Regulation of the ompC Gene of Escherichia coli

IN Volvement of THREE TANDEM PROMOTERS

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ompC expression in Escherichia coli K-12 is known to be regulated by the ompB locus, comprising the ompR and envZ genes, and the OmpR protein is believed to act as a positive transcriptional factor. We examined the transcriptional capability of the ompC gene in vitro and found that RNA polymerase could transcribe ompC without a requirement for other transcriptional factors. Furthermore, transcripts from three tandem promoters in ompC were identified in vitro. We employed oligonucleotide-directed site-specific mutagenesis to dissect the promoter region of the gene and assayed the promoters separately for transcriptional ability using fusions to the lacZ gene. The levels of β-galactosidase indicate that ompC expression in vivo is dependent on the function of at least one of the upstream promoters. The function of OmpR appears to be the enhancement of a basal level of ompC expression in vitro and in vivo. Our results show that ompC is expressed at a low constitutive basal level that is subject to activation by OmpR, and the site of this interaction appears to be the upstream tandem promoters.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases were obtained from Bethesda Research Laboratories or from International Biotechnologies, Inc., New Haven, CT. HindIII 10-mer and XbaI 8-mer linkers were from New England Biolabs, Beverly, MA. Bal31, T4 polynucleotide kinase, T4 DNA ligase, and Klenow enzyme were from Bethesda Research Laboratories. E. coli RNA polymerase for in vitro transcription was from Boehringer Mannheim. [γ-32P]ATP and [α-32P]UTP were from Amersham Corp.

Bacterial Strains and Plasmids—E. coli K-12 strains used in this work are MC4100 (ΔargF-lacU189 araD139 rpsL rpsL1 thiA thiA rpsL1 ΔrbsR deoC ptsF) and MH1160 (MC4100 ompR101) (6, 11). Plasmid pMY150, an ompC clone (12), was the parent plasmid for construction of the various plasmids. Plasmid pOMpC5 has been described (Plasmid I in Ref. 12). Plasmid pKM007 is a vector derivative of pKM005 (13), carrying additional cloning sites as shown in Fig. 1C. Plasmid pJDC406 (14) was used as vector for oligonucleotide-directed site-specific mutagenesis. pYM140 is a plasmid carrying the tcp gene under control of two promoters and was used to generate control transcripts in in vitro transcription reactions.

Media—Cells were grown and maintained in Luria broth. Nutrient broth was supplemented with 20% sucrose and used as high osmolar broth. The abbreviation used is: bp, base pair(s).
ompC Promoter of Escherichia coli

A

B

C

Fig. 1. Construction of plasmids. A, construction of ompC-lacZ operon fusion plasmids and the plasmid used in mutagenesis. B, site-directed mutagenesis of the ompC promoter region. C, dissection of the ompC promoter region and construction of lacZ fusions. p(Hind)9317 of ompC (see below). Synthetic oligonucleotides were employed to generate the mutant pKI0643, in which a C residue at −94 from the start of transcription (see Fig. 4) was replaced by an A residue, thus generating a restriction site for the enzyme AhaIII. pKI0643 served as parent plasmid for the generation of two additional mutants, pKI0644 in which a C at −131 is substituted with a G to generate a NruI site and pKI0645 in which a C at −83 replaced with a G generates a BglII site (see Fig. 4).

In order to test in vivo activities of the dissected ompC promoter, each of the mutant plasmids pKI0643, pKI0644, and pKI0645 was digested with XbaI and the particular enzyme whose site was introduced by mutagenesis, and the fragments carrying the start of transcription of the ompC gene were cloned into pKM007 to produce plasmids pKI0743, pKI0744, and pKI0745, respectively (Fig. 1C). These plasmids are ompC-lacZ fusions essentially identical to pKL007 at the site of fusion but carrying varying lengths of the 5' region of the ompC promoter (see Fig. 2).

Oligonucleotide-directed Site-specific Mutagenesis—Oligodeoxynucleotides for creating the mutations were synthesized by automated phosphoramidate chemistry (15) in a Systec Microsyn 1160 DNA synthesizer. The protocol for introducing mutations at specific sites was essentially as described (16). Heteroduplexes were made by heat denaturation and annealing between plasmids pDC406 cut at the XbaI site and pKI0641 cut at PstI. The oligomer 5'GGATTTAATTTTGA3' was used to introduce the AhaIII site (TTTAAA) and pKI0643 thus constructed. Plasmid pKI0643 was used with pDC406 to generate, in a similar manner, plasmids pKI0644 and pKI0645, and pKI0645 carries a NruI site (TCGCGA) introduced by the oligomer 5'ATCGCGATTCCGC3' in addition to AhaIII, and the oligomer 5'TTAAGATCTTTCATTB' was used to introduce the BglII site (AGATCT) of plasmid pKI0645. The positions of the mutations in the ompC promoter and the resulting plasmids are indicated in Fig. 4.

In Vitro Transcription—DNA fragments were used as template in in vitro transcription reactions essentially as described (17) and analyzed on 8% polyacrylamide-urea gels.

β-Galactosidase Assays—Cells carrying various plasmids were grown overnight in Luria broth and transferred at a 1:100 dilution into nutrient broth containing 20% sucrose. β-Galactosidase activity was assayed as described (18) after 2–3 h of growth with shaking at 37°C.

RESULTS

In Vitro Transcriptional Analysis—Plasmid pompCpA5 is an ompC-lacZ gene fusion plasmid and represented in Fig. 2. The HpAI site upstream of ompC is changed to XbaI in the plasmids pompCpA5 and pGR111. In order to examine the transcriptional capacity of the ompC promoter, defined in vitro transcription reactions were carried out using the XbaI-BamHI fragment of pompCpA5 as template. A 390-bp HindIII-digested pYM140 (a lpp clone) fragment was used as control to generate RNA markers of 160 and 245 bases (a and b, respectively, in Fig. 3). Analysis of transcripts on a denaturing gel indicated three major transcripts (labeled 1, 2, and 3 in Fig. 3A, lane 1). When the shorter XbaI-HindIII fragment of pompCpA5 was used as template, all three major bands now migrated faster (Fig. 3A, lane 2) identifying them as run-off transcripts in the ompC direction. Consistent with this, a MspI-HindIII fragment (template cut at the 5' end) produced an identical set of three transcripts as XbaI-HindIII (data not shown). The three bands, 1, 2, and 3, were therefore clearly transcribed in the direction of ompC. Further shortening of the 3' end of the template by using the XbaI fragment of pompC and p(Xba) represent phosphorylated 10-mer and 8-mer linkers, respectively. BAP, bacterial alkaline phosphatase. Relevant restriction sites are indicated: A, AhaIII; B, BglII; E, EcoRI; H, HindIII; M, MspI; N, NruI; X, XbaI. bid represents the gene encoding ampicillin resistance. The ompC structural gene is indicated by the open box, and the micF and lacZ genes are shown. The arrows indicate the direction of transcription of the genes. 1 and 2 on A represent the lpp promoter lac promoter-operator.

[Diagram of plasmid construction and transcription analysis]
pGR111 resulted in predictably faster migrating bands corresponding to transcripts 1, 2, and 3 (Fig. 3A, lane 3). The shortest transcript 1 is estimated to be only about 30 bases in length and therefore the bands appear rather faint. A 4-base addition at the 3' end of the above template was effected when the XbaI fragment of plasmid pKL007 was used as template; the predicted resultant increase in the sizes of the three transcripts was observed (Fig. 3A, lane 4).

The transcript 1 corresponds to the ompC transcript identified by S1 mapping of in vivo synthesized RNA (12) and is believed to be directed by the P1 promoter ((12) see Fig. 4). This particular band is more intense when a corresponding fragment carrying an up-promoter mutation of ompC is used as template; this mutation allows a high ompC expression independent of mutations in ompR. The two additional transcripts, 2 and 3, suggest the presence of additional promoters in the system.

Identification of Three Tandem Promoters—The sequence of the ompC promoter region was scrutinized in order to identify any possible upstream promoter sequences that could initiate transcription and generate transcripts 2 and 3 described above. Two overlapping putative promoters (P2 and P3, Fig. 4) were identified with possible −35 and −10 regions in good agreement with the consensus sequences of TTGACA and TATAAT, respectively (19). The −10 region of P1 (TATAAT) is separated by 17 bases, and the P3 −10 region (AAACAT) is separated from the −35 region (ATGAAA) by 18 bases from the −35 region (TTGAAA) (Fig. 4).

In order to enable dissection of these promoters, the XbaI fragment of pKL007 was subcloned into pJDC406 (plasmid pKI0641) and used as the target for site-directed mutagenesis. In this manner, an AhaIII site was introduced immediately upstream of the −35 region of promoter P3 (plasmid pKI0643). The XbaI fragments of pKI0641 and pKI0643 were used as templates for in vitro transcription and showed an essentially identical transcription pattern (Fig. 3A, lanes 4 and 5). The introduction of the mutation, therefore, did not significantly alter the generated transcript pattern; an enhancement of band 2 was observed, but the reason for this is not clear.

In order to perform a finer dissection of the promoter region, additional restriction sites were created in pKI0643, as shown in Fig. 1B, and Fig. 4, generating pKI0644 that has a NruI site and pKI0645 that has a BglII site. When the 3' NruI-XbaI fragment from pKI0644 was used as a template in transcription, the three transcripts, 1, 2, and 3, were observed (Fig. 3B, lane 2), identical to the pKI0643 fragment (Fig. 3B, lane 1); however, the bands are relatively weaker, probably because the shorter DNA fragment is a poor template for transcription.

When the 3' AhaIII-XbaI ompC fragment of pKI0643 was used at the template, only two identifiable transcripts, 1 and 2, were generated. Production of both transcripts, however,


was very poor. This was expected as the AhaIII site is too close to the -35 region of promoter P3 to allow efficient transcription. The BglII-XbaI fragment pKI0645 does not carry either of the promoters P2 and P3; and as expected, only transcript I was observed when the fragment was used in an in vitro transcription assay (Fig. 3B, lane 4). These results clearly demonstrate that there are three functional promoters for ompC transcription in vitro.

In Vivo Analysis of Tandem Promoters—In order to test if the P2 and P3 promoters, in addition to P1, are functional in vivo, the fragments used for in vitro transcription in Fig. 3B were inserted into pKM007, a promoter-proving vector, and as expected, only transcript I was observed when the fragment was used in an in vivo expression system. The plasmids pKI0743, pKI0744, and pKI0745 carryompC-lacZ fusions of the fragments derived from pKI0643, 0644, and 0645, respectively. MC4100 (ompR+), pKM005, and pKI0745 cells were transformed with these plasmids, and the β-galactosidase produced by the fusion was then assayed after growth in nutrient broth supplemented with 20% sucrose; ompC is normally expressed at a high level in this medium. Cells were transformed with vector pKM005 as control for background production of β-galactosidase.

Table 1 lists the β-galactosidase activities of MC4100 cells transformed with various plasmids. Comparison of the activities of pGR111 and pKI007 shows a 66% reduction in activity following shortening of the promoter fragment at the 5' end. pKI0744 and pKI0743 have essentially the same activity, similar to pKI007, although they have different 5' ends to the ompC promoter. pKI0745 which lacks promoters P2 and P3 shows another 80% reduction in activity.

MH1160 is an ompR mutant cell strain that is phenotypically OmpF− OmpC− (6). We measured the β-galactosidase activities of the various ompC-lacZ fusions in this strain in order to determine if any of the fusions still retained an activity against OmpR. Table 1 shows that pGR111, pKI0743, and pKI0745 in MH1160 cells have essentially the same low β-galactosidase activity as pKI0745 in MC4100, the ompR+ strain. pKI0743 has all three promoters, P1, P2, and P3, while pKI0745 has only P1. It is important to note that pKI0743 is capable of activation by OmpR whereas pKI0745 is not. This result indicates that the region of the P2 and P3 promoters is required for OmpR function.

**DISCUSSION**

In this paper, we have presented results showing that, in a defined transcription system, three distinct transcripts are produced from the DNA fragment carrying the ompC promoter. Since the lengths of these transcripts were equated by shortening the DNA fragment at the 3' end (Fig. 3A), we concluded that these transcripts were produced from three tandem promoters, P1, P2, and P3 (Fig. 4). The P1 promoter has been previously identified by S1 mapping of the ompC mRNA produced in vitro (19). Promoters P2 and P3 have been identified upstream of P1. The -35 and -10 regions of these promoters are separated by 17 bp in the case of P2 and 18 bp in the case of P3, which is consistent with the 17 ± 1 bp spacing for the E. coli promoter (19). There is no overlap of the -10 region of P3 and the -35 region of P2. The promoters P2 and P3 lie 36 and 56 bp, respectively, upstream of the promoter P1, and the sizes of the transcripts generated in vitro are consistent with this spacing.

DNase I protection studies of the ompC promoter fragments with RNA polymerase show this entire stretch of three tandem promoter sequences to be protected in the absence of any external factors. These results suggest that the ompC gene can be transcribed at a basal level without requirement for OmpR. Similar attempts at in vitro transcription with the ompF promoter failed to yield the predicted mapped transcript. This indicates that a positive factor is absolutely required for activation of ompF transcription, whereas ompC gene transcription occurs, although at a low level, in the absence of factors besides RNA polymerase. The role played by the ompB products must therefore be different in regulation of the two porin genes. These results are consistent with the observation that ompC, when cloned into multicyclop plasmids, is expressed in ompR mutants of OmpC− phenotype, whereas OmpF is not similarly produced in ompR mutants of OmpF− phenotype (20). There was a stepwise decrease in ompC expression in vivo, as measured by β-galactosidase activity, when the length of the region upstream of the ompC promoter was shortened. pKL007 had 33% of the activity of pGR111, which is probably due to the absence of a functional micF gene in pKL007. In the cells harboring pGR111, in which micF is active, OmpF synthesis should decrease (21), which may then make conditions favorable for ompC expression. The plasmids pKI0744 and pKI0743 showed essentially the same activity as pKL007 (Table I). However, pKI0745 showed a further 80% reduction in β-galactosidase activity, indicating that the promoters P2

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1. S. Norioka, G. Ramakrishnan, K. Ikenaka, and M. Inouye, manuscript in preparation.
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and P3 are indeed functional in vivo.

Multiple promoters are commonly found to be present preceding genes and operons in E. coli that are subject to regulation, such as gal (21), uvrB (22), rnrE (23), rpsA (23), carAB (24, 25), lac (26), and glnA (27). It has been proposed that the activities of the different promoters are sensitive to different signals and thus coordinate the control of gene expression in response to multiple factors (22). It is possible that in a similar manner the different promoters of the ompC gene render it responsive to multiple factors.

The region upstream of the transcriptional start is also the probable site for interaction with the regulatory factor OmpR as has been suggested previously for ompF (28). β-Galactosidase activities in ompR* or ompR1 cells harboring an ompC-lacZ fusion plasmid with only the P1 promoter were similar to each other. However, when ompC-lacZ fusion plasmids with all three promoters (pK10743 or the plasmids with longer fragments in Fig. 2) were used, the activities were much higher in ompR* cells than in ompR1 cells (Table I). These results are consistent with the notion that OmpR interacts with the probable site for interaction with the regulatory factor OmpR, which render it responsive to multiple factors.

To explain the finding that the major OmpR interaction with the P3 region and to specifically bind at the P3 region and to specifically inhibit the production of the P3 transcript in vitro. Interaction of OmpR with P3 in vivo might result in the activation of transcription from the downstream promoter(s). This would explain the finding that the major ompC transcript in vivo was found by S1 mapping to be directed by the P1 promoter (12). In the absence of OmpR, all three promoters may be active in transcription in vivo.

There are other possibilities for OmpR function. OmpR may be involved in removal of some inhibitor of transcription from either of the promoters. Re-examination of the DNA sequence between −50 and −80 (Fig. 4) reveals several interesting aspects of this region. First, sequences “TAGCGA” (−78 to −73) and “AAAGTT” (−56 to −51) are quite similar to “TAGCGA” and “AAAGAT,” which are the −35 and −10 sequences of ompF (28), respectively. Second, a sequence “TTTTGAC” overlapping the −35 region of P3 is also present at about 70 bp upstream of the ompF transcription initiation site. This common sequence may be important for recognition by OmpR. We are currently investigating the importance of these sequences to OmpR binding and action.

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