the consensus sequence are underlined.

initiation assay Malan has shown that CAP does increase the initial binding of RNA polymerase, i.e., the formation of the "closed complex" rather than the subsequent isomerization of the closed complex into the "open complex." Further work will be required to decide whether the same holds true for the *malT* promoter.

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