Volume 14 Number 6 1986 Nucleic Acids Research

The promoters of the genes for colicin production, release and immunity in the ColA plasmid: effects of convergent transcription and Lex A protein

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Received 24 January 1986; Accepted 19 February 1986

ABSTRACT

The initiation sites of transcription in vivo for the three genes caa, cai and cal encoding respectively colicin A (Caa), the immunity protein (Cai) and the pColA lysis protein (Cal) have been analysed by nuclease S1 mapping. This analysis demonstrates that caa and cal form an operon. cai is located between these two genes and transcribed in the opposite direction from its own promoter. The start sites for caa and cai have also been determined in vitro. For caa, the same start site was found in vivo and in vitro. In constrast, for cai the most efficient start site in vitro was not used in vivo. LexA protein strongly repressed the in vivo and in vitro transcription of the caa-cal operon. As determined by DNase I protection experiments, LexA protein binds with a high affinity to an approximately 40 bp long sequence just downstream of the Pribnow box. The sequence of the binding site is composed of two overlapped "SOS boxes". Two transcripts of the caa-cal operon were detected by blot hybridization. The longer mRNA can direct the synthesis of both Caa and Cal while the shorter one is terminated at the end of caa. When the transcription of the caa-cal operon is induced, there is a strong interference with cai transcription.

INTRODUCTION

pColA is a small colicinogenic plasmid of 6.9 Kb (1). The best characterized proteins encoded by this plasmid are the colicin A (Caa), an antibiotic protein of 63,000 daltons, the immunity protein (Cai), of 20,500 daltons which protects producing cells from colicin A action, and the lysis protein (Cal) of 5,800 daltons, the expression of which is required for secretion of colicin A (2). The nucleotide sequences of the genes for these three proteins have been previously reported (2, 3, 4) as well as the existence of a putative LexA binding site downstream of the caa promoter accounting for the induction of transcription of this gene by treatments that damage DNA or inhibit DNA replication (5).

In this paper, we present a detailed account of the gene organization and regulation of transcription of the three genes. We present evidence that the LexA protein is indeed a repressor of the colicin A gene. The LexA binding site of the caa operator region is described and the effect of LexA protein on the transcription of this gene in vitro is demonstrated.

In order to further elucidate the mechanism of regulation of the three genes caa, cai

and cal, their transcriptional start sites have been determined. The construction of plasmids (containing these three genes) which differ by the modification of the upstream and -35 box of the promoter of caa gene, demonstrates the existence of a LexA regulated operon formed by caa and cal. The intercistronic region of 592 bp between caa and cal comprises cai which is transcribed constitutively in the opposite direction. Moreover, it is demonstrated that transcription from the cai promoter is blocked by convergent transcription when the expression of the caa-cal operon is induced.

MATERIALS AND METHODS

Bacterial strains, growth conditions and plasmids.

The bacterial host strain W3110 and C600, plasmids pBR322 and pColA have been previously described (6). The strain W3110 (pColA9, pColA or pDV5) was grown in the M9 minimal medium supplemented with glucose (4 g/l), casamino acids (4 g/l), thiamin (5 mg/l), and ampicillin (50 mg/l). Colicin A synthesis was induced with 300 ng/ml of mitomycin C (MTC) in early log phase ($A_{600\ nm}$ = 0.1).

Construction of recombinant plasmids.

pVC60 (Fig. 1) was constructed as follows : pBR322 was linearized with BamHI. After treatment with DNA polymerase I (in the presence of deoxyribonucleoside triphosphates) to make blunt ends, the linearized plasmid was ligated to the HincII fragment of pColA containing the entire coding sequence of the colicin A gene. pColA9 (Fig. 2) was constructed by exchanging the EcoRV-NdeI fragment of pColA which contains the caa, cai, and cal genes for the EcoRV-NdeI fragment of pBR322 containing the tetracycline gene. To introduce the cai and cal genes in pVC60, the SmaI-EcoRV fragment of pColA9 was exchanged for the SmaI-EcoRV fragment of pVC60 and the resulting recombinant was called pDV5.

Preparation of E. coli total RNA.

Total E. coli RNA was prepared essentially as previously described (7). Briefly, a volume of culture was harvested such that the total absorbance at 600 nm corresponded to 6 OD units and an equal volume of cold M9 medium containing 20 mM NaN_3 was added. The cells were collected by centrifugation and resuspended in 1 ml of 20 mM sodium acetate (pH 5.5), 0.5% SDS and 1 mM EDTA (SSE buffer). After addition of 2 ml of redistilled phenol (equilibrated with the SSE buffer), the mixture was shaken vigourously at 60°C for 10 min. After centrifugation, the aqueous phase was extracted at room temperature with 1 ml of phenol. The aqueous phase was finally extracted with $CHCl_3$ and precipitated twice with ethanol. After centrifugation, the pellet was washed with 70% ethanol, dried in vacuo, resuspended

in 0.2 ml of water and stored at -20°C. The E. coli total RNA was stable for several weeks. The RNA concentration was determined by measuring the absorbance at 260 nm.

S1 nuclease quantitative analysis.

E. coli total RNA was analysed by quantitative S1 nuclease mapping using ^{32}P-5'-end labelled single stranded probes (described in fig. 2). An amount of 20 µg total RNA (E. coli tRNA was added to bring the total amount to 20 µg when this was required) was mixed with an excess of probe (as determined by testing different amounts of E. coli total RNA) and dried in a speed vac concentrator. The pellet was dissolved in a hybridization buffer (35 µl) containing 50% desionized formamide, 400 mM NaCl, 40 mM 1-4 piperazine-N, N'-bis (2-ethane sulfonic acid) pH 6.5 and 1 mM EDTA. After denaturation for 10 minutes at 85°C, the solution was incubated at 42°C for 12 hours. The samples were diluted 10 fold in a buffer containing 400 mM NaCl, 30 mM sodium acetate, 3 mM $ZnSO_4$ and 200 units of S1 nuclease (BRL). After incubation at 25°C for 4 hours, the reaction was stopped with 70 µl of 2 M Tris-HCl pH 8.0, 20 mM EDTA. The samples were precipitated twice, washed with 70% ethanol and dissolved in loading buffer containing 80% formamide. After denaturation at 90°C for 3 minutes, electrophoresis was carried out in 7 M urea-6% polyacrylamide gels. The dried gels were autoradiographed and the amount of each RNA transcript was measured by scanning of suitably exposed autoradiographs. These amounts were corrected using the internal standard (transcription of the bla gene).

DNase I protection experiments.

The reaction mixture (200 µl) contained 10 mM Tris-HCl at pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 100 µg/ml bovine serum albumin, 5 µg/ml Salmon sperm DNA, labelled DNA ($\leqslant$ 0.22 nM) and various amounts of LexA protein (0 to 80 nM). After preincubation for 15 minutes at 37°C, DNase I (2 ng/ml) was added to the reaction mixture and the incubation was continued at 37°C for 15 minutes. The reaction was stopped by the addition of cold sodium acetate pH 4.5 (0.3 M) and tRNA (50 µg/ml). The DNA was precipitated and washed with 70% ethanol. The pellet was resuspended in loading buffer and analyzed by electrophoresis on a 7 M urea-6% polyacrylamide gel, followed by autoradiography. The ^{32}P-5'-end-labelling of DNA and nucleotidic sequencing were standard techniques, as previously described (8).

In vitro transcription.

3 to 5 nM of DNA template (NcoI (259)-PvuII (-147) fragment for caa or BglII (2190)-HindIII (from pBR 322) fragment for cai) were incubated with or without 150 nM of LexA protein in 50 µl of 150 mM KCl, 6 mM $MgCl_2$, 30 mM Tris-HCl pH 7.9,

6 mM 2-mercaptoethanol, 2% glycerol, 100 µg/ml BSA for 10 minutes at 37°C. Then 200 µM of nucleoside triphosphates and 1 µg of E. coli RNA polymerase (Boerhinger) were added and incubated for 20 minutes at 37°C. The reaction was stopped by addition of one volume of phenol and the aqueous phase was precipitated twice with 5 µg of tRNA as carrier and washed with 70% ethanol. The dried pellet was solubilised in water before S1 mapping analysis.

Blot hybridization of cellular RNA.

Total RNA was first electrophoresed on a 10 mM methylmercury-1.1% agarose gel (9), then transferred to nitrocellulose and hybridized with nick-translated restriction fragments, as previously described (10).

Enzymes.

Restriction and other enzymes were obtained from commercial suppliers and used according to their instructions. LexA protein was kindly provided by M. Schnarr (IBMC, Strasbourg).

RESULTS

Construction of recombinant plasmids.

In order to carry out quantitatively the S1 nuclease analysis, we required an internal "invariable" gene not regulated by the inducers of cellular "SOS functions". The β-lactamase gene (bla) is transcribed from two well defined promoters P1 and P3 (11), and could fulfill this function. Moreover, the ampicillin resistance provides an easy selection of transformants; pColA9 was thus constructed as described in Materials and Methods. To obtain conclusive evidence about the existence of caa-cal operon, pDV5 was constructed to modify the promoter region of the presumed operon. This was achieved by changing the TTGACA sequence to TCGACA, using the HincII restriction site located in the -35 box of the promoter in pColA, as described in Fig. 1 and Materials and Methods. Besides this main difference with respect to our goal, pColA9 and pDV5 differ in that there is a deletion of the fragment -250 to -1 in the promoter region of caa in the pDV5 construction (Fig. 1).

Probes for the initiation sites of transcription of caa, cai and cal.

S1 nuclease-mapping for transcription initiation sites required the use of various ^{32}P-5'-end labelled probes as described in Fig. 2. For caa, the NcoI (259)-PvuII (-147) probe was used. The HpaII (2656)-HaeIII (2467), HincII (2603)-HincII (2124), and BglII (2190)-NdeI (1816) fragments were used to locate the start site of transcription for cal. For cai, the BglII (2190)-HindIII (from pBR322) and HaeIII (2467)-HpaII (2656) probes were used. As mentioned above, the bla gene was used as an internal standard in quantitative S1 mapping experiments. The AvaII-EcoRI and TaqI-EcoRI restriction

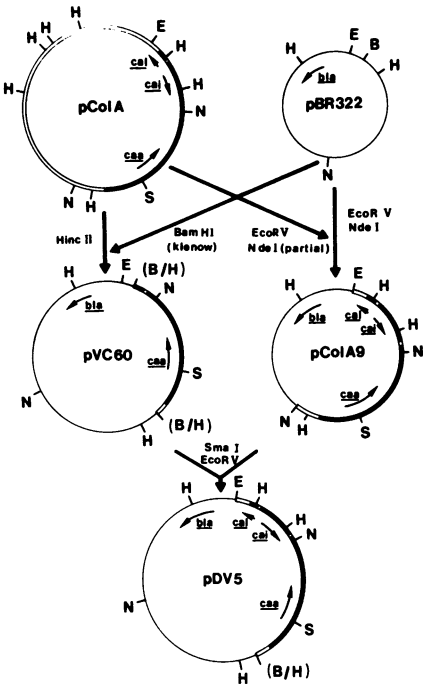

Fig. 1 : Scheme for construction of pColA9 and pDV5 plasmids.
The restriction sites are abbreviated as follows : B, BamHl; E, EcoRV; H, HincII; N, NdeI; S, SmaI. Bracketed restriction sites were destroyed in the constructs. Arrows indicate the direction of transcription of the genes and bold lines indicate coding sequences for caa, cai and cal genes.

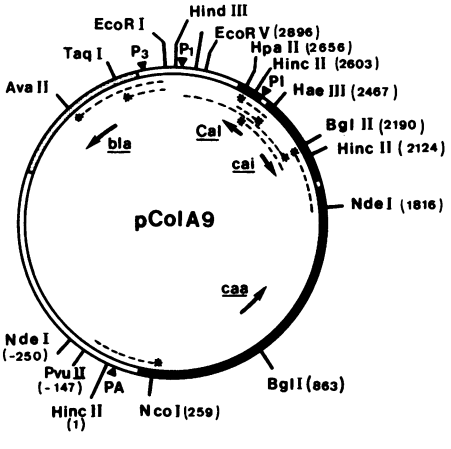

Fig. 2 : Probes used in nuclease S1 mapping experiments.
The probes (dashed lines) are the 32P-5'-end-labelled (star) single-strand fragments of the non-coding strand for the bla, caa, cai and cal genes. Bracketed numbers correspond to the nucleotide just after the site of cleavage of the various restriction enzymes. Restriction sites which are not numbered belong to pBR322. PA and PI indicate the location of the Pribnow boxes for caa and cai respectively. P1 and P3 indicate the locations of Pribnow boxes for bla. Bold lines indicate the coding sequences for caa, cai and cal. The stippled line corresponds to the coding sequence of bla. Arrows show the direction of transcription for the various genes.

fragments from pBR322 were used for this purpose. The 5'-end labelled probes were chosen such that they overlapped the promoter region and the beginning of the coding sequence of the different genes. The length of the probe was determined knowing the nucleotide sequence of the gene.

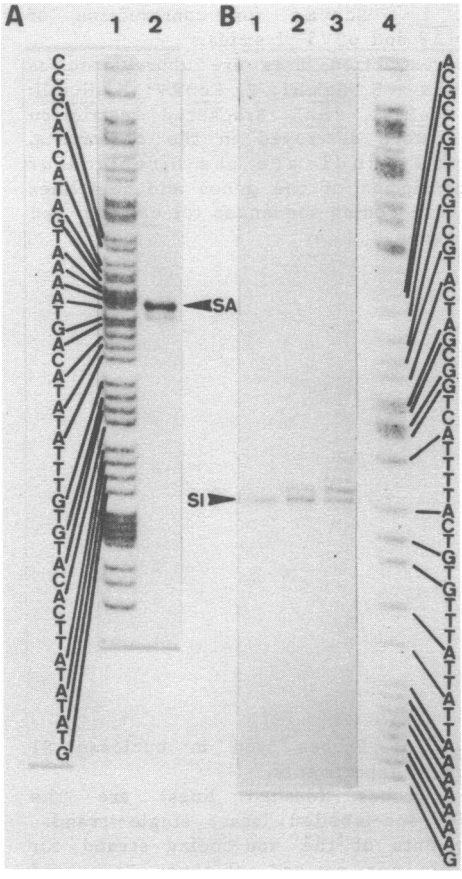

Fig. 3 : S1 mapping analysis of transcription start sites in vivo for caa and cai.
A) The 5'-end labelled NcoI-PvuII probe was hybridized with total cellular RNA from MTC treated W3110 (pColA) cells. The arrow corresponds to the major start site for caa (SA). B) The 5'-end-labelled HaeIII-HpaII probe was hybridized with total RNA from uninduced cells W3110 (pColA). The amounts of S1 nuclease used were : 400 units (lane 1), 200 units (lane 2) and 100 units (lane 3). The arrow marked SI indicates the transcriptional start site for cai.
Nucleotide sequence from chemical cleavage at the purines of the caa and cai probes are shown in A): lane 1 and B):lane 4, respectively.

The in vivo transcription initiation site of caa.

Production of colicin A in E. coli and related bacteria is highly increased when cells are grown in the presence of MTC, an inducer of SOS responses (12). The RNA from induced W3110 (pColA) cells was thus hybridized with the labelled NcoI-PvuII probe. The hybrid was treated with S1 nuclease to remove the unhybridized RNA and DNA tails. After denaturation of the hybrid, the protected DNA probe was analyzed (Fig. 3A, lane 2). By comparison with the bands of the nucleotide sequence of the probe, the start site can be determined. The position of the S1-resistant band indicated takes into account the 1.5 nucleotide faster migration of the Maxam and Gilbert sequence bands (13, 14). The major and minor fragments correspond respectively to an adenine nucleotide at position 35 and a thymine nucleotide at position 37 (Fig. 4, upper line). Whether there are two mRNAs initiated at the 35 and 37 positions or whether the minor fragment is due to an artifact from the S1 mapping

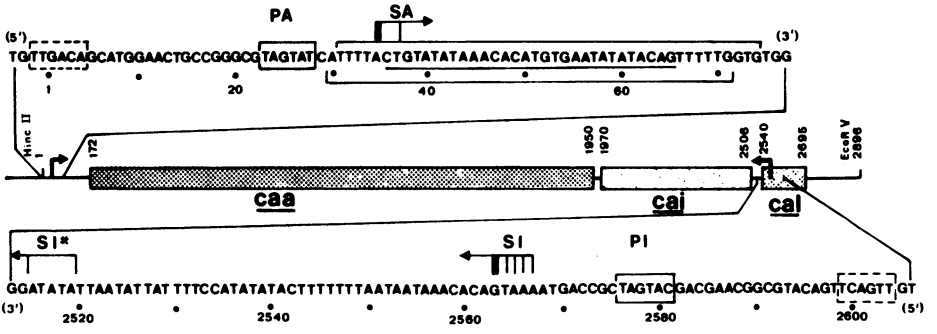

Fig. 4 : Organization of the caa-cal operon and the cai gene.
The nucleotide sequences for the promoters are presented : the recognition and binding sequences for RNA polymerase are boxed with dotted lines (-35 sequence) and solid lines (Pribnow box). PA and SA indicate the Pribnow box and transcriptional start site for the caa-cal operon while PI and SI indicate those for the cai gene. SI* indicates the start site for cai in vitro. Arrows above the start sites indicate the direction of transcription, the bold line indicates the major band detected by S1 mapping. In the case of SI*, one of the nucleotides within the bracket corresponds to the start site. The binding site for LexA in the operator region for caa is indicated by brackets overlining the protected region for the sense-strand and brackets underlining the sequence (not represented) for the anti-sense strand. The two overlapped consensus "SOS sequences" are underlined. The sequence is numbered taking as the +1 nucleotide the cleavage site for HincII. Coding sequences for caa, cai and cal are indicated by stipples and hatched boxes. Numbers above the boxes indicate the first and last nucleotides of the coding sequence, and arrows indicate the direction of transcription.

is not known. Using twice as much or less S1 nuclease per assay did not make any significant difference in the pattern obtained (not shown). A Pribnow box TAGTAT (PA, Fig. 4), and a -35 box TTGACA, the latter of which is a perfect consensus sequence, are found upstream of the start sites of transciption (Fig. 4).

Transcriptional start site of cai in vivo.

Total RNA from W3110 (pColA) was hybridized with the HaeIII-HpaII probe described in Fig. 2. The number of S1-resistant bands depended upon the nuclease concentration. Using 100 units, five protected DNA fragments were detected (Fig. 3B, lane 3); with 200 units, 2 bands were observed (lane 2) and with 400 units only the lower fragment was protected (lane 1). This latter corresponds to the G nucleotide at position 2563 and the other fragments correspond to T,A,A,A nucleotides at positions 2564 to 2567 (Fig. 4). Upstream of these nucleotides a Pribnow box CATGAT (PI, Fig. 4) and a 35 box TTGACT are found (Fig. 4). However, the distance between the Pribnow box (PI) and the apparent major start site of transcription (nucleotide 2563) is probably too long (12 nucleotides) to be correct. Indeed, the longest reported distance between the start site of transcription

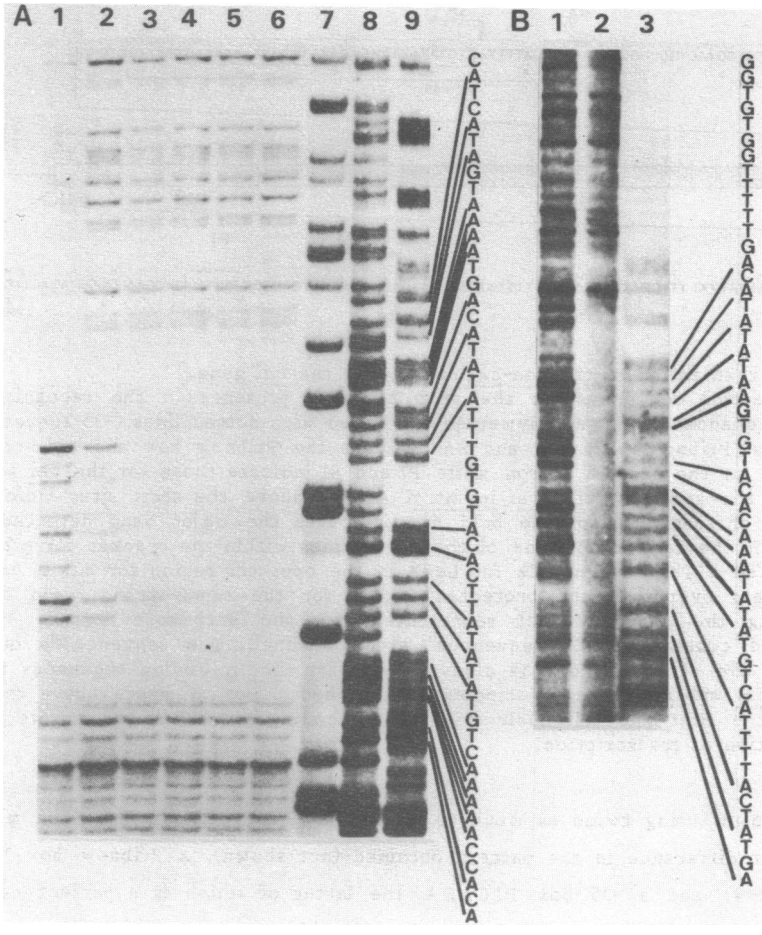

Fig. 5 : Binding of LexA to the operator of the caa-cal operon.
Footprinting was carried out as described in Materials and Methods. The promoter, operator region for caa-cal is contained in the PvuII-NcoI restriction fragment. A) Various concentrations of LexA were incubated (15 min) with the fragment labelled at the 5'-end of NcoI: lane 1, 0; lane 2, 2.5 nM; lane 3, 10 nM; lane 4, 20 nM; lane 5, 40 nM; lane 6, 80 nM. Nucleotide sequence from chemical cleavage at G (lane 7) at G+A (lane 8) and at A>C (lane 9), are also shown. B) The 406 bp fragment labelled at the 5'-end of the PvuII restriction site was used for the footprinting without LexA (lane 1) or with 20 nM LexA (lane 2). The pattern from chemical cleavage at A+G is shown in lane 3.

and the Pribnow box for an E. coli promoter is 8 nucleotides (15). The observation of the kinetic of S1 nuclease digestion and the AT rich character of the sequence at this point suggest that the real start site of transcription could be the A nucleotide

at position 2567. This artifact of the S1 mapping technique has also been observed with the start sites of transcription of the early promoter of SV40 (16).

Lex A protein represses the expression of the caa gene.

As previously reported (5), just downstream the promoter region of caa there is a possible LexA binding site (Fig. 4). To assess the role of this site in the regulation of caa expression, LexA binding was assayed by footprinting experiments. The LexA protein was indeed found to bind this site (Fig. 5 A and B). The coding strand was protected from DNase action from nucleotide 31 to 74 (Fig. 4 and Fig. 5B) and the non-coding strand was protected from nucleotide 30 to 71 (Fig. 4 and Fig. 5A). The region that is protected might be slightly shorter than that actually shown in Fig. 4 since short DNA segments appear to be resistant to DNase I (Fig. 5A and B, lane 1). However, the protected region cannot be shorter than the sequence underlined in Fig. 4. By using densitometer scanning of the protected bands (with appropriate corrections for differences in the amount of samples applied in the various lanes), 70% protection was measured with 2.5 nM of LexA protein (Fig. 5A, lane 2). Similar results were found with the 5'-end labelled PvuII-NcoI fragment (not shown). Thus a dissociation constant value of LexA protein for the operator of lower than 2.5 nM can be estimated.

The caa and cal genes are organized in an operon.

The expression of cal, like that of caa, is increased in MTC-treated cells (2, 17). Thus, RNA from untreated or MTC-treated cells from the strains W3110 (pColA), W3110 (pColA9) and W3110 (pDV5) was hybridized with various suitable probes, described in Fig. 2, and digested with S1 nuclease to determine the transcriptional start site of cal. The full length of the HpaII-HaeIII fragment hybridized with a specific messenger RNA (EPC, Fig. 6A, lanes 1 to 6). In fact, a very slight difference (about two nucleotides) was detected between the full-length probe (minor band) and a major band (see for example Fig. 6A, lane 4). In this situation, it is hard to decide whether this major band corresponds to the transcriptional start site of cal or if it results from an artifact of the S1 mapping. The HpaII-HaeIII (189 bp) probe covers 73 nucleotides upstream of the ATG translation initiation codon of cal. From the results shown above, the transcriptional start site might have been near the HaeIII site (2467). To check this hypothesis, the HincII-HincII (479 bp) fragment (Fig. 2), covering 426 nucleotides upstream of the ATG initiator was used. Again, the probe fully hybridized to the mRNA (EPC, Fig. 6A, lanes 7, 8) implying that the transcriptional start site was not near the HaeIII site but upstream of the HincII (2124) site. Another probe, BglII-NdeI (Fig. 2) was thus used, but again the fragment protected from S1 nuclease corresponded to the full length probe (not shown).

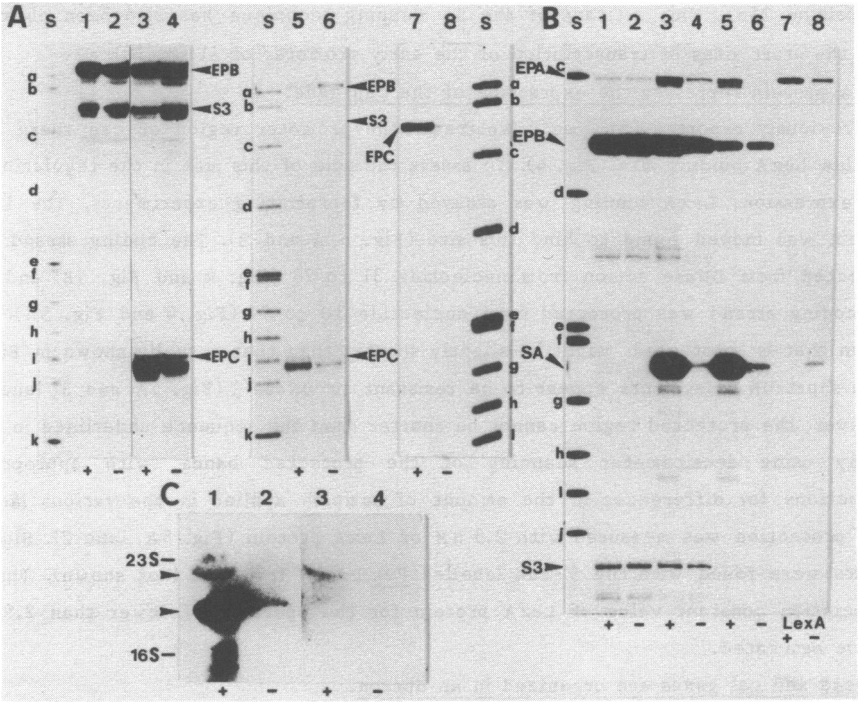

Fig. 6 : The caa-cal operon. Quantitative S1 nuclease mapping of transcriptional start sites in vivo for the caa and cal genes in pColA, pColA9 and pDV5.
A, B) Total RNA was isolated from W3110 (pDV5) (lanes 1 and 2), W3110 (pColA9) (lanes 3 and 4) and W3110 (pcolA) + C600 (pBR322) (lanes 5 and 6) cells, either untreated (-) or treated for 5 hours with MTC (+). SA and S3 indicate the transcription start sites for caa and bla respectively. EP represents the entire probe. EPA, EPB and EPC indicate the S1 nuclease resistant bands corresponding to the full-length of the caa, bla and cal probes. **A) S1 mapping of transcription of cal (lanes 1 to 6).** The probes used for cal and bla were respectively : HpaII-HaeIII and AvaII-EcoRI single strand 5'- end labelled fragments. For cal, the HincII-HincII probe described in Fig. 2 was also used (lanes 7 and 8). **B) S1-mapping of transcription of caa in vivo (lanes 1 to 6) and in vitro (lanes 7 and 8).** The NcoI-PvuII and TaqI-EcoRI probes were used for caa and bla respectively. LexA protein was either added (+) or omitted (-) in the reaction mixture. **C) Evidence for two mRNA transcripts for the caa- cal operon as detected by blot hybridization.** Total cellular RNA (5 ug) isolated from W3110 (pColA) cell untreated (-) or treated 5 hours with MTC (+) was fractionated on a methylmercury-agarose gel and transferred to nitrocellulose paper. The paper was hybridized with the nick translated probes: NcoI-BglI (lanes 1, 2) and HaeIII-HpaII (lanes 3, 4). Arrows indicate the RNA transcripts. The 16S and 23S ribosomal RNAs of E. coli migrated to the indicated locations. In lanes S, the HpaII digestion products of pBR322 were used as Mr standards: a (622 bp), b (527 bp), c (404 bp), d (309 bp), e (242 bp), f (238 bp), g (217 bp), h (201 bp), i (190 bp), j (180 bp), k (160 bp).

Comparing the intensity of protected fragments from MTC-induced (Fig. 6A, lanes 3, 5 and 7) and uninduced cells (lanes 4, 6 and 8) indicates that induction results in an increase in the amount of mRNA produced. The only possible LexA binding site immediately upstream of the NdeI site (1816) is the sequence 1522-1537 inside of caa, homologous to LexA, but which is not protected from DNase I action (see Discussion). Thus we are led to conclude that the caa promoter and operator are also used for cal transcription. To check this hypothesis, we used pDV5 in which the -250 to -1 region of the caa promoter has been deleted, removing a possible CRP (cyclic AMP receptor) binding site (unpublished result) and modifying the -35 box.

As previously mentioned, the transcript from bla was used as an internal standard. Only the S3 hybrid from the P3 bla promoter (and not the EPB hybrid) could be used as a reliable internal standard (Fig. 6, A and B). Indeed, the bla probes map only the transcript initiated by P3 (S3) because the full-length of these probes hybridizes with the transcript initiated by P1 (EPB). The transcription of bla was not found to be altered at all in MTC induced cells (data not shown). Given these facts, the only difference between the samples applied to the various lanes is in their amounts. Furthermore, traces of DNA contaminating the RNA preparations would also hybridize with all the probes used. Thus, despite the use of an internal standard, we could not rely completely upon the intensity of the full-length hybrids of cal (EPC). However, it is clear that EPC is highly decreased in RNA from W3110 (pDV5) cells compared to RNA from W3110 (pColA9) cells.

In order to compare the level of transcription of caa to that of cal, RNA from W3110 (pColA9), W3110 (pDV5) and W3110 (pColA) was hybridized with the caa probe and analyzed by S1 mapping. The bla transcript was again used as an internal standard. In the case of W3110 (pColA), we added the internal standard as follows: one volume of MTC treated or untreated W3110 (pColA) cells ($A_{600\ nm}$ = 0.6) was mixed with one volume of C600 (pBR322) cells (at the same optical density) before purification of total cellular RNA. Thus, after scanning the autoradiograph, the optical density corresponding to the hybrid of the caa transcript (SA, Fig. 6, B) was corrected according to the internal standard band (S3, Fig. 6, B). Thus, we could estimate that 5 hours after MTC induction there was about a 25 fold increase in the level of caa transcription with pColA or pColA9. With RNA from W3110 (pDV5), the amount of caa hybrid resistant to S1 nuclease was much lower regardless of induction, than that observed from W3110 (pColA9) cells. Since for both caa and cal, the level of transcription was severely reduced in the pDV5 construction (which affects only the promoter region of caa), these results indicate that cal forms an operon with caa.

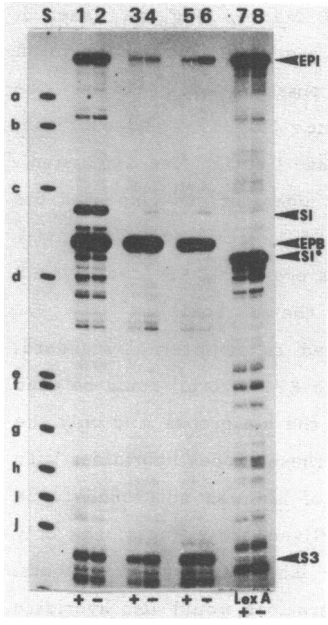

Fig. 7 : S1 nuclease mapping of transcription initiation of cai gene.
Total cellular RNA was isolated from W3110 (pDV5) (lanes 1, 2), W3110 (pColA9) (lanes 3,4) and W3110 (pColA) + C600 (pBR322) (lanes 5, 6) cells treated for 5 hours with MTC (+) or untreated (-). SI and S3 indicate the transcriptional start site in vivo for cai and bla respectively. EPB and EPI are the S1 nuclease resistant bands corresponding to the full-length of bla and cai respectively. For the transcription in vitro, the same probe was used as a template and the LexA protein was either added (lane 7) or omitted (lane 8) in the reaction mixture. SI* is the start site for transcription in vitro.

Transcription start site in vitro for caa and cai.

The NcoI-PvuII fragment (Fig. 2) was used as a template for RNA polymerase in the absence or presence of the LexA protein. A hybrid resistant to nuclease S1 was detected only in the absence of LexA protein (SA, Fig. 6 B, lane 8). The transcriptional start site corresponds to that of the major start site in vivo. When the autoradiograph was overexposed (not shown), one could also detect the minor hybrid (observed in vivo) initiated at the T nucleotide (at position 37, see fig. 4).
For transcription of cai in vitro, the BglII-HindIII probe was used as a template for RNA polymerase, in the presence or absence of LexA. A hybrid resistant to S1 nuclease was detected regardless of the addition of LexA (SI*, Fig. 7 and Fig. 4). This in vitro start site (SI*) located between nucleotides 2514 and 2520 is different of the in vivo start site (SI). Thus the cai promoter in vitro is located in a DNA region that contains 90 % of A and T nucleotides.

Evidence for two mRNA transcripts for the caa-cal operon.

Total cellular RNA was also analyzed by blot hybridization (Fig. 6C). To this end, a ^{32}P-labelled nick-translated probe NcoI-BglI corresponding to a coding fragment of caa (Fig. 2), was hybridized to blotted RNAs. Two RNA bands were found to be hybridized (Fig. 6C), the major one (approximately 2000 nucleotides) corresponds to colicin A mRNA, the minor one corresponds to a longer transcript (about 2700 nucleotides) probably directing synthesis of both colicin A and Cal protein. The

difference between MTC-treated (+) and untreated (-) cells (Fig. 6C, lanes 1, 2) can be clearly detected. In uninduced cells only a small amount of the shorter transcript was detected. The amount of larger transcript was probably too low to be detected. The second point of interest is the existence of two mRNA species which confirms the organization of caa and cal in an operon with a possible attenuation occuring at a previously proposed Rho-independent terminator (3). When the ^{32}P-labelled HaeIII-HpaII nick-translated probe was used, only the longer transcript was detected and only in MTC-treated cells (Fig. 6C, lane 3), a result expected from the location of the probe.

Effect of convergent transcription of caa, cal and cai.

The effects of convergent transcription between trp and λP_L promoters have been previously reported (18). In particular, it has been proposed that collision of RNA polymerase molecules moving in opposing directions can terminate transcription (18). Such a situation was encountered in this study for transcription of the caa-cal operon and the cai gene. In order to assess the effect of convergent transcription in this system, the expression of cai was studied with the three plasmids : pDV5, pColA9 and pColA, in MTC-treated and untreated cells (Fig. 7).

When the transcription of the caa-cal operon is induced in pColA and pColA9, the start site of cai transcript cannot be detected (Fig. 7, lanes 3 and 5) while in non-induced cells a weak band is observed (SI, Fig. 7, lanes 4 and 6). In pDV5, in which the transcription of the caa-cal operon is decreased (see Fig. 6B, lanes 1, 2), an intense band of cai transcript was detected regardless of MTC treatment (Fig. 7, lanes 1, 2). One can thus conclude that when the caa-cal operon is induced, there is an interference with cai transcription. It is of interest to note that the cai transcription even in untreated cells, containing pColA or pColA9, is much lower than that observed in pDV5 (using the internal standard , about a 50 fold difference can be measured). At present, the explanation for this is not entirely clear. However, from a practical point of view, a high cai expression is not required since this gene is constitutively expressed under normal growth conditions and since the immunity protein is only aimed at external colicin A, a low expression does not constitute a problem for cell survival (19).

DISCUSSION

In this work, the organization of the caa, cai and cal genes has been analyzed. It has been demonstrated that caa and cal form an operon. However the cai gene, transcribed in the opposite direction, is located between these two cistrons. A detailed analysis of the transcriptional start sites for the caa-cal operon and for cai has also been carried out.

The caa-cal operon promoter has many features which define a strong promoter : i) correct nucleotide sequence in the -10 and -35 consensus regions, ii) correct spacing between the -10 and -35 regions, iii) availability of a purine initiating nucleotide at the correct distance from the Pribnow box. According to the homology score (HS) algorithm for E. coli promoters, as described by Mulligan et al. (20), the HS for the caa-cal promoter is 57.4%, similar to that of the lac UV5 promoter. However the strength of this operon promoter is probably further increased by the presence of a possible CRP (cyclic AMP receptor protein) binding site (two CRP binding sites have also been described for the ceaA gene of pColE1 (21)). This would be consistent with the observation that deleting the -250 to -1 fragment of the caa-cal promoter in pDV5 has such a strong effect on transcription. However, the HS for the modified operon promoter in pDV5 is 39.6%. Since the calculation of HS takes into account only 9 nucleotides upstream of the -35 region, this suggests that the deletion of only these 9 nucleotides (and the first 2 of the - 35 region) might be sufficient to cause the observed decrease in promoter strength.

The promoter for cai used in vivo features a -35 box which is almost consensus, but the Pribnow box has a C in the first position, which is known to cause a severe effect on promoter efficiency (22). Initiation of transcription probably occurs at the adenine (position 2567), located at the maximal distance of 8 nucleotides (15) downstream from the Pribnow box. By S1 mapping experiments, we detect the transcription of the cai gene of pColA or pColA9 only when the caa-cal operon is repressed. However, the level of transcription of cai is much higher in pDV5, which suggests that the very low extent of transcription that occurs from the repressed caa-cal operon of pColA (or pColA9) might be sufficient to prevent to a large extent initiation at the cai promoter (see further).

While the start site of transcription for the caa-cal operon in vivo and in vitro is the same, it is quite different for cai under these two conditions. Although we have not determined exactly (by nucleotide sequencing) the in vitro start site of cai, a five nucleotides region where it should occur according to the size of the transcript is shown in Fig. 4. A possible in vitro promoter (which has a HS of 69.8%) could be defined (position 2558 to 2518) : 5' AAATAATAATTTTTT - 17 nucleotides - TATAATTAT 3' (hypothetical -35 and -10 boxes are underlined). It is striking that this promoter is not used at all in vivo. This suggests that the DNA conformation might create some constraint in the cai region which results in the use of an alternative promoter. The efficiency of the cai promoter in vitro is not influenced at all by the LexA protein.

In contrast, the LexA protein binds to an operator overlapping the transcriptional start site of the caa-cal promoter. This is likely to effectively reduce promoter

occupancy by RNA polymerase since the LexA binding site overlaps one of the major contact regions of this enzyme (23, 24). For the ceaA gene, a similar situation to that reported here has been described (25). A LexA operator downstream from the Pribnow box of ceaA was also found to be protected. In both cases, this region is composed of two overlapped "SOS boxes", one nucleotide overlaps for the ceaA operators whereas two nucleotides do for caa. The affinity of LexA for both caa and ceaA operator also seems to be in the same range.

A putative internal "SOS box" within caa (from nucleotide 1522 to 1537 in the sequence reported by Morlon et al. (3): 5' CTGAATAAAATCACAG 3') is not a LexA binding site, since we did not find any protection in footprinting experiments. In fact, this result is not surprising considering the consensus nucleotide sequence of LexA operator (26) which differs by one nucleotide (CTGA instead of CTGT).

Two mRNA species of about 2000 and 2700 nucleotides from the caa-cal operon which were transcribed in large amounts in vivo under induced conditions have been demonstrated. Since the transcription of the caa-cal operon is initiated at one site in vivo, these two mRNAs must be due to transcription termination at two different sites. The longer transcript (corresponding to caa-cal mRNA) is much less abundant (Fig. 6C) than the short one (caa mRNA). Two mRNA transcripts starting from ceaA promoter in pColE1 have also been described (27). The difference with the case reported here concerns the nature of the first terminator which is Rho-dependent for ceaA (27) and probably Rho-independent for caa (3).

The last point of interest concerns the effect of convergent transcription on gene expression. When caa-cal transcription is induced there is an interference with the transcription of cai such that cai mRNA cannot be detected by S1 mapping experiments. To our knowledge, only one similar case has been reported, in bacteriophage λ (18). The results have been interpreted as meaning that collision of RNA polymerase molecules moving in opposing directions can terminate transcription. Alternately, a promoter occlusion for cai might occur when the caa-cal operon is actively transcribed since this promoter is located in cal. It is striking to note that transcription from the cai promoter is more efficient in pDV5 than in pColA9 (or pColA) under non induced conditions for the caa-cal transcription. This suggests that even under these conditions, there is some interference with the basal level of transcription of the wild type caa-cal operon.

ACKNOWLEDGEMENTS

The authors wish to thank M. Schnarr for the generous gift of pure LexA protein and for helpful discussions. They are also grateful to D. Job for introducing them to the in vitro transcription system, to J.C. Daghorn for help in blot hybridization

experiments, to V. Crozel and D. Cavard for the gift of pVC60, to P. Howard for a critical reading and to M. Payan for carefully preparing the manuscript. This work was supported by grants from the C.N.R.S., INSERM (Contrat de recherche externe n° 83107) and the "Fondation pour la Recherche Médicale".

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