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Sequence elements determining *ampC* promoter strength in *E. coli*

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A number of spontaneous up-promoter mutations have been isolated in the *ampC* β-lactamase gene of *Escherichia coli*. The mutants were analyzed by DNA sequencing, and the level of *ampC* gene expression was determined. Six mutants with a 21-fold increase in promoter strength compared with the wild-type were mutated in the −35 promoter region from TTGTCA to the consensus sequence TTGACA. The −10 region sequence TACAAT was mutated to the consensus sequence TATAAT in three mutants exhibiting an *ampC* promoter seven times stronger than the wild-type. We have previously described a 1-bp insertion mutant (Jaurin *et al.*, 1981) that changes the inter-region distance to the consensus 17 bp. Thus, all the up-mutations found in the *ampC* promoter represent corrections of the three major discrepancies between the *ampC* promoter and the consensus *E. coli* promoter. We conclude that the three consensus elements of *E. coli* promoters, the −35 and −10 regions and an optimal inter-region distance of 17 bp, are the main elements determining the promoter strength.

Key words: ampC β-lactamase gene/initiation of transcription/up-promoter mutants

Introduction

Since gene expression is primarily controlled at the level of initiation of transcription, a detailed analysis of promoter-RNA polymerase interactions is central for understanding gene regulation. The nucleotide sequences of ~60 different promoters for *Escherichia coli* RNA polymerase have been compiled. This has revealed two regions of homology, the −35 region and the −10 region (the Pribnow box) (Pribnow, 1975; Schaller *et al.*, 1975; Maniatis *et al.*, 1975; Gilbert, 1976; Siebenlist *et al.*, 1980). They are located ~35 and 10 bp upstream from the first nucleotide of the transcript, and have consensus sequences of TTGACA and TATAAT, respectively. In the −10 region sequence, TATAAT, the T in the sixth position is found in all promoters investigated so far (Siebenlist *et al.*, 1980). The first two positions of TATAAT are also very conserved (i.e., found in 90% of investigated promoters), whereas the other three bases of the hexanucleotide show a somewhat larger variation (Siebenlist *et al.*, 1980). The very conserved TTG sequence of the −35 region is followed downstream by three less stringently conserved nucleotides. The sequences upstream and downstream from the −35 region are often AT-rich (Siebenlist *et al.*, 1980). The −10 and −35 regions are separated by 16–19 bp with 17 bp being most frequent (i.e., 60% of the investigated promoters) (Siebenlist *et al.*, 1980). Comparison of various mRNA start points shows that transcription initiates predominantly from a single position 4–8 bp downstream from the −10 region (Siebenlist *et al.*, 1980). The starting nucleotide is usually a purine with A being predominant. In certain cases, a 5′-terminal mRNA heterogeneity has been observed (Rosenberg and Court, 1979). Homologies have been found in the nucleotides surrounding the initiation start point, with a CAT sequence as the most common one (Siebenlist *et al.*, 1980).

Attempts have been made to correlate promoter strength with promoter DNA sequence. This has in part been possible by the analysis of ~30 promoter mutations (reviewed in Rosenberg and Court, 1979; Siebenlist *et al.*, 1980). The majority of these mutations adversely affect promoter function (i.e., promoter down-mutations) and are located either in the −10 or the −35 region. A limited number of mutations have been characterized that enhance the rate at which RNA polymerase interacts with the promoter site (reviewed in Rosenberg and Court, 1979; Siebenlist *et al.*, 1980). The majority of these up-promoter mutations are also found in the −10 and −35 regions. The mutations are base substitutions that increase the homology of the conserved regions to the consensus sequence. These results are not surprising since the promoters in which the mutations arose have a relatively low initiation frequency and relatively low homology to the consensus promoter sequence. Two up-promoter mutations which have been described in the *ampC* β-lactamase and *lacZ* promoters, respectively, have a change in the distance between the two conserved regions to the most commonly found 17 bp (Jaurin *et al.*, 1981; Stefano and Gralla, 1982).

Wild-type *E. coli* cells produce constitutively low amounts of a chromosomally encoded β-lactamase (~10^{-4} of total cell protein). The structural gene, denoted *ampC*, was cloned and DNA sequenced (Edlund *et al.*, 1979; Jaurin and Grundström, 1981). The control region *ampA* was shown to map adjacent to *ampC* (Grundström *et al.*, 1980). Jaurin *et al.* (1981) demonstrated that the *ampA* region consists of two control regions, a promoter and an attenuator. The attenuator was shown to mediate the growth rate dependent regulation of the enzyme. The promoter was found to have a −35 region with the sequence TTGTCA and a −10 region with the sequence TACAAT separated by 16 bp. An up-promoter mutation, denoted *ampP15G16*, which leads to a 16-fold increase in β-lactamase synthesis, was shown to have an insertion of a G-C base pair between the two conserved regions (Jaurin *et al.*, 1981). Recently it was demonstrated that the *ampC* promoter is located within the last structural gene of the fumarate reductase *(frd)* operon and that the *ampC* attenuator serves as a terminator for transcription of this preceding operon (Grundström and Jaurin, 1982).

The strict linear relationship between the levels of β-lactamase production and ampicillin resistance enables isolation of mutants with an increased production of β-lactamase by selection on broth plates containing different concentrations of ampicillin. We describe herein the isolation of a number of up-promoter mutants and thereby analyze the distribution of mutations that can increase the efficiency of initiation of transcription from the *ampC* promoter.

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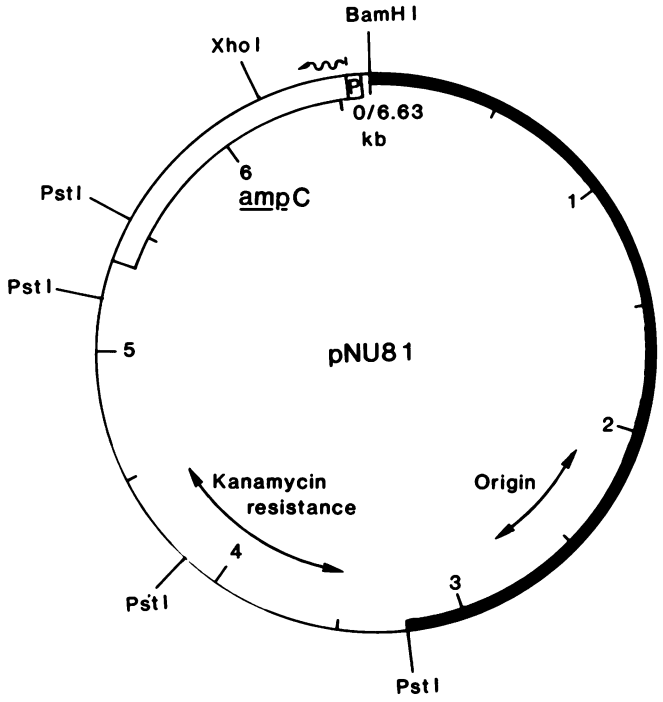

Fig. 1. Physical map of plasmid pNU81. The relevant restriction sites, location of the origin of replication, the kanamycin resistance gene, and the *ampC* gene (box) are given. P denotes the *ampC* promoter and the wavy arrow shows the start of *ampC* transcription.

Results

Isolation of spontaneous up-promoter mutants of ampC

To facilitate the isolation and analysis of spontaneous up-promoter mutants of *ampC* (see Discussion) plasmid pNU81 was constructed (Figure 1). pNU81 carries the kanamycin resistance gene of the transposon Tn*5* and the *ampC* gene coding for β-lactamase. A *Bam*HI linker was inserted into the *Rsa*I site located 81-84 bp upstream from the point of initiation of transcription of the *ampC* gene. The strategy to isolate and sequence up-promoter mutants was as follows:

1. Single cells of *E. coli* JC12768 carrying plasmid pNU81 were grown and plated on broth plates containing high levels of ampicillin.

2. Spontaneous mutants derived from independent single cells and resistant to the selected ampicillin concentration were analyzed by a total lysate assay. Plasmids that showed an increase in copy number or a detectable change in size were sorted out.

3. In order both to obtain cells containing plasmids exclusively carrying the mutant alleles and to avoid homologous recombination between the chromosome and the plasmid, isolated plasmid DNAs from mutants were transformed into the recombination defective *E. coli* strain SN01.

4. Plasmid DNA was purified from the transformants and linearized with *Bam*HI. α-^{32}P-labeled dGTP was incorporated into the 3′-end of the *Bam*HI site with the use of the Klenow fragment of DNA polymerase I.

5. After recleavage with *Xho*I, the 436-bp *Bam*HI-*Xho*I fragment was isolated and subjected to DNA sequencing (Maxam and Gilbert, 1980).

Up-promoter mutants were selected at concentrations of 50, 75, and 100 μg/ml ampicillin. The incidence of up-promoter mutations was only 5 – 13% of the total incidence of mutations (Table I). The main reason for this is that copy number mutants were far more common than up-promoter mutations. However, DNA rearrangments (insertions, deletions, and gene amplification) were also rather frequently obtained. The total incidence of mutations leading to an increased ampicillin resistance decreases by approximately one order of magnitude between selection at 50 μg/ml and 75 μg/ml and between 75 μg/ml and 100 μg/ml of ampicillin as shown in Table I. The incidences of up-promoter mutations were quite low, 4 x 10^{-11} at 50 μg/ml, 1 x 10^{-11} at 75 μg/ml, and 1 x 10^{-12} at 100 μg/ml of ampicillin.

Table I. Incidence of mutations

Selection level[a] (μg/ml ampicillin)	Incidence of mutations	Incidence of *ampC* up-promoter mutations
50	8 x 10^{-10}	4 x 10^{-11}
75	1 x 10^{-10}	1 x 10^{-11}
100	8 x 10^{-12}	1 x 10^{-12}

[a]Single cell cultures were grown as described in Materials and methods. A total of 60, 107, and 44 cultures were used in the selections at 50, 75, and 100 μg/ml ampicillin, respectively.

Table II. Ampicillin resistance of strains containing wild-type and up-promoter mutant plasmids

Plasmid present	Selection level (μg/ml ampicillin)	LD_{50} (μg/ml ampicillin)	Mutation
pNU101	75	186	*ampP32A*
pNU102	75	182	*ampP32A*
pNU103	75	186	*ampP32A*
pNU104	75	190	*ampP32A*
pNU105	75	184	*ampP32A*
pNU106	100	183	*ampP32A*
pNU107	50	56	*ampP11T*
pNU108	50	53	*ampP11T*
pNU109	50	55	*ampP11T*
pNU113	–	130	*ampP15G16*
pNU81	–	8.5	wild-type

Analysis of ampicillin resistance levels required to kill 50% of the cells (LD_{50}) of strains containing *ampC* wild-type or up-promoter mutant plasmids. The strains were tested as described in Materials and methods. The nucleotide sequences of the *ampC* promoter of the plasmids are displayed in Figure 2B.

Ampicillin resistance of isolated mutants

Using the strategy outlined above, three mutants, denoted pNU107, pNU108, and pNU109, were isolated at 50 μg/ml of ampicillin and six mutants, denoted pNU101, pNU102, pNU103, pNU104, pNU105, and pNU106, were isolated at 75 and 100 μg/ml of ampicillin. As a comparison, in this study the up-promoter mutant *ampP15G16* (Jaurin *et al.*, 1981) was also included. We have shown that this mutation, which leads to a 16-fold increase in β-lactamase synthesis, changes the distance between the two conserved regions in the *ampC* promoter by an insertion of a G-C base pair into the spacer region. The *ampP15G16* mutation was transferred to plasmid pNU81 by means of homologous recombination between plasmid pNU81 and the strain LA51 carrying the mutation on the chromosome. The *in vivo* recombined plasmid, denoted pNU113, was transformed into the *recA* strain SN01.

Table II shows the LD_{50} values against ampicillin for

plasmid pNU81 and the 10 mutant derivatives. Plasmid pNU81 carrying the wild-type *ampC* promoter mediates an ampicillin resistance of 8.5 μg/ml (LD_{50}). The mutants pNU101–106 show LD_{50} values of ~185 μg/ml of ampicillin, whereas the mutants pNU107, pNU108, and pNU109 exhibit LD_{50} values of ~55 μg/ml of ampicillin (Table II). The mutation *ampP15G16* when present on plasmid pNU81 mediates an ampicillin resistance of 130 μg/ml.

Thus, the mutants fall into three different classes with respect to ampicillin resistance. All mutants show a considerably higher ampicillin resistance than wild-type cells and attenuator mutants. This points to the fact that each class of the mutants represents a separate DNA sequence change in the *ampC* promoter.

Nucleotide sequence of up-promoter mutants

Figure 2A displays the autoradiographs obtained from DNA sequencing experiments with plasmids pNU81, pNU103, pNU109, and pNU113. The DNA sequence changes of all mutants are summarized in Figure 2B. Mutants pNU101–106 all showed the same mutation: a T-A to A-T transversion at position −32 changing TTGTCA to TTGACA. Thus this mutation, denoted *ampP32A*, creates a perfect −35 region with respect to the consensus sequence (Siebenlist *et al.*, 1980). A C-G to T-A transition at position −11 was found in mutants pNU107, pNU108, and pNU109. This mutation, denoted *ampP11T*, changes the −10 region from TACAAT to TATAAT with the latter sequence constituting a perfect −10 region with regard to the consensus promoter sequence (Siebenlist *et al.*, 1980). As expected, plasmid pNU113 carrying the mutation *ampP15G16* showed an insertion of a G-C base pair between positions −16 and −15 (Jaurin *et al.*, 1981).

From the results described above it seems likely that three elements may be altered in the *ampC* promoter to obtain high levels of expression; changes of the −35 and the −10 regions and a change in the distance between these two conserved regions.

In vivo expression of up-promoter mutants

It was of importance to investigate the levels of expression of the *ampC* β-lactamase gene of the up-promoter mutants and compare the values obtained with the levels of ampicillin resistance in the mutants. Two different assay systems were used. First, the relative amounts of β-lactamase made per total protein in wild-type cells and in the different up-promoter mutants were measured by rocket immunoelectrophoresis (Table III). The values correspond well with the LD_{50} values, confirming a linear relationship between amount of β-lactamase and ampicillin resistance.

The minicell system was used to confirm that the increased β-lactamase production was plasmid encoded. Each plasmid was transformed into a minicell producing *E. coli* strain and the polypeptides synthesized were labeled by [^{35}S]methionine and analyzed by autoradiography after gel electrophoresis. Figure 3 shows the protein pattern obtained from wild-type cells and up-promoter mutants. A protein with an approximate mol. wt. of 27 K is produced at the same level from all plasmids analyzed. It corresponds to the neomycin phosphotransferase II (NPTII) of transposon Tn*5* which confers resistance to kanamycin and neomycin upon the cell. The mol. wt. found by us is 1–2 K higher than reported by others (Matsuhashi *et al.*, 1976; Rothstein *et al.*, 1980). The protein with a mobility corresponding to a mol. wt. of 37 K migrated with purified *ampC* β-lactamase. As shown in Figure 3, the wild-type and the different classes of mutants produced different amounts of *ampC* β-lactamase. By scanning the autoradiograph, relative amounts of β-lactamase (taking the amount of NPTII made as an internal standard) could be calculated (Table III). The values show a good correspondence to the LD_{50} values and the rocket immunoelectrophoresis data.

Table III. Relative expression of the *ampC* gene from wild-type and up-promoter mutant plasmids

Mutation	Relative amounts			
	LD_{50} (μg/ml ampicillin)	Rocket immunoelectrophoresis	Minicells	*In vitro* transcription
Wild-type	1.0	1.0	1.0	1.0
ampP32A	21.8	21.5	18.6	22.2
ampP11T	6.5	6.5	6.3	8.0
ampP15G16	15.3	17.7	13.9	15.8

The relative expression compared to the wild-type was determined as described in Materials and methods. In the rocket immunoelectrophoresis analysis strains carrying plasmids pNU101, pNU107, pNU113, and pNU81 were used. The *in vitro* transcription analysis used plasmids pNU106, pNU107, pNU113, and pNU81 as templates. In the other expression measurements all plasmids were analyzed.

In vitro transcription of up-promoter mutants

The relative strength of wild-type and mutant promoters was also established by *in vitro* transcription. The system used utilized covalently closed circular (CCC) plasmid DNA as a template in contrast to the common use of linear DNA fragments as templates. Preliminary results indicate that initiation of transcription from *ampC* templates is considerably more efficient with CCC templates than with linear templates (unpublished data). In an earlier report (Jaurin *et al.*, 1981) it was shown that the *ampC* gene was regulated by antitermination at an attenuator site. Thus, *in vitro* transcription gave rise to two RNA species; one 41 bases long that terminates at an attenuator site and a read-through transcript. About 93% of the transcripts were reported to be terminated at the attenuator both with wild-type and *ampP15G16* templates (Jaurin *et al.*, 1981). Thus, measurements of the 41-base attenuated transcript will reflect the efficiency of initiation of transcription *in vitro* at the *ampC* promoter.

Figure 4 displays the RNAs synthesized using wild-type (pNU81) and pNU106, pNU107, and pNU113 CCC DNA as templates in the reaction mixture. Also included is a *Sal*I-*Xho*I fragment of plasmid pNU28 carrying the *ampP15G16* mutation to show the location of the 41-base long transcript previously reported (Jaurin *et al.*, 1981). Two transcripts were made from all the CCC DNA templates tested. A 108-base transcript was synthesized at about the same level from all the CCC templates. This RNA corresponds to the 108-base transcript of the pBR322 origin of replication that is present in pNU81 and its derivatives (Morita and Oka, 1979; Sutcliffe, 1978). The shorter transcript previously shown to be 41 bases in length (Jaurin *et al.*, 1981) was synthesized at different levels from wild-type template and the different classes of up-promoter templates. By scanning the autoradiograph and taking the 108-base transcript as an internal standard, the relative amount of the 41-base transcript could be determined (see Table III). Again, the data match well the values from the other expression measurements in Table III.

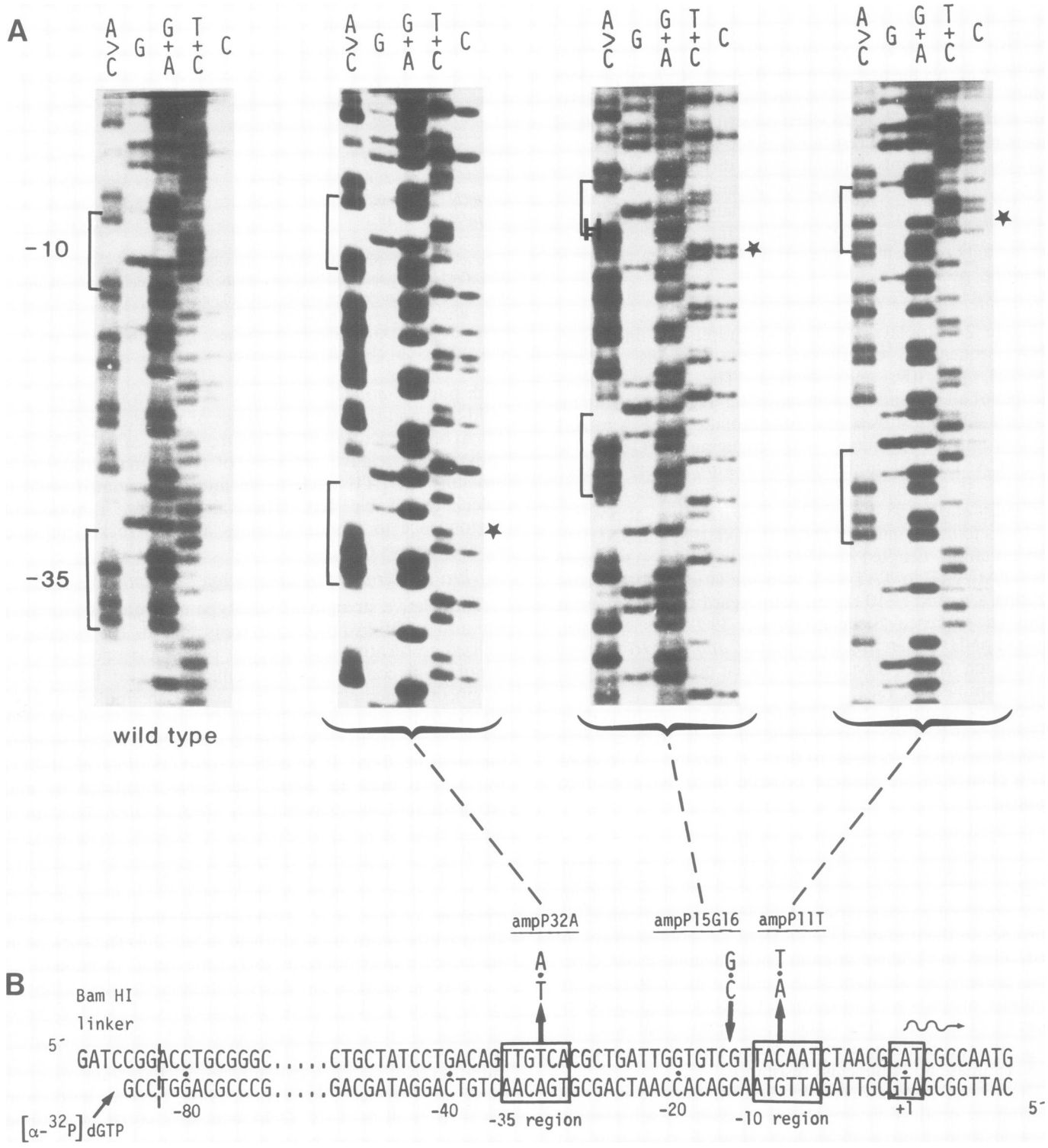

Fig. 2. Nucleotide sequence of *ampC* wild-type and up-promoter mutants. **A.** The autoradiographs from DNA sequencing of *ampC* wild-type and the three classes of up-promoter mutant plasmids (pNU81, pNU103, pNU109, and pNU113) are shown. Only the relevant parts of the gels are given. The specificities of the different chemical degradation reactions (Maxam and Gilbert, 1980) are shown on the top of each gel. The brackets show the positions of the −10 and −35 regions. The stars indicate the location of the three types of up-promoter mutations. **B.** Nucleotide sequence of wild-type and the three different classes of up-promoter mutations. Start of transcription is marked by +1 and the wavy arrow. Every twentieth base pair is marked with a dot between the strands. The −35 region, the −10 region, and the CAT sequence are within boxes. The boundary between the *Bam*HI linker and the *ampC* promoter region is marked by a vertical dashed line. The point of the [α-^{32}P]dGTP 3′-end labeling is shown by a diagonal arrow. The positions of the *ampP32A-*, *ampP15G16-* and *ampP11T*-mutations are shown by vertical arrows.

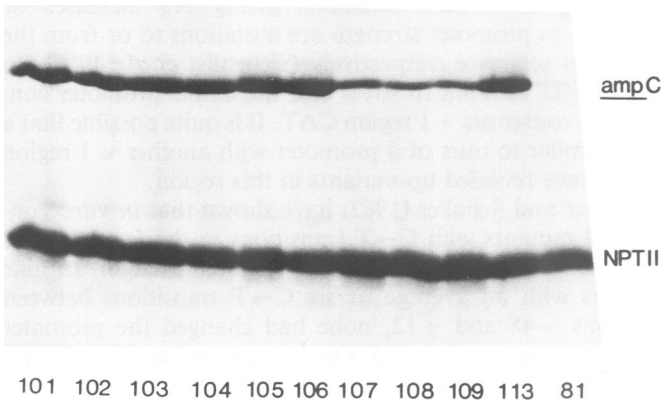

Fig. 3. Proteins expressed by the *ampC* plasmids. Minicells containing the different *ampC* plasmids were labeled with [^{35}S]methionine. The proteins were electrophoresed on an 11% SDS-polyacrylamide gel, and the relevant part of the autoradiograph is shown. *ampC* and NPTII stand for *ampC* β-lactamase and neomycin phosphotransferase II, respectively. The plasmids analyzed are given by their numbers.

Thus, the mutants show increased ampicillin resistance due to increased initiation of transcription from the *ampC* promoter. The *ampP11T*-mutation increases the promoter strength 7-fold and the *amp32A*-mutation 21-fold compared with a 16-fold increase with the *ampP15G16*-mutation.

Discussion

The aim of this study was to elucidate the sequence changes which could lead to more than a slight increase in the strength of the *ampC* promoter of *E. coli* K-12. Studies of promoter structure by isolation of up-mutations are few (reviewed in Rosenberg and Court, 1979; Siebenlist *et al.*, 1980). This is mainly because of the very limited number of possible sequence changes that can give this effect, necessitating an easily selectable phenotype. The few isolated up-promoter mutations that do exist are not in the same system, making it hard to draw conclusions about the distribution of possible up-mutations in a single promoter. The promoters used in these previous mutant studies are also binding sites for proteins regulating the RNA polymerase-promoter interaction positively and/or negatively. This complicates the quantitative interpretation of the effect of the mutation on the direct RNA polymerase-promoter interaction. The *ampP15G16* up-mutation (Jaurin *et al.*, 1981) is the only one described that is located within a purely constitutive promoter. We have chosen to isolate up-mutations in the *ampC* promoter both because no regulatory protein is known to be involved in the initiation from this promoter, and because of the strict correlation between the levels of ampicillin resistance and β-lactamase production enabling direct isolation of β-lactamase overproducing strains occurring at frequencies as low as $<10^{-11}$ per viable cell.

The *ampC* attenuator, which has been shown to mediate the growth rate-dependent regulation of the operon, leads to premature termination of only about three-quarters of the transcripts in rich media (Jaurin *et al.*, 1981). Thus, by selection on broth plates, background attenuator mutants will not be obtained when selection is made for more than a 4-fold increase in β-lactamase expression. In preliminary experiments selection was made for increased β-lactamase production from a chromosomally located *ampC* gene in a number of different strains. Selection for a 10-fold increase in β-lactamase production yielded only two mutants out of $>10^{12}$ cells. Both mutations were moved to plasmids and found to be gene rearrangements (data not shown). Selection with a strain carrying *ampC* on a pBR322 derivative with a high copy number was preferred because of the higher number of *ampC* genes that could be mutated in each cell. A mutation in one plasmid copy will, after a few generations, give a

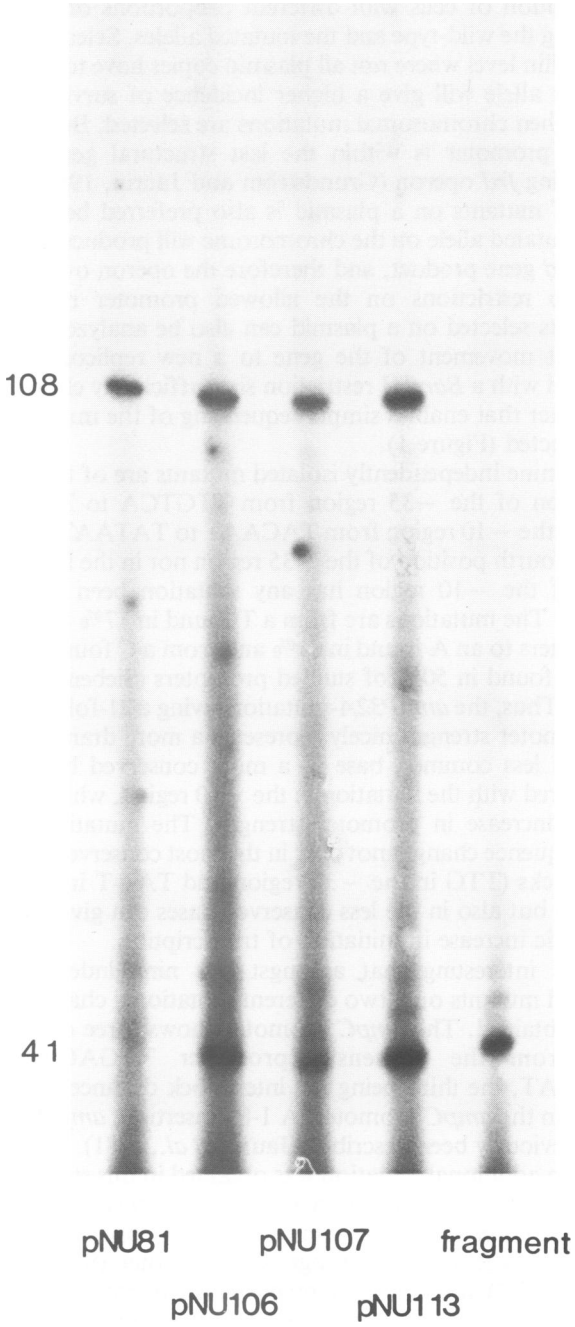

Fig. 4. *In vitro* transcription with *ampC* plasmids. The conditions for *in vitro* transcription are described in Materials and methods. The RNA products were electrophoresed on a 6% polyacrylamide/8 M urea gel followed by autoradiography. 108 and 41 designate the 108-base transcript of the pBR322 origin of replication (Morita and Oka, 1979; Sutcliffe, 1978) and the 41-base long *ampC* transcript (Jaurin *et al.*, 1981), respectively. CCC plasmids were used as templates when analyzing pNU81, pNU106, pNU107, and pNU113. The fragment used was a *Sal*I-*Xho*I fragment of plasmid pNU28 carrying the *ampP15G16*-mutation to show the location of the 41-base long transcript previously reported (Jaurin *et al.*, 1981).

distribution of cells with different proportions of plasmids carrying the wild-type and the mutated alleles. Selection at an ampicillin level where not all plasmid copies have to carry the mutant allele will give a higher incidence of surviving cells than when chromosomal mutations are selected. Because the *ampC* promoter is within the last structural gene of the preceding *frd* operon (Grundström and Jaurin, 1982), selection of mutants on a plasmid is also preferred because the non-mutated allele on the chromosome will produce the wild-type *frd* gene product, and therefore the operon overlap will put no restrictions on the allowed promoter mutations. Mutants selected on a plasmid can also be analyzed directly without movement of the gene to a new replicon. A new plasmid with a *Bam*HI restriction site sufficiently close to the promoter that enables simple sequencing of the mutants was constructed (Figure 1).

The nine independently isolated mutants are of two types, mutation of the −35 region from TTGTCA to TTGACA and of the −10 region from TACAAT to TATAAT. Neither in the fourth position of the −35 region nor in the third position of the −10 region has any mutation been described before. The mutations are from a T found in 17% of studied promoters to an A found in 61% and from a C found in 19% to a T found in 50% of studied promoters (Siebenlist *et al.*, 1980). Thus, the *ampP32A*-mutation giving a 21-fold increase in promoter strength nicely represents a more dramatic shift from a less common base to a more conserved base when compared with the mutation in the −10 region, which gives a 7-fold increase in promoter strength. The mutations show that sequence changes not only in the most conserved bases in the blocks (TTG in the −35 region and TA--T in the −10 region) but also in the less conserved bases can give rise to a dramatic increase in initiation of transcription.

It is interesting that amongst our nine independently isolated mutants only two different mutational changes have been obtained. The *ampC* promoter shows three discrepancies from the consensus promoter TTGACA-17bp-TATAAT, the third being the inter-block distance, which is 16 bp in the *ampC* promoter. A 1-bp insertion, *ampP15G16*, has previously been described (Jaurin *et al.*, 1981). However, no such additional mutation was obtained in this study. A lot of different 1-bp insertions in a 16-bp segment can occur. The finding that *in vitro* expansion from 16 to 17 bp in inter-block distance gives a much stronger *lac* promoter (Stefano and Gralla, 1982) argues that many possible 1-bp insertions in the *ampC* promoter would increase the promoter strength. We argue that either 1-bp insertions in a short DNA segment are rare compared with a specific single base substitution or that a very limited number of the possible 1-bp insertions in the 16-bp DNA segment can increase the promoter strength sufficiently much or both. At the lowest selection level (50 μg/ml) three *ampP11T* and no *ampP32A* were obtained. This indicates that the C→T transition is more common than the T→A transversion.

The 10 promoter mutants fall into three groups representing each of the three discrepancies of the *ampC* promoter from the consensus promoter. This indicates that the three concensus elements are not only common elements of promoters but also the main elements determining the promoter strength. Our data indicate that no other single sequence change in the promoter can increase the strength of the promoter sufficiently to give the selected ampicillin resistance level. Promoter mutations previously isolated by others favor this hypothesis. The mutations giving big increases or decreases in promoter strength are mutations to or from the consensus sequence, respectively (Siebenlist *et al.*, 1980). In this context we want to stress that the *ampC* promoter contains the consensus +1 region CAT. It is quite possible that a study similar to ours of a promoter with another +1 region would have revealed up-mutants in this region.

Weiher and Schaller (1982) have shown that *in vitro* constructed mutants with C→T transitions in the *lac* promoter had relatively little effect on the promoter strength. In nine mutants with an average of six C→T transitions between positions −48 and +12, none had changed the promoter strength more than 2.5-fold. On the contrary, a single mutant with a base substitution in the −35 region had a 10-fold decrease in promoter strength. Therefore, our data and that of others (Jaurin *et al.*, 1981; Stefano and Gralla, 1982; mutations reviewed in Siebenlist *et al.*, 1980) can be generalized as follows: the gain or loss of one base homology to the −35 or −10 consensus blocks or the change to or from a 17-bp inter-block distance represents roughly a factor of 10 in promoter strength and other types of single sequence changes only give minor effects on promoter strength.

Materials and methods

Materials

All chemicals were of the highest grade commercially available. The enzymes used in this study were from New England Biolabs and Boehringer Mannheim. The *Bam*HI linker was from Collaborative Research. [α-^{32}P]dGTP (3000 Ci/mmol), [γ-^{32}P]ATP (>5000 Ci/mmol) and [^{35}S]methionine (1200 Ci/mmol) were from Amersham. Ampicillin was kindly provided by AB Astra, Sweden.

Construction of plasmid pNU81

The two *Pst*I fragments from plasmid pBY06 obtained from A.Byström, this laboratory, carrying the kanamycin-resistance gene was inserted in the *Pst*I site which delimits the pBR322 DNA and the *ampC* DNA of plasmid pNU79 (Grundström and Jaurin, 1982). The construction of this plasmid, denoted pNU80, was carried out by O.Olsson, this laboratory. pNU81 is a derivative of pNU80 where a *Bam*HI linker (5'CCGGATCCGG 3') was inserted into the *Rsa*I site at position −84 to −81 of *ampC* and this *Bam*HI site was fused to the *Bam*HI site in the tetracycline resistance gene of plasmid pNU80. A physical map of the obtained plasmid is shown in Figure 1.

Isolation of spontaneous mutants

Approximately 1 x 10^{10}−2 x 10^{10} cells of an overnight culture of *E. coli* JC12768 (a C600-derivative: *rpsL, thr, leu, del(srlR-recA)306::*Tn*10*, kindly provided by Bernt Eric Uhlin, this laboratory) containing plasmid pNU81 were plated per LA plate (Bertani, 1951) containing 20 μg/ml of kanamycin and 50, 75, or 100 μg/ml ampicillin and grown at 37°C for ~30 h. Mutants obtained were checked for their markers and restreaked twice on LA plates containing 20 μg/ml kanamycin and the selected level of ampicillin. To detect copy number mutants and DNA rearranged mutants a total lysate assay was made: ~10^8 cells were incubated for 30 min at 65°C in 50 μl of 10% glycerol, 1% SDS, 0.01% bromophenol blue, 2 mM EDTA, 20 mM Na-acetate, 33 mM Tris-HAc. 10 μl was applied to a 0.7% agarose gel. Plasmid DNA was purified (Jaurin and Grundström, 1981) from mutants that showed the same plasmid size and the same plasmid content compared with a total lysate of JC12768/pNU81. *E. coli* SN01 (*pyrB, thr, leu, recA, rpsL* (Normark and Burman, 1977) was transformed (Mandel and Higa, 1970) and selection was made on LA plates containing 100 μg/ml streptomycin, 20 μg/ml kanamycin, and 30 μg/ml ampicillin. The transformants were checked for their markers and restreaked twice on LA plates containing streptomycin, kanamycin, and 50 μg/ml ampicillin. Mutant plasmid DNA was purified as previously described (Jaurin and Grundström, 1981).

To construct plasmid pNU113, the *E. coli* strain LA51 (*pyrB, thr, leu, his, rpsL, ampP15G16*, Normark and Burman, 1977) was transformed with plasmid pNU81 as described (Mandel and Higa, 1970). Transformants were selected on LA plates containing 100 μg/ml streptomycin, 20 μg/ml kanamycin, and 20 μg/ml ampicillin. Transformants were grown in 1 ml Luria Broth (Bertani, 1951) for 3 h and ~10^8 cells were plated on an LA plate containing 20 μg/ml kanamycin and 100 μg/ml ampicillin. Recombinants

were obtained at a frequency of $\sim 10^{-8}$ per viable cell. This low frequency may be due to the limited length of homology between the *ampC* allele of the chromosome and plasmid pNU81 (i.e., there are only 68 bp between the *Bam*HI linker and the *ampP15G16* mutation). After testing the markers, plasmid DNA was purified from the *in vivo* generated recombinant and transformed into strain SN01 as described above.

DNA sequence analysis

Approximately 5 pmol plasmid DNA was linearized with *Bam*HI. The 3′ end labeling was in principle as described by Schwarz *et al.*, 1978. 15 pmol [α-^{32}P]dGTP (3000 Ci/mmol) was incorporated with the use of 5 units of DNA polymerase I (Klenow fragment) (Boehringer Mannheim). After recleavage with *Xho*I, the two fragments were separated on a 5% polyacrylamide gel and the 436-bp *Bam*HI-*Xho*I fragment was recovered by diffusion as described by Maxam and Gilbert (1980). The nucleotide sequencing procedure was essentially according to Maxam and Gilbert (1980). The degradation products were separated on 0.5 mm thick 8% and 20% sequencing gels with subsequent autoradiography at −80°C using intensifying screens (DuPont Hi-Plus).

Measurement of ampicillin resistance levels

The level of ampicillin resistance mainly conferred by the pNU81 plasmids in SN01 was measured. An identical number of cells (100–400) was plated on LA plates containing different levels of ampicillin. The number of surviving colonies was counted and the concentration of ampicillin necessary to kill 50% of the cells was determined graphically, this value being the LD_{50} of the strain.

Rocket immunoelectrophoresis

The immunoelectrophoresis method of Laurell (1966) was used with the modifications described by Jaurin and Normark (1979). The same amount of protein (determined according to Lowry *et al.*, 1951) was applied to each well in the agarose gel. The area under the precipitation line was taken as a relative value of the amount of *ampC* β-lactamase.

Analysis of protein expression in minicells

Plasmid pNU81 and its derivatives were transformed into the minicell-producing strain M2141 (*del(pro-lac), rpsL, minA minB*, Thompson and Achtman, 1978). Minicells containing pNU107, pNU108, and pNU109 were grown in the presence of 10 μg/ml ampicillin and those containing pNU101–106 and pNU113 were grown in 50 μg/ml ampicillin. Preparation and labeling of plasmid-containing minicells with [^{35}S]methionine were as described by Grundström *et al.* (1980). The radioactive samples were electrophoresed on an 11% SDS-polyacrylamide gel (Laemmli, 1970). Mol. wt. standards and pure *ampC* β-lactamase were electrophoresed in parallel. Fixation, staining, destaining, and autoradiography was performed as described by Grundstöm *et al.* (1980).

In vitro transcription

All templates were first deproteinized. 10–25 μg of DNA in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA was treated for 20 min at 37°C with 40 μg of Proteinase K. After phenol and diethylether extractions and ethanol precipitation, the DNA was dissolved in distilled H_2O. The *in vitro* transcription assay system contained 3.4 nM of CCC DNA or 115 nM of linear DNA, 100 μCi of lyophilized [γ-^{32}P]ATP, 400 μM CTP and UTP, 100 μM ATP and GTP, 100 nM of RNA polymerase (Boehringer Mannheim) in 40 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol. The buffer was according to McClure (1980).

The RNA polymerase was added to 5 μl of reaction mixture, which had been prewarmed to 37°C. After 20 min of synthesis at 37°C, 5 μl formamide-buffer dyes (Maxam and Gilbert, 1980) were added. A 6% acrylamide:bis (19:1) gel with 8 M urea was run and autoradiographed. All plastic wares and solutions used were autoclaved.

Scanning of autoradiographs

The autoradiographs were scanned using a Zeiss KM3 Chromatogram Spectrophotometer. Two autoradiographs with different intensities were scanned. The peak areas were taken as the values of the amount of protein and RNA. In the minicell experiment, the relative amount of *ampC* β-lactamase was determined by taking the amount of NPTII as an internal standard. In the *in vitro* transcription experiment, the relative amount of *ampC* β-lactamase RNA (41-base transcript) was measured by taking the 108-base transcript as an internal standard.

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