MalR-mediated Regulation of the Streptococcus pneumoniae malMP Operon at Promoter $P_M$

INFLUENCE OF A PROXIMAL DIVERGENT PROMOTER REGION AND COMPETITION BETWEEN MalR AND RNA POLYMERASE PROTEINS*

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The Streptococcus pneumoniae mal regulon contains two operons, malXCD and malMP involved in the uptake and utilization of maltosaccharides. Both operons are transcribed from two divergent promoters, $P_X$ and $P_M$, and are negatively regulated by the MalR transcriptional repressor. Purified MalR protein binds to two DNA regions that encompasses both promoters, thus occupying its two operators, $O_M$ and $O_X$. However, the levels of occupation and repression were different, being higher when MalR was bound to $O_M$ than when it was anchored to $O_X$. Competition experiments between MalR and the Escherichia coli RNA polymerase on promoters $P_M$ and $P_X$ showed that the affinity of either protein for the promoter/operator DNA sequences was important to determine the frequency of transcription initiation. In addition to the control exerted by MalR, expression from promoter $P_M$ was affected by upstream sequences located within or close to $P_X$ promoter.

Initiation of transcription in prokaryotes is the stage usually controlled by positive and negative regulators (1–3). In many of the known instances of repressor proteins, they generally bind to specific DNA sequences, which are located close to or within the promoter, although the binding position of the repressors seems to vary, in contrast to the relatively fixed binding positions of the activator sites (4). As a consequence, repressors may hinder the binding of the RNA polymerase (RNAP)† to the promoter, thus interfering with the transcription initiation process (3, 5). The mechanisms of transcription inhibition by repressor proteins have been mainly studied in the case of Gram-negative bacteria, especially in Escherichia coli (6) and in some of its extrachromosomal genetic elements, in which examples of inhibition of transcription at different stages have been reported. Repression by phages P22-Arc and λ-cl proteins is exerted at the first step of initiation. These proteins compete with RNAP for binding to the free promoter, although mutational analyses have shown that both proteins may exhibit a more complex behavior (7, 8). A similar picture has been reported for the lac repressor, in which the binding of the protein to its DNA target hinders the access of RNAP to the promoter (9), whereas in the case of plasmid R6K-encoded KorB repressor, inhibition occurs at the isomerization stage (10). Repression mediated by GalR is due to a GalR-induced DNA loop at the promoter region, the protein inhibiting synthesis of both abortive products, and complete transcripts (11). There are more complex situations, like the AraC regulatory protein, in which the three AraC-DNA binding sites are positioned so that the protein can repress or activate the promoters located in the araCBAD region via DNA looping (see Ref. 12, and references therein). In the case of Gram-positive bacteria, several chromosome-encoded transcriptional repressors have been described, mainly from Bacillus subtilis (13–15), Staphylococcus aureus (16–18), and Streptomyces coelicolor (19). However, information on their mechanism of repression at the molecular level is still scarce.

Over a number of years, we have been studying the mal regulon of the Gram-positive bacterium Streptococcus pneumoniae (Fig. 1). This regulon is composed of three operons, two of them involved in maltosaccharide uptake (malXCD) and its utilization (malMP), the third one (malAR) being involved in regulation of the other two operons (20, 21). The two former operons are transcribed from two divergently oriented promoters, termed $P_M$ (for the malMP operon) and $P_X$ (for the malXCD operon), which are negatively regulated by the product of gene malR (22). Protein MalR belongs to the LacI-GalR family of transcriptional repressors (23) and binds specifically to two operator sequences located in the intergenic region between operons malXCD and malMP (22). However, purified MalR protein was shown to bind more tightly to the malMP operator sequence ($O_M$) than to the malXCD ($O_X$), even though both operators differ only by two nucleotides. The binding of MalR to its DNA target is inactivated by the addition of maltose (22).

In the present work, we have studied the occupancy of the promoter/operator regions of the malMP and malXCD operons by purified MalR and RNAP proteins through in vitro transcription, electrophoretic mobility shift assays (EMSA), and DNase I protection. Identification of the initiation of transcription sites for both operons showed that promoters $P_M$ and $P_X$ are in the vicinity of two palindromic DNA sequences (the $O_M$ and $O_X$ operators, respectively), which are the sites where MalR protein binds (22). The target sites of MalR and RNAP overlapped, and both proteins competed for their binding to DNA. Affinities of MalR and RNAP for binding to their respec-
ative DNA sequences were important for the level of gene expression of both operons. In addition, gene fusions showed that neither promoter Pm nor operator Ox were needed for in vivo expression of the malMP operon, and that this region was also unnecessary for MalR-mediated repression of Pm. However, the DNA region upstream of the malMP promoter/operator region may play a role in modulation of transcription from Pm, as shown by mutational analyses of the Pm/Ox DNA region.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—** *S. pneumoniae* R61 (wild type) harboring plasmid pLS70 was used for preparation of total RNA. Over expression of the malR gene was performed in *E. coli* BL21(DE3) using the pET21b (Novagen)-derived plasmid pMRrey, in which the malR gene is cloned as a fusion protein harboring a C-terminal His6 tag. Plasmid pLS70 contains a 3.5-kilobase pair PstI DNA fragment of the *S. pneumoniae* chromosome cloned into the streptococcal plasmid pMV158 and harbors part of the mal region including promoters Pm and Pm (24). Plasmid pLS1MGFP was constructed like the previously described pLS1GFP (25), but digesting the parental pCL1GFP with *SalI* and *HindIII*, cloning this fragment into plasmid pJD9 (26), and then into pLS1 (27). In plasmid pLS1MGFP, the sequence encompassing promoter Pm is removed, and the reporter gfp gene (encoding a green fluorescent protein (GFP)) is placed under the control of promoter Pm. The nucleotide sequences of the pneumococcal inserts in these plasmids, pLS1GFP and pLS1MGFP, were determined and shown to be identical to the corresponding pneumococcal chromosomal sequence. In addition to the above, plasmid pLS1Er was constructed by substitution of the EcoRl-HindIII fragment of pLS1 by a C1at-Smal fragment of pJDC9, so that the cloned fragment harbors the eren gene (encoding resistance to erythromycin (Er)). Selection was applied for resistance to tetracycline (1 µg/ml; pLS70, pLS1GFP, pLS1MGFP, and pLS1mGXFP), erythromycin (1 µg/ml; pLS1Er, pLS1GFP, pLS1MGFP, and pLS1mGXFP), or ampicillin (200 µg/ml; pMRerp).

**Oligonucleotides and Polymerase Chain Reaction Amplification—** Mutagenic oligonucleotides were used in the PCR amplification reactions to obtain DNA templates, either for EMSA assays (oligonucleotides 1–4) or for site-directed mutagenesis (mut1, mut2, mut3, and mut4). Their co-ordinates (22) are given in parentheses: 1, 5'-GTGTA-ACAGTTCAACGACC-3' (1170–1190); 2, 5'-TCCAGTCTGATCC-TTCGTTG-3' (1852–1812); 3, 5'-GGGATTAGAACAGGAGGATTG-3' (1457–1487); 4, 5'-TACCTCCTGGTGTTCAAATCC-3' (1467–1487); mut1, 5'-GCAAGCTTCTTCCTATGCATGCCAA-3' (1313–1275); mut2, 5'-CTTTATGACTGTTAGGTGGCTAA-GAAGCGTGTC-3' (1275–1313); mut3, 5'-GCAAGTTACCTTTTATGGTAAAGAAAC (1241–1262); mut4, 5'-CGGGAGTGTCATCTCG-AGATTTGTTCGACGACAGCA-3' (1730–1711).

The latter two oligonucleotides contained recognition sites (underlined) for the restriction enzymes EcoRI (mut3), and BamHI and XbaI (mut4).

The three DNA fragments used, namely *Xm, X*, and *M*, were amplified by PCR using oligonucleotides 1-2, 1-3, or 2-4, respectively. Amplification was done during 20 cycles using the *Pfu* DNA polymerase (Stratagene) and, as template, plasmid pLS70 DNA (22). The fragments obtained had blunt ends. When the fragments were used for DNase I footprinting assays, synthesis of the DNA fragments by PCR was carried out after 5'-end labeling of one of the primers with [γ-32P]ATP and polynucleotide kinase (28).

**Overproduction and Purification of MalR—** To increase the solubility of the MalR protein, we modified the method previously described (22). To this end, *E. coli* BL21(DE3) cells harboring plasmid pMRrey were induced as described (21). Cells were suspended in buffer A containing 20 mM Na2HPO4, pH 7.6, supplemented with 1 mM NaCl, and disrupted by passage through a French pressure cell. The cell lysate was cleared by low and high speed centrifugation, and the supernatant was passed through an immobilized metal ion affinity chromatography column (chelating sepharose fast flow, Amersham Pharmacia Biotech), eluting the protein with buffer B (1 mM NaCl, 20 mM Na2HPO4, pH 7.6) containing 500 µM imidazole. The eluate, containing MalR purified almost to homogeneity, was dialyzed against buffer C (200 mM NaCl, 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and stored at −80°C. No loss of DNA binding activity was observed during 1-year storage.

**Mapping of Initiation of Transcription Start Points and in Vitro Transcription Assays—** Total RNA was isolated from *S. pneumoniae* R61, and endonuclease S1 protection and primer extension assays were performed as described (23). The in vitro transcription assays were carried out as templates the three DNA fragments (*X, Xm, M*), which harbor promoters Pm, Pm, or both, respectively. Transcription reactions (50 µl) contained 2 mM amounts of template DNA, ATP, CTP, GTP (200 µM each), UTP (50 µM), and 0.25 µM [γ-32P]UTP (2.5 µCi), 12 units of the bacteriophage T7 RNA polymerase (Stratagene) at 15°C, 10 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 200 mM NaCl, 0.1 mM EDTA, 5% glycerol, and 1 µg of poly(dI-dC) (Amersham Pharmacia Biotech). Reactions were started by the addition of RNAP (purchased from Roche Molecular Biochemicals) (0.165 units; 22). When the effect of MalR on transcription was assayed, the mixtures received this protein at the concentrations indicated under “Results and Discussion.” When RNAP was replaced by a mixture of MalR, the proteins were added in the reaction mixtures, and they were added simultaneously to the MalR protein. Reaction mixtures were incubated 15 min at 37°C, and then stop buffer (final concentration of 300 mM sodium acetate, pH 8.0, 15 mM EDTA, and 0.1 µg/µl of tRNA) was added. Nucleic acids were ethanol-precipitated, and the transcripts were separated by 6% PAGE, 8 µa urea sequencing gels. Results were quantified from three different experiments by means of the storage phosphor technology, with the aid of a PhosphoImager equipment and the ImageQuant software (Molecular Dynamics). The approximate size of the run-off transcripts were determined by comparison to the length of the sequence ladder of the same DNA template.

**DNase I Footprint Experiment—** The XM-DNA fragment (synthesis of Pm) was 5'-end labeled by PCR. Reactions were done in the presence of buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 2.75 mM CaCl2, 1 mM dithiothreitol, 0.1 mM EDTA), supplemented with 200 mM NaCl and 500 ng of calf thymus DNA. Mixtures were incubated with RNAP (Roche Molecular Biochemicals, at the specific activity of 1 unit/µl; 200–400 units/µg of protein) and/or MalR proteins (37°C, 15 min), prior to digestion with 0.042 units of DNase I (Worthington, 2.15 units/µl), 5 min at room temperature. Reactions were stopped by addition of 25 µl of stop buffer (2 mM ammonium acetate pH 7.5, 0.15 M EDTA, 0.8 M sodium acetate, pH 7, 0.1 µg/µl calf thymus DNA, and 0.4 µg/µl tRNA). Samples were ethanol-precipitated, and the DNA products were separated by 6% denaturing polyacrylamide gel electrophoresis (28).

**EMSA with MalR and/or RNAP Proteins—** DNA binding reactions (30 µl) contained 20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 10 mM MgCl2, 200 mM NaCl, and 5% glycerol. Purified MalR protein (0.25–0.900 µM) and/or RNAP (36 nM) was mixed with 32P-labeled DNA (0.9 µM), and 1 µg of poly(dI-dC). Reaction mixtures were incubated at 37°C, 15 min. Free and bound DNA forms were separated by native PAGE, using 5% gels for the assay with the X (318-bp) or the M (365-bp) DNA fragments, and 4% gels when the XM (684-bp) DNA fragment was used. To separate the half-lives of RNAP-DNA, 30 µl reaction mix were added to a solution containing 0.9 nM 32P-labeled X or M DNA fragments, and incubated 15 min at 37°C. Then, an excess of heparin (10 µg) or 300-fold molar excess of competing unlabeled DNA fragment was added. Samples were withdrawn and applied to a running 5% polyacrylamide gel at 7°C. The samples were analyzed by autoradiography, and the DNA bands were quantified by densitometry.

**Measurement of GFP Activity—** Cells harboring plasmids (pLS1Er, pLS1GFP, pLS1MGFP, or pLS1mGXFP) were grown in medium containing 0.8% sucrose or maltose to middle exponential phase (OD600 of 0.4, about 3 × 106 colony-forming units/ml). Cells were pelleted by centrifugation and suspended in PBS buffer (10 mM Na2HPO4, 140 mM
NaCl, 3 mM KCl), pH 7.2. Fluorescence was determined on a LS-50B spectrophotometer (PerkinElmer Life Sciences) by excitation at 488 nm and detection of emission at 510 nm. All experiments were performed at least three times. To measure GFP synthesis in a real-time scale, a background fluorescence, cells harboring plasmid pLS1Er were used, and their fluorescence during various periods of time. As the control for background fluorescence, the values were subtracted in each experiment.

RESULTS AND DISCUSSION

Transcription Initiation from Promoter $P_M$—Promoters $P_X$ and $P_M$ are placed in a divergent orientation within the non-coding intergenic region between operons malXCD and malMP (Fig. 1A). Hydroxyl radical interference assays showed that the MalR binding sites, the operators O$_X$ and O$_M$, are placed just downstream of the promoters (22). The DNA sequence around $P_X$ (but not around $P_M$) has a perfectly conserved “extended –10 region” (5’-TgTGcTATAcT-3’) and a good –35 region (5’-TTGcaA-3’). Such an extension of the –10 region is commonly associated to strong promoters that may lack the –35 region both in S. pneumoniae (29) and E. coli (3). Initiation of transcription from $P_X$ (23) showed the target of MalR protein at this promoter, the O$_X$ operator (22), was located downstream of the +1 start point (Fig. 1B).

To map the initiation of transcription from promoter $P_M$, total mRNA was prepared from S. pneumoniae, and primer extension and endonuclease S1 assays were performed. Primer extension assays yielded a 130-nt protected band (Fig. 2A), whereas the major band observed by S1 mapping was 129 nt long (Fig. 2B). These results positioned the initiation of the malMP mRNA just at the beginning of the O$_M$ operator at this promoter (Fig. 1C). It was important to determine whether initiation of transcription of the malMP operon in both S. pneumoniae and E. coli occurred at the same position. Since the pneumococcal RNAP is not available to us, E. coli RNAP-directed in vitro transcription assays were carried out. As templates, the DNA fragments M (Fig. 2C) or XM (Fig. 3), were used. The former fragment contained only promoter $P_M$, whereas the latter harbored both $P_M$ and $P_X$. The results showed the synthesis of a run-off transcript of 130–131 nt (Fig. 2C), which placed the transcription initiation point from promoter $P_M$ at the same position than that obtained for S. pneumoniae. In the case of $P_X$ in vitro transcription assays, using the X or the XM DNA fragments, showed synthesis of two run-off products of 110 and 112 nt (Fig. 3). The sizes of these transcripts are in accordance with the transcription initiation
point previously determined for the malXCD operon in pneumococcal cells (23). We conclude that: (i) promoters $P_X$ and $P_M$ are functional in S. pneumoniae and E. coli, (ii) the promoters are equally recognized by both bacterial RNAP, and (iii) initiation of transcription takes place at the same position in both hosts. An interesting feature of the DNA regions upstream of promoters $P_X$ and $P_M$, is the very high A+T-content (over 90%) from positions −40 to −65 (Fig. 1). These upstream regions share the consensus sequence found for UP elements in several promoters, both at their distal (AAA(a/t)(a/t)TTTT) and proximal (AAAA) regions (30), indicating that promoters $P_X$ and $P_M$ may have UP elements with which the α-subunit of RNAP would contact (3).

MalR Differentially Represses Transcription from Promoters $P_M$ and $P_X$ in Vitro—Previous results obtained by transcriptional fusions between promoters $P_X$ and $P_M$ with two reporter genes indicated a preferential MalR repression on $P_M$ (22). To determine the degree of transcriptional repression by MalR protein on these promoters, direct measurements were performed by in vitro transcription assays. To this end, DNA fragments containing $P_X$, $P_M$, or both promoters (fragments X, M, or XM, respectively, as schematized in Fig. 3A) were used as templates to support RNAP-directed synthesis of run-off RNA products in the presence and absence of MalR. Reaction mixtures received increasing amounts of purified MalR protein, and transcription assays were initiated by addition of RNAP.

The results showed that synthesis of the run-off transcripts (130–131 nt) from $P_M$ was strongly inhibited by MalR, even at the lowest protein concentration tested (Fig. 3B). The levels of MalR-mediated repression from $P_M$ were similar for both the M or the XM DNA fragments. In the case of promoter $P_X$ two main transcripts (110 and 112 nt) were synthesized, as expected from transcription initiating from the previously determined start point (23). However, MalR-mediated inhibition was only observed at the highest protein concentration (2.7 μM), a result found for both X or XM DNA fragments (Fig. 3B).

Quantification of the results indicated that full MalR-mediated repression of transcription from $P_M$ was achieved at protein concentrations above 1.62 μM, whereas repression from $P_X$ (at MalR concentrations of 2.7 μM) was at most 35% (fragment X) or 45% (fragment XM) of the value obtained in the absence of the repressor. In the absence of MalR, RNA synthesis from $P_M$ was about 6 times more efficient when the template carried only this promoter (fragment M) than when both promoters were present (fragment XM), which was not the case for transcription from $P_X$ (Fig. 3B). These findings suggested that RNAP may have a preferential recognition of promoter $P_X$. If this were the case, transcription of the malMP operon could be reduced when transcription of the malXCD operon was fully functional, due to sequestering of RNAP. Thus, a delicate interplay between MalR and RNAP in the recognition of the
promoters/operators sequences may take place, so that MalR-mediated repression would be much stronger on promoter \( P_M \) than on \( P_X \), whereas RNAP recognition would be the opposite. Consequently, we can postulate that the main operator for the binding of MalR is \( O_M \), although the presence of auxiliary operators (like \( O_X \)) could be required for maximum repression in vivo, as in the case of the LacI repressor and its operator sequences \( O_1, O_2, \) and \( O_3 \) (31). Alternatively, existence of DNA sequences leading to promoter interference could also explain the above results (see below).

MalR repression was preferential on promoter \( P_M \), and the regions protected by the repressor were previously located downstream \( P_M \) (22). To know the relative position of the RNAP-binding sites within the \( P_M/O_M \) region, the regions protected by RNAP were determined by DNase I footprinting assays, and these protected regions were compared with those generated by MalR. The results (not shown) indicated that RNAP covered \(-69 \) nt on the promoter \( P_M \) region, the footprints spanning from positions \(-47 \) to \(+22 \) (see Fig. 1C). This protection pattern is typical for most of the RP\( _O \) complexes, and lies within the upstream limits found for short contacted areas and the normal downstream contact border (32). Bands showing hypersensitivity to DNase I cleavage were located at \(-37 \) and \(-45 \), plus an additional band at \(+18 \) (see Figs. 5, A and C). Appearance of enhanced bands at these positions could be due to the formation of a DNA bend at this region (see Ref. 5, and references therein). Consequently, we conclude that the regions protected by RNAP on promoter \( P_M \) overlap with the \( O_M \) operator.

**Displacement of MalR by RNAP at Promoter \( P_M \)—**To determine the differential affinity of MalR and RNAP for binding to promoter \( P_M \), we performed in vitro transcription experiments in the presence of increasing amounts of either protein, and using as template the M-DNA fragment (Fig. 3). In these assays, MalR or RNAP were first incubated with the template DNA prior to the addition of the competing protein to the reaction mixtures, and transcription was initiated by addition of the NTPs. When the competing protein was RNAP, it was apparent that the polymerase was able to displace the already bound MalR to its \( O_M \) target (Fig. 4A) despite the long half-life of MalR-\( O_M \) complexes (more than 90 min). Quantification of the results indicated that about 264 nm of RNAP were required to reach 90% of the synthesis obtained with RNAP alone (Fig. 4B). Thus, the rate of dissociation of the MalR-\( O_M \) complex, in the presence of high concentrations of RNAP, would account for the frequency of transcription initiation from \( P_M \). When the converse experiments were performed, it was found that MalR was able to displace the RNAP already bound to \( P_M \) (Fig. 4C), although transcription was reduced at most to about 40% in the presence of 2.7 \( \mu M \) of MalR (Fig. 4D). Comparison of these results with those obtained when RNAP and MalR were added simultaneously (Fig. 3B) showed that, at 1.08 \( \mu M \) MalR, transcription decreased only to 60% when RNAP was already bound to its target (Fig. 4D), whereas simultaneous addition of MalR and RNAP reduced transcription efficiency to 10% of the control (Fig. 3B, fragment \( M, \) lane 2). Thus, once a stable complex RNAP promoter is formed, displacement by MalR is inefficient, indicating that effective repression is exerted during the first
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steps of transcription. In the absence of MalR, RNAP would bind to promoter $P_M$, generating mostly RPO stable complexes, productive for transcription, and only the unstable complexes would be susceptible to competition by MalR and, as a consequence, to repression.

Competition between MalR and RNAP for Promoter $P_M$ Occupancy—The overlap of the RNAP- and MalR-induced footprints, and the in vitro transcription competition assays, suggested to us that the mechanism of MalR repression at $P_M$ could be due to competition between both proteins for promoter occupancy. To test this assumption, DNase I footprint and EMSA assays were performed in the presence of both proteins.

**TABLE I**

| Plasmid      | Fluorescence | Ratio (M/S)* |
|--------------|--------------|--------------|
|              | S            | M            |               |
| pLS1Er       | 0            | 0            | —             |
| pLS1GFP*     | 68           | 383          | 5.67          |
| pLS1mXGFP*   | 73.8         | 437          | 5.92          |
| pLS1MGFP*    | 41.9         | 493          | 11.7          |

* M/S corresponds to the ratio of fluorescence between cells grown in maltose versus cells grown in sucrose.

* Harbors promoters $P_M$ and $P_X$.

* Harbors promoters $P_M$ and mutated promoter $P_X$.

* Harbors only promoter $P_M$.

**Competition between MalR and RNAP for Promoter $P_M$ Occupancy**—The overlap of the RNAP- and MalR-induced footprints, and the in vitro transcription competition assays, suggested to us that the mechanism of MalR repression at $P_M$ could be due to competition between both proteins for promoter occupancy. To test this assumption, DNase I footprint and EMSA assays were performed in the presence of both proteins. In the DNase I footprint exclusion assay (Fig. 5), the XM-DNA fragment was terminally labeled in the coding strand of the malMP operon (Fig. 1B). RNAP (88 nM), and increasing amounts of MalR (0.54–1.62 μM) were added simultaneously, and the protection patterns were compared with those generated by MalR or RNAP to the two sites are indicated as $M_2$ or $R_2$, respectively. The band $R/P$ indicates the binding of both MalR and RNAP to the same fragment. In all cases, $F$ denotes the unbound DNA.
ing $P_M$ (fragment M), $P_X$ (fragment X), or both promoters (fragment XM). When fragment X was tested (Fig. 6, panel X), retarded bands due to binding of MalR (bands M) or to RNAP (bands R) were distinguishable and, when both proteins were present, the specific complex generated by RNAP (36 nM) was almost insensitive to increasing amounts (0.22–0.9 μM) of MalR. This was not the case when fragment M was assayed (Fig. 6, panel M), since the intensity of the single band generated by RNAP bound to $P_M$ was reduced as the concentration of MalR in the assay was increased. These results indicated to us that RNAP binds preferentially to the $P_X$/$O_X$ region, whereas MalR would bind preferentially to the region encompassing $P_M$/$O_M$.

A more complex pattern was found when the DNA fragment employed contained both promoters (Fig. 6, panel XM). In this case, two retarded bands were detected when RNAP alone was used (bands R1 and R2). These bands may correspond to RNAP bound to one promoter (either $P_X$ or $P_M$) or to both promoters. In that sense, when the amount of RNAP was increased, the RNAP-specific band of higher mobility (RNAP bound to $P_X$ or to $P_M$; band R1) was shifted to the position of lower mobility, corresponding to the RNAP bound to both promoters (band R2). MalR protein alone generated also two retarded bands, corresponding to its binding to operator $O_M$ (for which it has higher affinity; band M1), and to both operators ($O_M$ and $O_X$; band M2). Addition of increasing amounts of MalR to RNAP-DNA complexes showed the gradual disappearance of both RNAP-DNA complexes, and the appearance of a new complex migrating between both RNAP-promoter complexes (band R/P). We interpreted this latter band as corresponding to a ternary complex which involved RNAP bound mainly to promoter $P_X$ and MalR to the $O_M$ operator, within the same DNA molecules. To evaluate the involvement of MalR in the formation of these ternary complex, we took advantage of the inhibition of MalR-binding to DNA by maltose (22). We performed a similar EMSA assay with fragment XM but increasing amounts of maltose (from 0.35 to 5.6 mM) were added to the reaction mixtures. As expected, maltose gradually reduced the binding of MalR alone or in the presence of RNAP, so that the ternary MalR-RNAP-DNA complexes were decreased, this reduction being paralleled by a concomitant increase in the RNAP-DNA complexes (results not shown).

![Figure 7](http://www.jbc.org/)

**Fig. 7.** Expression of GFP in cells of *S. pneumoniae* as a function of the time of growth. Data were collected every 10 min, and fluorescence was detected in media containing maltose (open symbols) or sucrose (closed symbols). Plasmids used were pLS1GFP, containing both promoters (C, ●), pLS1MGFP, harboring a deletion in promoter $P_X$ (△, ▲), and pLS1mXGFP (□, ■), containing the wild type promoter $P_M$ and the mutated promoter $P_X$. 

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Taking the above results together, we may conclude that MalR represses transcription from promoter $P_X$ by binding to its operator $O_M$, and hindering the binding of RNAP to its target, both proteins competing for the same DNA region. Competition between MalR and RNAP was more evident at the malMP operon than at the malXCD operon, because of the higher affinity of RNAP for $P_X$. However, quantification of the half-lives of the RNAP-DNA complexes by challenging the complexes with an excess of competitor, showed no significant differences between the half-life of RNAP at $P_X$ (26 min) or at $P_M$ (25 min). This may reflect that sequences within or near the $P_X$-O$_M$ DNA region may influence the binding of RNAP to its target DNA (see below).

Role of the O$_X$ Operator in the Level of MalR-mediated Repression of the malMP Operon—Although MalR binds more weakly to operator O$_X$ than to O$_M$, our results do not rule out that the former operator is required in vivo (in conjunction with O$_M$) for either an optimal expression of the malMP operon or for effective repression, as shown for the LacI repressor (31). To test this hypothesis, transcriptional fusions were assayed in S. pneumoniae by placing the gene encoding GFP under the control of promoter $P_M$. We used three plasmid constructions, namely pLS1GFP (25), pLS1mXGFP, and pLS1MGFP. Plasmid pLS1mXGFP carried a defective $P_X$ promoter in which the −10 region and the TG extension were altered. This mutation led to a 20-fold reduction in the binding of RNAP to the $P_X$-O$_X$ DNA region, as compared with the wild type sequence (data not shown). In the case of plasmid pLS1MGFP, the sequence encompassing promoter $P_X$ was removed. As a control, plasmid pLS1Er (lacking the gfp gene) was used. Pneumococcal cells harboring plasmids were grown in media containing either sucrose (repressed conditions) or maltose (induced cultures). As a control, plasmid harboring plasmids were grown in media containing either sucrose (repressed conditions) or maltose (induced cultures) as carbon source, and the fluorescence of the cultures were measured. The values obtained (Table 1) showed that the fluorescence of the maltose-induced cultures harboring pLS1GFP increased by a factor of about 6, as compared with the uninduced cells, a value that agrees with previous results (25). Mutations in the −10 region of $P_X$ (plasmid pLS1mXGFP) affected this value only slightly if at all. However, the ratio observed in the induced versus uninduced cultures increased nearly 12-fold in cells harboring pLS1MGFP (deletion of $P_X$). Such an increase in fluorescence should be due to a 60% increase in the transcription rate from $P_M$ itself, there is a DNA region proximal to it, which is responsible for the interference with transcription from $P_M$. Curiously, deletion of the $P_X$ promoter region led to a reduction in the basal levels of GFP synthesis observed in the uninduced cultures. This difference could be due to a weak promoter activity, within the deleted region, reading in orientation toward promoter $P_M$. Although we have not found indications of the existence of such a putative promoter, the presence of an extremely AT-rich region makes it possible to find several sequences that resemble −10 regions. The existence of a weak promoter in this region was previously suggested (26).

Taking the above results together, we propose that the different affinities of RNAP and MalR for $O_X$ and $O_M$ may play a role in the induction of both promoters so that, under non-induced conditions, MalR would bind preferentially to $O_M$, leading to a basal expression from $P_M$. When the system is induced by maltose, promoters $P_X$ and $P_M$ would compete for RNAP binding, with RNAP having a higher affinity for $P_X$ than for $P_M$. An alternative explanation for our results would be that an interference between $P_X$ and $P_M$ may occur. This phenomenon is well characterized in phage λ promoters $P_R$ and $P_M$, which have start sites separated by 83 phosphodiester bonds (33). However, in the $P_X$ and $P_M$ pneumococcal promoters, such a distance is of 424 phosphodiester bonds. So far, we have been unable to show experimentally the possible existence of a MalR-mediated DNA loop (not shown), which would bring about both promoters shortening this distance.

We conclude that MalR represses transcription at promoter $P_M$ at an early stage, prior to $R_P$ formation, most likely by binding to the $O_M$ operator and hindering the access of RNAP to the promoter. Two observations support the hypothesis that there is a greater exclusion between MalR and RNAP at promoter $P_M$ than at $P_X$. First, the position of $O_M$ within the malMP operon is such that it almost overlaps with the +1 position, which is not the case for the $O_X$ operator. Second, the MalR footprints at the $P_M$/$O_M$ DNA region are totally included within those generated by RNAP. In vivo and in vitro observations indicate that there is a differential and opposite affinity of MalR and RNAP for the promoter/operator regions of the pneumococcal malMP and malXCD operons (22). In addition, measurements of amylo maltase (the product of gene malM) activity in pneumococcal cells showed a 20-fold induction by maltose when the operon was in a single copy (24), whereas partial de-repression was found when the cells harbored multiple copies of the malR gene (21). The implications of these findings are that the malMP operon, involved in the metabolism of maltodextrins, should be shut off in the absence of the inducer, whereas the malXCD, involved in the uptake processes, would be functioning, at least at a basal level, in all growth conditions. In this sense, the −10 extension of promoter $P_X$ could account for a rather high basal level of transcription. As a consequence, pneumococcal cells would always be ready for the uptake of nutrients, whereas their metabolism would be operative only when needed. Thus, regulation of the two operons may depend on the interplay between the relative affinities of MalR and RNAP for their operator/promoter regions.

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