Volume 13 Number 4 1985 Nucleic Acids Research

The *mac* promoters: functional hybrid promoters activated by the *malT* product and repressed by the *lacI* product

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Received 4 December 1984; Accepted 1 February 1985

ABSTRACT

Using in vitro techniques we have fused upstream sequences from the malPp promoter (normally activated by the MalT protein) to downstream sequences from the lacZp promoter (normally repressed by the LacI protein). Several hybrid promoters were thus obtained, which were controlled by the MalT protein, but were poorly active. More efficient promoters were then isolated using in vivo selection. Three main conclusions could be derived from the analysis of all of these hybrid promoters. Firstly, the MalT protein seems able to force RNA polymerase to start transcription at any DNA sequence, albeit with a low efficiency. Secondly, the strength of the hybrid promoters is considerably increased if a Pribnow Box is positioned at a precise location with respect to the MalT binding site. Thirdly, the presence of the lac operator, even when properly positioned with respect to the transcription startpoint, does not suffice to permit full repression by the lacI product.

INTRODUCTION

A few hybrid promoters have already been constructed (1,2,3,4,5). They involve elements from constitutive and negatively controlled promoters, and provided information on the structure of what constitutes an efficient transcription starting signal for the RNA polymerase of Escherichia coli. We now report the construction of hybrid promoters involving elements from a positively controlled promoter, malPp, and from a negatively controlled promoter, lacZp.

malPp, the promoter of the malPQ operon of E.coli, is activated by the product of gene malT in the presence of maltose (6). Deletion analysis, and the characterization of point mutations, has shown that the site of action of the MalT protein is located between positions -33 and -72 with respect to the transcription startpoint (7). lacZp, the promoter of the lac operon of E.coli, is repressed by the product of lacI. Numerous studies have shown that repression of this promoter results from the binding of the LacI protein to a sequence, the operator, located between positions +1 and +21

with respect to the transcription startpoint (reviewed in 8).

In principle, by combining the upstream elements of malPp with downstream elements of lacZp, we expected to obtain hybrid promoters which would be activated by the MalT protein, and repressed by the LacI protein. We have constructed such hybrid promoters (mac promoters), and studied their activity when they were present at a single copy on the E.coli chromosome. From this, we obtained information on the sequences which are needed, in addition to the MalT binding site, in order to obtain an efficient MalT controlled promoter, and we found that the presence of a properly positioned operator sequence does not constitute a sufficient condition to obtain full repression of a promoter by the lac repressor.

MATERIALS AND METHODS

Two bacterial strains were used : pop3, which is F⁻araD139 Δ (lac)U169 thiA rpsL relA flaB (9) and HfrG6, which is Hfr his (10). All media and most techniques were previously described (11).

For DNA sequencing, the chromosomal mac promoters were transferred onto plasmid pOM1 as described previously (12). DNA sequencing was according to Maxam and Gilbert (13) after labelling the DNA fragments at the 3'-end using α-^{32}P d ATP and the Klenow fragment of DNA polymerase.

Reverse transcriptase mapping of the transcription startpoints was as previously described (14), except that the hybridization between mRNA and labelled DNA primer was accomplished in the presence of 40% (rather than 80%) formamide, and that after the denaturation step (10 min at 75°C) the temperature was slowly decreased to 30°C (in about 6h) and then maintained at this temperature for a 10h period.

RESULTS

The hybrid promoters were constructed as follows (fig.1). The upstream elements of malPp (upstream from position -26) were first fused at random with a population of Bal31 digested DNA fragments containing downstream elements from lacZp (including the Pribnow box, the transcription startpoint, and the operator). The promoter activity of these constructions was usually very low, as evidenced from the fact that they failed to activate the tet gene of the vector plasmid to a level sufficient to render the cells Tet^R. Three of these constructions, called mac1, 2 and 3, were transferred onto the chromosome of E.coli, in place of the wild type malPp promoter. The resulting strains were Mal⁻, due to the low promoter activity

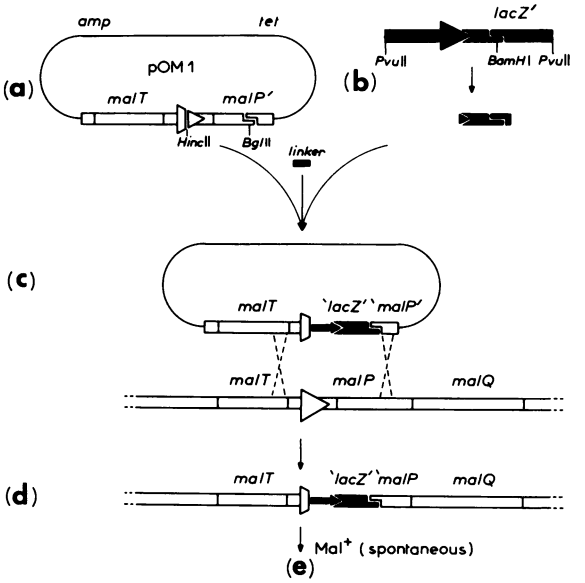

Figure 1. Construction of the mac promoters.
(a) Plasmid pOM1 is a derivative of pBR322 into which a 6kb EcoRI-HindIII DNA fragment has been cloned (17). This fragment carries gene malT, and the beginning of the malPQ operon, including its promoter malPp (open triangle). pOM1 contains a single BglII site located at codon 432 in malP (18) and five HincII sites one of which, shown in the figure, is located at position -26 with respect to the transcription startpoint in malPp. Limited digestion with HincII and complete digestion with BglII yielded a derivative of pOM1 deleted for the small HincII-BglII fragment, which was purified on agarose gel.
(b) Phage M13mp7 contains a 310 bp PvuII DNA fragment which extends from the end of lacI to the beginning of lacZ, with a polylinker in the 4th codon of lacZ (19). The BamH1 site present in the polylinker is shown on the figure, as well as the lacZp promoter (closed triangle). The PvuII fragment was purified on polyacrylamide gel, digested for various periods of time with Bal31, and cleaved with BamH1.
(c) The Bal31 digested fragments were then cloned between the HincII and BglII sites of the deleted pOM1 plasmid, in the presence of an excess of SalI linker. These constructions generally had a very low promoter activity. Three of them were called mac1, 2 and 3.
(d) The mac1,2 and 3 constructions were transferred onto the chromosome of pop3, in place of the malPp$^+$ promoter, as previously described (12). The resulting strains were Mal$^-$.
(e) Spontaneous Mal$^+$ derivatives were selected . Four inducible derivatives were retained for further study. They carried the efficient and maltose inducible promoters mac11, 12, 21 and 31.

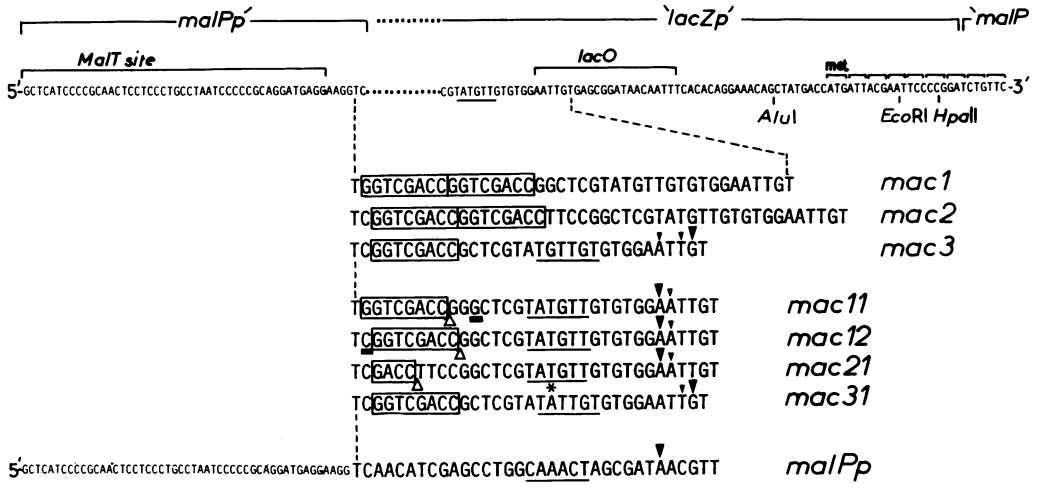

Figure 2. Sequences of the mac promoters . The general structure of the constructions is shown on the first line with, from left to right: the upstream sequence of the malPp promoter, including the binding site(s) for protein MalT; a sequence which differs in the different promoters, shown by an interrupted line; the downstream sequence of lacZp, including the Pribnow box (underlined), the operator, and the initiation codon for lacZ; the end of malP, from codon 432, fused in phase with the beginning of lacZ.

On the following lines are shown the segments of the mac promoter sequences which vary between the different promoters. The SalI linkers, or what remains of them, are boxed. The different DNA rearrangements in mac11,12,21,31 are indicated as follows: an empty arrowhead indicates the position of a deletion, a heavily underlined nucleotide corresponds to an insertion, an asterisk indicates an altered nucleotide. The last line corresponds to the sequence of the intact malPp promoter. The transcription startpoints, when known (14 and fig.3) are shown by closed arrowheads (large ones for the major startpoints, small ones for the minor startpoints). The Pribnow boxes, as deduced from the position of the transcription startpoints, are underlined.

Table 1. malQ expression from the hybrid promoters in the absence of lac repressor

strains	Relevant genotype[a]	amylomaltase[b](u/mg)	
		glycerol	glycerol-maltose
pop3	malPp$^+$	10	340
pop2341	mac1	2.9	16
pop2342	mac2	0.8	14
pop2343	mac3	0.9	22
pop2344	mac11	2.8	175
pop2345	mac12	2.2	189
pop2346	mac21	2.9	193
pop2347	mac31	4	221

(a) Strains pop2341 to 2347 all derived from pop3, which is deleted for the whole lac region, including lacI. (b) Amylomaltase was assayed after growth at 37°C in minimal medium containing glycerol or glycerol and maltose (0.4% each), according to the method of Raibaud et al (7).

of mac1, 2 and 3, and not to the absence of the malP product (maltodextrin phosphorylase), which is not necessary for growth on maltose (15). Spontaneous Mal$^+$ derivatives of these strains were then selected and tested for inducibility of malQ expression as previously described (16). Approximately half of these synthesized amylomaltase (malQ product) constitutively, and were discarded. Of the other half, which synthesized amylomaltase in a maltose inducible manner, four were further studied. The hybrid promoters present in these maltose inducible Mal$^+$ derivatives were called mac11 and 12 (derived from mac1), mac21 (from mac2) and mac31 (from mac3).

The DNA sequences corresponding to the original constructions (mac1,2 and 3), and to their derivatives (mac11, 12,21 and 31), are shown in fig.2. The rearrangements which converted the original constructions into efficient promoters are seen to correspond to an 8bp deletion plus a 1bp insertion (mac11 and 12), a 12 bp deletion (mac21) or a point mutation (mac31).

The promoter activity of the original constructions and their derivatives, when present at a single copy on the chromosome , was determined by assaying amylomaltase. (The constructions introduced no polar effect on malQ expression since lacZ is fused in phase to malP, as shown in

Table 2. malQ expression from the hybrid promoters in the presence of lac repressor.

Strains	relevant genotype[a]	amylomaltase[b](u/mg)		
		glycerol	glycerol-maltose	glycerol-maltose-IPTG
pop2324	mac11	<0.5	26	159
pop2325	mac12	<0.5	28	176
pop2326	mac21	<0.5	33	211
pop2327	mac31	<0.5	37	176

(a) Strains pop2324 to 2327 all derived from HfrG6, which is lac^+. They were constructed by transducing the mac promoter of strains pop2344 to 2347 into pop2170, a derivative of Hfr G6 deleted for the malPp promoter (11), the selection being for growth on maltose in the presence of isopropyl-thio-β-D-galactoside (IPTG). (b) Amylomaltase was assayed as described in the legend of table 1. IPTG, when added, was present at 10^{-3}M. Beta-galactosidase was assayed according to Miller (20): In glycerol and maltose medium the strains synthesized approximately 18 and 8000 units of β-galactosidase in the absence and presence of IPTG, respectively.

fig.2). malQ expression was first determined in the absence of the lac repressor (table 1). For the three original constructions (mac1, 2 and 3) the promoter activity was low but detectable (approximately 5% of $malPp^+$) and was maltose inducible. With the derivatives (mac11,12,21 and 31) the induced promoter activity ranged from 50 to 70% of that of $malPp^+$, the induction ratio being slightly higher than with wild type. The mac promoters were then transferred into a $lacI^+$ strain, so that the effect of the lac repressor could be studied (table 2). Surprisingly it was found that the induction ratio (±isopropyl-thio-β-D-galactoside), in the presence of maltose, was only about 5 for amylomaltase, whereas it was approximately 500 for the lac operon present in the same strain.

The transcription startpoints corresponding to the different mac promoters were determined by reverse transcriptase mapping (fig.3). If one takes as +1 the A where transcription starts in the wild type lacZp promoter (21), the startpoints for mac11,12, and 21 are mainly at +1 and +2. With mac3 and mac31, however, transcription starts mainly at +4 and +5, with a preference for the latter. With mac1 and 2 no transcription startpoint could be detected. The position of the transcription startpoints, when known, is shown in the sequences in fig.2.

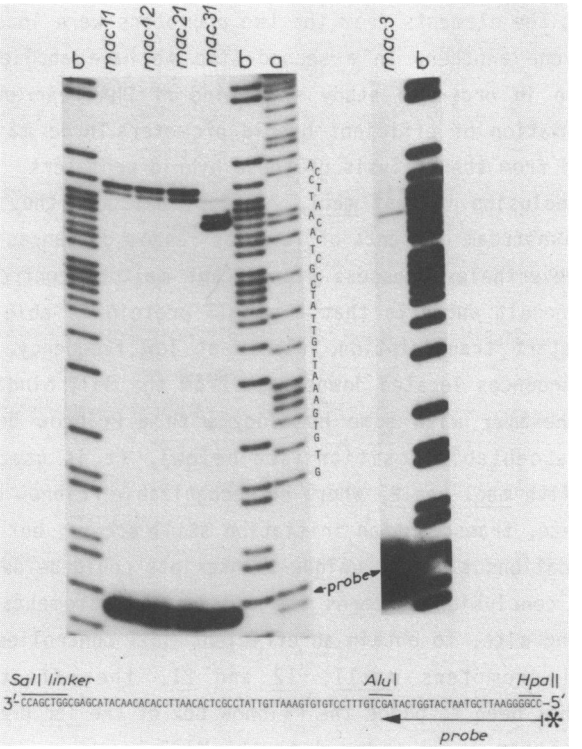

Figure.3. Reverse transcriptase mapping of the transcription startpoints in the mac promoters. An HpaII fragment, approximately 700 bp long, extending from the beginning of malT (11) to the beginning of lacZ (fig.2) was purified from pOM1 mac3, 5'-labelled using γ-^{32}P-ATP and T4 polynucleotide kinase, and cleaved either with AluI or with SalI. The two SalI-HpaII fragments were partially cleaved at purines (13) and used as molecular weight markers:(a) SalI-HpaII fragment containing the beginning of malT, (b) SalI-HpaII fragment containing the beginning of lacZ (sequence at the bottom of the figure). The 27 bp AluI-HpaII fragment was purified and hybridized with RNA extracted from strains pop2343 to 2347, which carry the different mac promoters. After elongation with reverse transcriptase the fragments were analyzed on urea containing 7% polyacrylamide gels, as previously described (14).

DISCUSSION

The construction of previously described hybrid promoters was performed in vitro, and was based on precise assumptions concerning the characteristics of an active promoter (1,2,3,4,5). We have chosen a different approach, in which such precise assumptions were not needed. In a first step we have fused DNA sequences from the two promoters, lacZp and

malPp, such that the elements from the two promoters were located at random distances from one another. In a second step we have applied a selection pressure in vivo in order to study what kind of DNA rearrangements could lead to the formation of efficient hybrid promoters.Three main conclusions could be derived from the analysis of these hybrid promoters.

A first conclusion is that mac1, 2 and 3, although they were obtained by fusing the downstream elements of lacZp at random distances from the MalT binding site, nevertheless possess significant maltose controlled promoter activity. This result suggests that the MalT protein is able to force RNA polymerase to start transcription, albeit at low frequency, whatever the nature of the sequences located downstream from the MalT binding site. With mac3, where a hexamer with some homology with a Pribnow box (TGTTGT) is present at an acceptable position (see below), it is used to start transcription. With mac1 and 2, where no recognizable Pribnow box is present at the right place, transcription initiation still occurs, but presumably at a variety of locations since no unique transcripts could be detected.

The second conclusion concerns the sequence requirements, in addition to a MalT binding site, to obtain an efficient MalT controlled promoter. In the high level promoters mac11, 12 and 21, the effect of the DNA rearrangements has been to place the Pribnow box of the lac promoter exactly at the same location, with respect to the MalT binding site, as the one occupied by the Pribnow box in malPp$^+$ (fig.2). For mac31, the point mutation which made it an efficient promoter led to the formation of a good Pribnow box (TATTGT) one nucleotide downstream from the position of the Pribnow box in malPp$^+$. Previous work has already shown that such a position is acceptable for a MalT controlled promoter (7). On the other hand, in mac3, the Pribnow box of the lac promoter is placed one nucleotide upstream from the normal location of the Pribnow box in malPp$^+$ (still with respect to the MalT binding site). Such a location is clearly not acceptable since i) mac3 is a rather poor promoter, and ii) the position of the transcription startpoint indicates that RNA polymerase favors the use of another Pribnow box, whose sequence is farther from consensus (TGTTGT), but which is located at a acceptable position. In conclusion, and consistent with previous data, a high level MalT controlled promoter must possess a "recognizable" Pribnow box either at the position, with respect to the MalT binding site, that it has in malPp$^+$, or one nucleotide downstream from this position. One nucleotide upstream is forbidden (22 and this work, mac3) and two nucleotides downstream is probably also forbidden since it was never

obtained in the total of 10 efficient MalT controlled promoter studied until now (this work, 7, 23). The most essential elements in the "recognizable" Pribnow box seem to be an A in second position, and a T in the sixth position, as found for most positively controlled promoters in enterobacteria (24).

The last conclusion concerns repression by the LacI protein. Despite the presence of a lac operator at its normal location with respect to the transcription startpoint, the mac promoters are repressed only 5 fold by the lac repressor. This is a small repression factor compared to the 500 fold observed with a wild type lac operon. This question of the repression exerted by the lac repressor could not be properly addressed for the previously characterized hybrid promoters, because these were carried on multicopy plasmids and the host cells contained variable amounts of repressor (1,2,3,5). In the present case one reason for the low repression might be that the LacI protein requires other sequences, in addition to the lac operator, in order to exert full repression. One such other sequence could be the second operator identified within the lacZ sequence (25). In favor of this hypothesis we recently reported that repression by the LacI protein is only 30-40 fold when a 203 bp DNA fragment extending from the end of lacI to the beginning of lacZ was inserted upstream of the malPQ operon (12). An additional reason for the low repression in the mac promoters might be that an RNA polymerase molecule primed for transcription by the MalT protein could override the repression exerted by the LacI protein. In this respect we note that a repression factor of about 5 was precisely that observed when the lac operator was located very much downstream from the transcription startpoint, in trp-lac fusions (26).

ACKNOWLEDGEMENTS

We thank M.Schwartz for his constant interest in this work and for his help in preparing the manuscript. This work was supported by grants from the "Centre National de la Recherche Scientifique" (LA 270), the Ministère de l'Industrie et de la Recherche (82 V 1279) and the "Fondation pour la Recherche Médicale".

REFERENCES

1. Amann,E., Brosius,J. and Ptashne,M. (1983) Gene 25,167-178.
2. De Boer,H.A., Comstock,L.J. and Vasser,M. (1983) Proc.Natl.Acad.Sci.USA 80,21-25
3. Russel,D.R. and Bennett,G.N. (1982) Gene 20, 231-243.

4. Stefano,J.E., Ackerson,J.W. and Gralla,J.D. (1980) Nucl.Acids Res. 8, 2709-2724.
5. Yansura,D.G. and Henner,D.J. (1984) Proc.Natl.Acad.Sci.USA. 81,439-443.
6. Débarbouillé,M., Shuman,H.A., Silhavy,T.J. and Schwartz,M. (1978) J.Mol.Biol. 124,359-371.
7. Raibaud,O., Gutierrez,C. and Schwartz,M. (1984), submitted.
8. Barkley,M.D. and Bourgeois,S. (1978) in The Operon, Miller,J.H.and Reznikoff Eds.,pp 177-220. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
9. Casadaban,M.J. (1976) J.Mol.Biol. 104, 541-555.
10. Matney,T.S., Goldschmidt,E.P., Erwin,N.S. and Scroggs,R.A. (1964) Biochem.Biophys.Res.Comm. 17, 278-281.
11. Raibaud,O., Débarbouillé,M. and Schwartz,M. (1983) J.Mol.Biol. 163, 395-408.
12. Raibaud,O., Mock,M. and Schwartz,M. (1984) Gene 29, 231-241.
13. Maxam,A. and Gilbert,W. (1980) in Methods in Enzymology, Grossman,L. and Moldave,K. Eds., pp 499-560. Academic Press, New York.
14. Débarbouillé,M. and Raibaud,O. (1983) J.Bacteriol. 153, 1221-1227.
15. Schwartz,M. Ann.Inst.Pasteur (1967) 112, 673-702.
16. Hofnung,M. and Schwartz,M. (1971) Molec.Gen.Genet. 112, 117-132.
17. Raibaud,O. and Schwartz,M. (1980) J.Bacteriol. 143, 761-771
18. Palm,D., Goerl,R., Burger,J.K., Bühner,M. and Schwartz,M. in Chemical and Biological aspects of vitamin B6 catalysis. Evangelopoulos,A.E. Eds. Alan Liss, N.Y. in press.
19. Messing,J., Crea,R. and Seeburg,P.H. (1981) Nucl. Acids Res. 9, 309-321.
20. Miller,J.H. (1972) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
21. Majors,J. (1975) Proc.Natl.Acad.Sci.USA 72, 4394-4398.
22. Gutierrez,C. and Raibaud,O. (1984) J.Mol.Biol. 117, 69-86.
23. Bédouelle,H., Schmeissner,U., Hofnung,M. and Rosenberg,M. (1982). J.Mol.Biol. 161, 519-531.
24. Raibaud,O. and Schwartz,M. (1984) Ann.Rev.Genet. 18, 173-206.
25. Reznikoff,W.S., Winter,R.B. and Hurley,C.K. (1974) Proc.Natl.Acad.Sci. 71, 2314-2318.
26. Reznikoff,W.S, Miller,J.H., Scaife,J.G., and Beckwith,J.R. (1969) J.Mol.Biol. 43, 201-213.