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Identification and sequence of gene *dicB:* translation of the division inhibitor from an in-phase internal start

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ABSTRACT

The dicA1 mutation, located in the replication termination region of Escherichia coli at 34.9 min, confers a temperature-sensitive, division defective phenotype to its hosts. Previous analysis had suggested that dicA codes for a repressor of a nearby division inhibition gene dicB. We show now that gene dicB is part of a complex operon. Five open reading frames (ORFs 1 to 5) preceeded by a promoter sensitive to dicA repression are found within a 1500 bp segment, and are organized into two clusters separated by a long untranslated region. Evidence for expression of these ORFs was obtained from in vitro or in vivo translation of plasmid-coded genes. IPTG-dependent cell filamentation was obtained when either the entire or the C-terminal part of the fourth ORF was placed under control of the lac promoter. In both cases, a 7 KD protein corresponding to translation from an in-frame ATG of ORF4 (dicB) was made. We propose that this C-terminal protein is the division inhibitor synthesized in dicA1 mutants.

INTRODUCTION

In the course of a search for division-defective mutations located in the region of the DNA replication terminators of Escherichia coli (1, 2), we isolated a mutant, JS1, which starts filamenting when cultures are raised above ca. 30°C. In this mutant, replication is not affected (3). The mutation responsible for this fts (filamentation temperature-sensitive) phenotype, designated dicA1, was mapped at 34.9 min and found to be complemented by plasmids carrying this region. Mutation dicA1 was suppressed by deletion of 10 Kb of DNA including part of the cryptic prophage kim (4), the relB operon (5), dicA and a further thousand base pairs, or by insertion of transposon Tn5 approximately one Kb clockwise of dicA. We concluded that dicA is a repressor of a nearby division inhibitor gene dicB (3).

Further analysis indicated that two genes, dicA and dicC, located close to each other, can complement mutation dicA1 in trans (6), although it was clear that dicC exerts complementation only when carried by multicopy plasmids. Genes dicA and dicC turned out to be structurally homologous to genes c2 and cro of phage P22, respectively (7). To a large extent, dicA and dicC function like their phage counterpart. Thus, in the $dicA^+$ state, dicA is self-activated and represses strongly

both dicCp and a promoter (referred to as dicBp) oriented toward the putative position of dicB. In the dicA1 context, dicA is repressed by dicC, whose expression is autoregulated. However, dicBp is almost entirely derepressed in the dicA1 mutant, exhibiting constant activity at all temperatures, as demonstrated by transcriptional fusions with the gene coding for amylomaltase (7). In the present study, we have sought the location of gene dicB by genetic analysis and sequencing. We now report that at least five genes organized in a complex operon are expressed as a consequence of dicBp derepression. Our results indicate that dicB is the fourth gene within this operon, and that it can be expressed from an internal in-phase start codon, possibly as a consequence of masking of the first ribosome binding site, or of mRNA processing.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains are listed in Table 1. To facilitate the construction of strains deleted for the dicABC region, a "tagged" deletion was made by the procedure of Jasin and Schimmel (11). A derivative of pBR322 (pJPB38) was constructed by linking together fragments originating from three different plasmids: an EcoRI-HindIII fragment from pBS28 (3) carrying tet, ori and 1.9 Kb from the relB region (5)(see Figure 1), a 1.7 Kb BamHI-EcoRI from pBS29 (3) corresponding to sequences at least 2 Kb beyond the dicB region, and the Ω interposon of pHP45Ω (13) as a BamHI-HindIII fragment. Plasmid pJPB38 DNA (4 µg) was cleaved at its unique PstI and EcoRI sites and used to transform strain JS120. Spectinomycin-resistant colonies that had lost resistance to kanamycin and had not gained that for tetracycline were selected. These colonies were presumed to have substituted the 5.5 Kb HindIII-BamHI $dicABC^{+}$ fragment (Figure 1) by the Omega interposon, following recombination within the flanking segments. One of these isolates (JS126) was checked for the loss of the chromosomal HindIII-BamHI fragment by Southern blot hybridization using pKC1 as a probe (not shown).

Strains carrying gene $lacI^{Q}$ integrated into malPp were constructed by the method of Raibaud et al (14). The details of these constructions will be published elsewhere.

Plasmids

The plasmids used in this study are derivatives of pBR322 or pBR325, pUC8 and pUC9 (15), pGB2 (16), pGEM plasmids (17) or pMC1403 (18). They are listed in Table 2. To avoid temperature-sensitivity resulting from DicA1 repressor titration, plasmids carrying dicBp were constructed in derivatives of JS115 or JS126. Plasmids carrying gene dicB and the lac promoter were constructed in JM101 or in derivatives of JS219.

Table 1. Bacterial strains.

Designation	Relevant genotype	Origin
CB0129	W1485 thi leu thyA deo	our collection
JS23	CB0129 (φ80) Δ(trpE) dicA1	(3)
JS54	JS23 dicB::Tn5	(3)
JS115	CB0129 Δ(dicABC)	(3)
JS119	CB0129 Δ(dicABC manA)	(3)
JC7623	rpsL recB21 recC22 sbcB15	(8)
JS119	CB0129 dicA1 dicB::Tn5	(3)
JS120	JC7623 dicB::Tn5	P1 transduction
JS126	JC7623 Δ(dicABC)::aadA	see text
MC1061	Δ(lacIOPZYA) rpsL	(9)
JS216	MC1061 Δ(dicABC)::aadA	P1 transduction
JS219	MC1061 malPpΔ534::lacI^{Q}	D. Gil
JM101	Δ(proB lac) /F'lacIq lacZΔM15	(10)
JS230	JS115 lac::Tn5 srlC::Tn10 recA1	this work
JS238	JS219 srlC::Tn10 recA1	this work
JS284	JS115 malPpΔ534::SalI-HpaI fragment	this work

Cloning and sequencing methods

Procedures for the construction and analysis of recombinant DNA were essentially those described by Maniatis et al (19). For sequencing, 5 μg of XhoI-EcoRI fragment from plasmid pBS37 were purified from a 1% low melting point agarose gel on a NACS PREPAC column (Bethesda Research Laboratories) and digested at 14°C with 100 ng/ml pancreatic DNase in the presence of MnCl2 as described by Anderson (20). Pieces about 200 bp-long were purified from a low melting point gel, end-repaired with the large fragment of DNA polymerase I and ligated at the SmaI site of M13mp9 RF DNA. Complementary sequence information was obtained by cloning the SalI-EcoRI and the EcoRI-BamHI fragments (Figure 1) in phage M13mp10, and by sequencing plasmid pJPB36 by the method of Chen and Seeburg (21). For sequence determination, DNA, labeled with [α^{35}S]-dATP, was run on thermostated 8% acrylamide, 8M urea gels with varying thickness (0.2 to 0.6 mm from top to bottom).

Analysis of plasmid-coded proteins

The coupled transcription translation system was purchased in kit form (Amersham or New England Nuclear) and used with supercoiled plasmid DNA and [^{35}S]-methionine according to the supplier's recommendations. For in vivo labeling, strains carrying different plasmids were grown in Vogel-Bonner medium containing 0.2% casaminoacids, 50 μg/ml ampicillin, and 500 μg/ml methicillin to reduce loss of

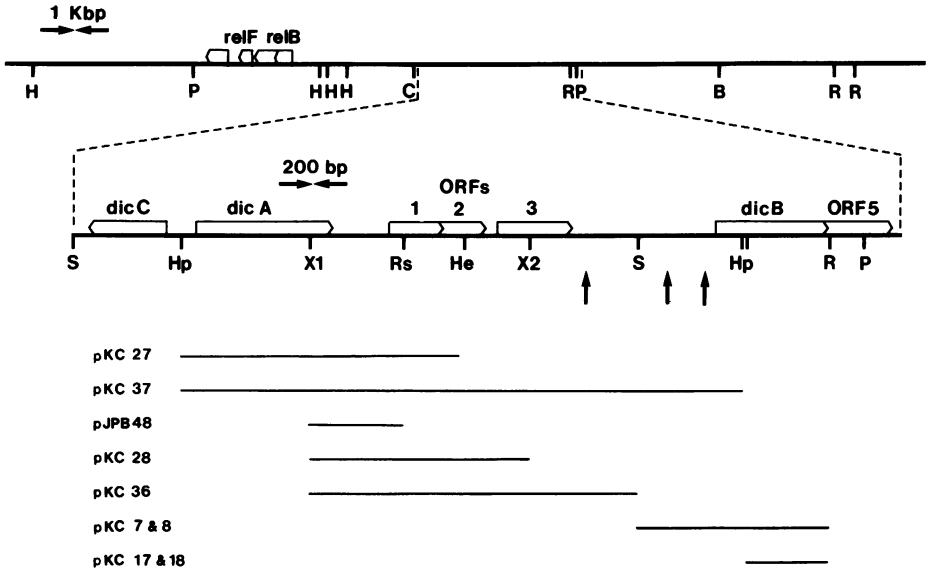

Figure 1. Physical-genetic map of the dicABC region. The map, in clockwise orientation, refers to the 399.1-411.3 kb interval of the map of Bouché (12). Restriction sites cited in the paper are abbreviated as follows: B, H, Hp, P, R, Rs, S and X stand for BamHI, HindIII, HpaI, PstI, EcoRI, RsaI, SalI and XhoI respectively. Vertical arrows indicate the approximate positions of Tn5 (3) or Tn5-lac (7) insertions which suppress the dicA1 phenotype. The position of rel genes in taken from Bech et al (5). The lower part of the figure indicates the chromosomal fragments carried by the plasmids used for the analysis of plasmid-coded proteins.

plasmids (23). Prior to UV irradiation at 30 J/m^2, cells were centrifuged and resuspended in Vogel-Bonner salts. Following UV irradiation, cells were treated essentially as described by Stocker et al (24).

Labeled proteins (5×10^4 to 10^5 cpm) were fractionated on 10% to 15% acrylamide gradient gels containing 6M urea made as described (Focus 6:3, 5). After electrophoresis, gels were equilibrated with a fluor solution (ENHANCE, NEN), dried and applied to Kodak X-AR5 films at -70°C.

Other methods

The PC/Gene sequence analysis package (distributed by Genofit SA and IntelliGenetics Inc.) and a program for promoter search written according to Mulligan et al (25) were used for sequence examination. Comparisons with protein banks were carried out at CITI2 (Palaiseau, France) using of the algorithm of Kanehisha (26) for the search of distantly related sequences. Cell number counting was done using a Model ZBI Coulter Counter with a 30 micrometer aperture.

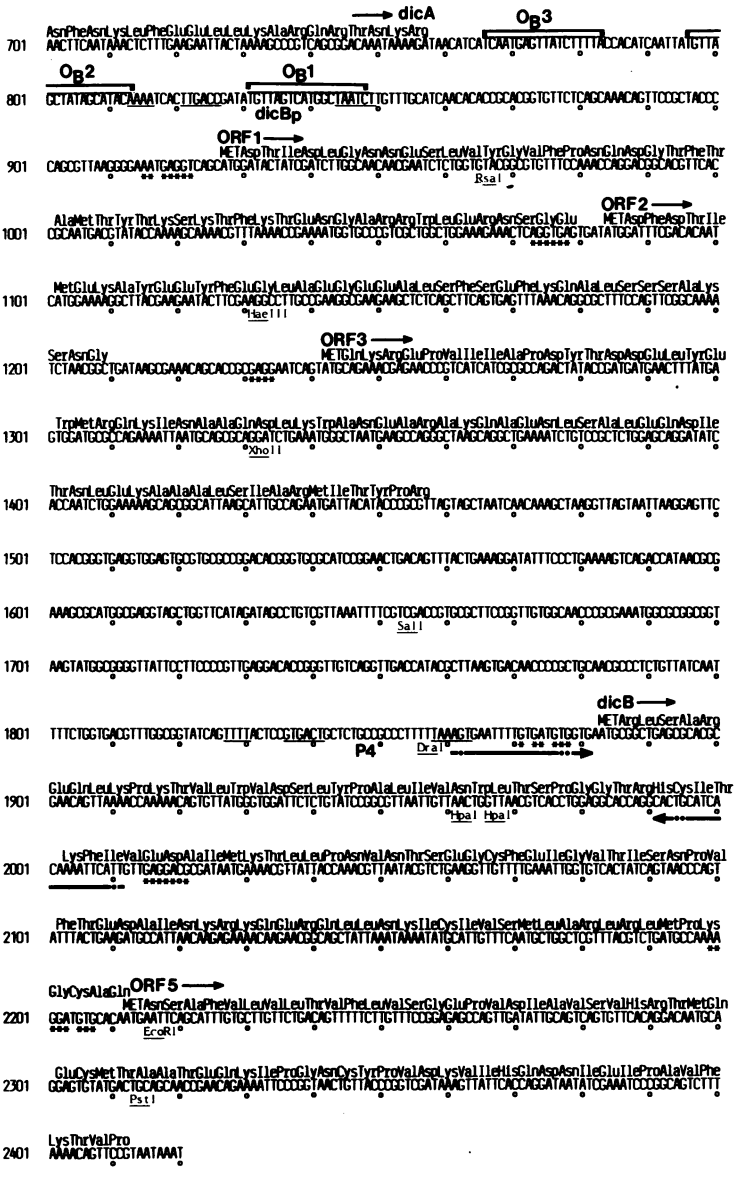

Figure 2. Sequence of part of the dicB operon including gene dicB. The sequence is shown from 5' to 3'. Intervals 751-1115, 1314-1450, 1487-1670 and 1748-2219 were established from sequencing both strands. Promoter consensus sequences are underlined. Data on dicBp and on operators O_B1 to O_B3 is from reference 7 and, for clarity, the possible dicA transcription terminator (6) is not shown. Bases of Shine-Dalgarno sequences that can pair with 16S RNA 5'-end are indicated by asterisks. The 21 nucleotide-long inverted repeat discussed in the text is shown by arrows beyond the putative promoter P4. G~U pairs are shown by dots.

RESULTS

DNA sequence of the dicB region

To avoid possible problems arising from the expression of gene dicB, the sequence of the XhoI-EcoRI fragment was determined by shotgun sequencing (see Materials and Methods). Approximately 200 bp beyond the EcoRI site were also sequenced. 66% of the sequence (Figure 2) was obtained from both strands.

Previous results had indicated that transposon Tn*5*-lac inserted at positions 1500 ± 50 or 1850 ± 50 bp suppresses the fts phenotype conferred by dicA1, and is sensitive to dicA regulation only when present in a clockwise orientation (7). Promoter dicBp, subjected to regulation by DicA and DicC repressors, and its three operator sequences (7), are shown in Figure 2. Promoter dicBp yields a score of 60 in a search according to Mulligan et al (25). When placed in front of gene malQ by the method of Raibaud et al, dicBp gave 1200 units/mg protein of amylomaltase activity in the absence of repressor and 2 units/mg in the $dicA^+$ state (7). The next best promoter candidate (reverse -44 sequence TTTT, -35 sequence GTGACT, -10 sequence TAAAGT, 17 bp spacing), designated P4, gives a score of 54. In an attempt to detect P4-promoted activity, fragment SalI-HpaI (1652-1980) was integrated upstream of malQ on the chromosome in the appropriate orientation. Less than 2 units/mg amylomaltase activity above background could be detected in the resulting strain (JS284), even in the presence of a multicopy plasmid carrying the same fragment, suggesting low intrinsic activity of P4 or a requirement for activation.

Homology between the dicB sequence and phage P22 immC P_L region stops at position 896 (7), located 18 bp upstream from the ribosome-binding site of a first open reading frame. Three ORFs (1 to 3) are then found within 530 bp. The proximity of ribosome binding sites to previous stop codons suggests translational coupling (27). These ORFs are predicted to code for low molecular weight acidic proteins of 5.8, 4.7 and 8.4 KD respectively. Their coding probabilities are 40%, 92% and 100% respectively, as indicated by an analysis according to Fickett (28). To obtain evidence for the synthesis of these proteins, plasmids containing dicBp and either none, one, two or three of these ORFs were constructed (Table 2). When tested in an in vitro coupled transcription-translation system, a single low molecular weight protein was detected, corresponding in size to the product of ORF1 (Figure 3, lanes A to E). In vivo, these plasmids, with the exception of pJPB48, were not maintained unless strong selective pressure was applied (see Materials and Methods), and pKC36 reduced the growth rate by 30% and caused the appearance of balloon-shaped cells (not shown). While proteins with the sizes expected for ORFs 1 and 2 could be detected in maxicells (Figure 3, lane H), the plasmid carrying all three ORFs repeatedly showed the intensive accumulation of a 6 KD protein

Table 2. Plasmids

Designation	Properties and/or reference.
pJPB19	An ampicillin-sensitive derivative of pBR322
pBS28	Chromosomal dicABC$^+$ PstI fragment in pBR325 (3)
pBS29	Chromosomal dicABC$^+$ HindIII fragment in pBR325 (3)
pBS32	Chromosomal HindIII-EcoRI dicABC$^+$ fragment in pBR325 (3)
pBS37	Chromosomal ClaI-EcoRI dicABC$^+$ fragment in pBR322 (3)
pBS39	Chromosomal dicAB$^+$ dicC::Omega fragment in pBR322 (6)
pJPB36	Sal1-Sau3A fragment (1-1331) in pUC9 (3)
pJPB38	See text
pKC1	Chromosomal dicABC$^+$ HindIII-BamH1 fragment in pGB2
pKC7	SalI-EcoRI fragment (1652-2214) in pUC9 SalI-EcoRI
pKC8	SalI-EcoRI fragment (1652-2214) in pUC8 SalI-EcoRI
pKC17	SalI-HpaI deletion from pKC7
pKC18	SalI-HpaI deletion from pKC8
pKC27	HpaI-HaeIII fragment (313-1131) in pGEM1 BamHI-HincII
pKC28	XhoI-XhoII fragment (691-1331) in pGEM1 EcoRI-BamHI
pKC36	XhoI-SalI fragment (691-1652) in pGEM2 SalI
pKC37	HpaI fragment (313-1960) in pGEM1 BamHI-HindIII
pJPB48	XhoI-RsaI fragment (691-965) in pGEM1 SalI-HindIII
pJPB50	SalI-AluI fragment (1652-2143) in pMC1403 SmaI

unexpected from the construction (lane I). The functions of ORFs 1, 2 and 3 are not known.

No ORF with a plausible Shine-Dalgarno sequence is present within the 422 bp following the end of ORF3. Although this segment contains long GC-rich streches and may form strong long-range secondary structures, no simple hairpin-loop with a free energy higher than 13 Kcal/mol is present. Next to this untranslated region are found two contiguous ORFs (4 and 5), that would code for proteins of 12.4 and 7.3 KD respectively. The Shine-Dalgarno sequence of ORF4 is located immediately after the P4 promoter. ORF4 (Fickett coding probability: 29%) contains 2 CGG, 2 GGA, 1 AGG, 2 AUA, 1 AGA, 1 CUA codons, amounting to 7% of total codons, a figure characteristic of weakly expressed genes (29).

Searches in the NBRF database failed to disclose significant similarities between the predicted aminoacid sequences and sequences of other proteins.

Identification of dicB

The locations of Tn5 (3) or of Tn5-lac (7) insertions that suppress temperature-sensitivity associated with the dicA1 mutation are given in Figure 1. They indicate that dicB is located beyond the 422 bp untranslated region. Another set of results suggested that dicB is located before the EcoRI site: when strains carrying either mutation dicA1 or a deletion of the dicABC region were transformed

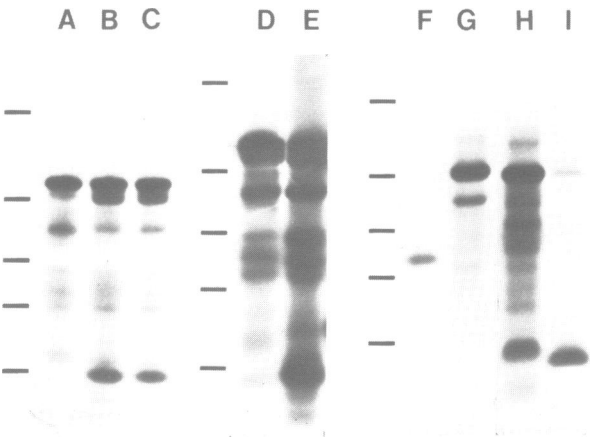

Figure 3. Analysis of plasmid-coded proteins from ORFs 1,2 and 3. Tracks A to E show proteins synthesized in vitro. A and D: pGEM1, B: pKC28, C: pKC27, E: pKC37. Lanes F to I refer to proteins labeled in vivo in the JS230 background. F: no plasmid, G: pJPB48, H: pKC28, I: pKC36. Indicated molecular weights are from a track containing pre-coloured markers. Bands discussed in the text are shown by arrows.

by dicAC$^+$ plasmids, low transformation efficiency was obtained if DNA containing ORF4 was also present (Table 3). Our results showing the analogy of the dicAC locus with a phage immunity region (7) suggested that in the absence of endogenous repressor, plasmid-coded DicC protein takes control of the system, permitting fairly stable expression of dicBp. Low transformation efficiency could thus be explained easily if the product of ORF4 inhibited division and colony-forming ability.

To test this hypothesis, the SalI-EcoRI fragment containing ORF4 was cloned in both orientations in front of the lac promoters of plasmids pUC8 and pUC9. When ORF4 was transcribed from lacZp, the plasmid (pKC7) could be

Table 3. Transformation efficiency of dicB$^+$ and dicB$^-$ plasmids.

	Recipient strain		
Plasmid	CB0129	JS119	JS115
Experiment 1			
pJPB19	2.5×10^6	1.8×10^3	1.7×10^3
pBS32	4×10^6	5×10^3	2×10^3
Experiment 2			
pJPB19	8×10^5	7×10^5	ND
pJPB36	2.7×10^6	2.6×10^6	ND

For each experiment, strains were transformed with the same equimolar mixture of plasmids. Selection was with 25 µg.ml tetracycline (pJPB19) or with 50 µg/ml ampicillin (other plasmids).

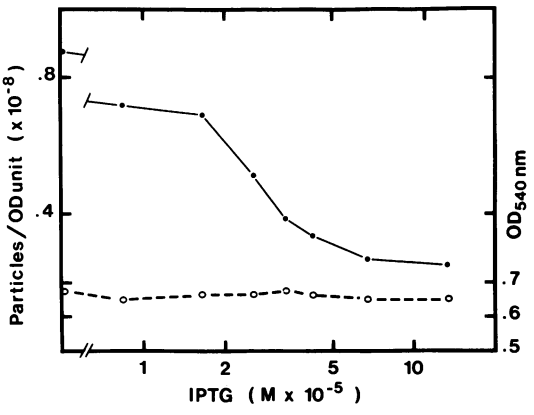

Figure 4. Division inhibition by lacZp-controled gene dicB. Strain JM101/pKC7 was grown in L broth supplemented with 50 µg/ml ampicillin for 6 generations with serial dilutions, then IPTG was added to subcultures at the IPTG concentrations shown in abscissa. After a time sufficient for 3 generations of the culture without IPTG, optical densities (dotted line) and particle number par $OD_{540\ nm}$ unit (continuous line) were measured.

maintained only in a $lacI^Q$ background, and the resulting strains were sensitive to addition of IPTG to the medium. Cloning of the same fragment in reverse orientation (pKC8) did not result in IPTG-sensitivity.

The arrest of cell division promoted by transcription of ORF4 is illustrated in Figure 4. Division of JM101/pKC7 grown in complex medium was half-inhibited for an IPTG concentration of 2×10^{-5} M. Maximal inhibition, corresponding to a residual increase of 40% in cell number, was obtained for $> 10^{-4}$ M IPTG. Under these conditions, cell growth (as inferred from optical density measurements) was not affected. As in dicA1 mutants, cells appeared under the light microscope as long filaments with no visible septa.

A notable feature of the DNA sequence shown in Figure 2 is the presence of a 14 bp inverted repeat between the beginning and the middle of gene dicB. This homology extends to 21 bp at the RNA level (G~U base pairs allowed). Folding by the algorithm of Zucker and Stiegler (30) of RNA pieces of various length including the 1860-2015 bp segment invariably predicted pairing between these regions (ΔG= -27 Kcal/mol). The first part of the repeat includes the Shine-Dalgarno sequence of dicB, while the second part is located just before a potential internal translation start (Figure 2). It was therefore conceivable that, in pKC7, the 5'-end ribosome binding site is not available for translation initiation, either due to pairing between the repeats or RNA processing. It should be noted that the RNA helix formed would meet the length criterion required for processing by RNase III (31, 32). In either case, synthesis of a protein from the internal AUG would still be possible. To test that the 3' half of dicB is sufficient for division inhibition, the 5' half was removed from pKC7 by digestion with SalI + HpaI, end-filling and religation, yielding pKC17. Plasmid pKC17 had no HpaI site left, indicating that the beginning of lacZ' was linked out of phase to dicB at position 1968. Nevertheless, pKC17 inhibited division

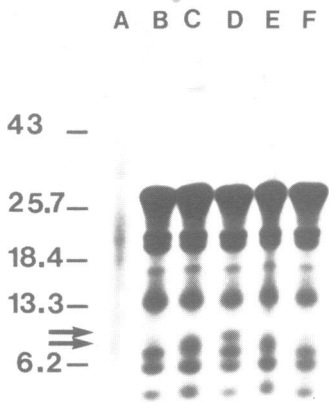

Figure 5. Proteins synthesized in vitro by dicB-containing plasmids. A: no DNA. B to F: pUC9, pKC7, pKC8, pKC17 and pKC18 respectively. Legend is as in Figure 3.

under the same conditions of lacZp induction as did pKC7. Additional evidence for the synthesis of a 7 KD C-terminal protein ($DicB_S$) was obtained from the analysis of plasmid-coded proteins synthesized in vitro (Figure 5). Compared to the pUC9 vector (track B), plasmids pKC7 and pKC17 (tracks C and E) suggested the presence of an additional band (apparent size: 9 KD). This band was not observed with plasmid pKC18 (track F) which lacks an upstream promoter for $DicB_S$. Plasmid pKC8 (track D), which contains dicB without the upstream lac promoter, differed from the vector by the synthesis of an 11 KD protein. This observation will be considered in the Discussion. Attempts to detect a complete ($DicB_L$) or C-terminal ($DicB_S$) form of dicB gene product in UV-irradiated cells were unsuccessful. This negative result may be due to instability of the division inhibitor.

DISCUSSION

The results reported in this paper confirm our original proposal that dicA represses a nearby gene dicB coding for an inhibitor of cell division. DicB has been identified as a gene coding for a 12.4 KD protein, $DicB_L$. However, a fusion to the lac promoter indicates that a smaller protein, $DicB_S$ (7KD), synthesized from an internal in-phase translation start, is sufficient to inhibit division. Indeed, there is some evidence that the 7 KD protein is the actual inhibitor in dicA1 mutants. Firstly, no difference could be seen between the pattern of proteins synthesized in vitro from the lac promoter, whether the entire gene or its 3'-end moiety were present (Figure 5). Secondly, our preliminary results indicate cleavage of dicB operon mRNA near positions 1880 and 2000 of the sequence, prohibiting $DicB_L$ (but not

$DicB_S$) synthesis. These cleavages may be easily explained by pairing between the repeats shown in Figure 2, followed by processing by a double-strand specific nuclease, namely RNase III. As a matter of fact, the question now raised is whether $DicB_L$ is made, and what function it may serve. The only conceivable instance where $DicB_L$ could be synthesized is if transcription starts near the middle of the first repeat, in such a way that no stable secondary structure may be formed but that the first ribosome-binding site is still present. Remarkably, functioning of promoter "P4" (Figure 2) would probably fulfill these requirements. In favor of P4 activity, we note that a distinct protein, with an apparent size of 11 KD, is synthesized from pKC8, for which dicB has no upstream lac promoter (Figure 5). This is only slightly less than the size predicted for $dicB_L$ (12.4 KD). Additional evidence in favor of a promoter located downstream from the SalI site came from the isolation of a dicB-lacZ fusion yielding 54 units/OD_{600nm} of β-galactosidase in the promoter-detection vector pMC1403 (Table 2). However, no obvious phenotype results from high level amplification of dicB associated with P4, and integration of "P4" in front of gene malQ did not result in increased expression of this gene (see Results). Thus a definitive assessment about P4 function must await further study.

Our previous studies had shown that dicB operon transcription is regulated in an all or none fashion by two genes, dicA and dicC, homologous to the immunity C region of phage P22. The first gene of the dicB operon has no significant homology with the gene at a corresponding location in lambdoïd phages, gene N, but this lack of resemblence cannot be taken as evidence against a phage origin for the 5' part of the dicB operon. In view of the large untranslated region between ORF3 and dicB and of the likely presence of a promoter on its own for this gene, it is tempting to consider that insertion of phage sequences (and subsequent dicAC regulation) has added itself to a more primordial expression system of the genes located in the 3' part of the dicB operon.

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REFERENCES

1. de Massy, B., Béjar, S., Louarn, J., Louarn, J.M. and Bouché, J.P. (1987). Proc. Natl. Acad. Sci. USA **84**, 1759-1763.
2. Hill, T.M., Henson, J.M. and Kuempel, P.L. (1987). Proc. Natl. Acad. Sci. USA **84**, 1754-1758.
3. Béjar, S. and Bouché, J.P. (1985). Mol. Gen. Genet. **201**, 146-150.
4. Bouché, J.P., Gélugne, J.P., Louarn, J.M., Louarn, J. and Kaiser, K. (1982). J. Mol. Biol. **154**, 21-32.
5. Bech, F.W., Jørgensen, S.T., Diderishen, B. and Karlström, O.H. (1985). EMBO. J. **4**, 1059-1065.
6. Béjar, S., Cam, K. and Bouché, J.P. (1986). Nucleic Acids Res. **16**, 6821-6833.
7. Béjar, S., Bouché, F. and Bouché, J.P. (1988). Mol. Gen. Genet. **212**, 11-19.
8. Kushner, S.R., Nagaishi, H., Templin, A. and Clark A.J. (1971). Proc. Natl. Acad. Sci. USA **68**, 824-827.
9. Casadaban, M.J. and Cohen S.N. (1980). J. Mol. Biol. **138**, 179-207.
10. Messing, J., Crea, R. and Seeburg, P.H. (1981). Nucleic Acids Res. **9**, 309-321.
11. Jasin, M. and Schimmel, P. (1984). J. Bacteriol. **159**, 783-786.
12. Bouché, J.P. (1982). J. Mol. Biol. **154**, 1-20.
13. Prentki, P. and Krisch, H.M. (1984). Gene **29**, 303-313.
14. Raibaud, O., Mock, M. and Schwartz, M. (1984). Gene **29**, 231-241.
15. Vieira, J. and Messing, J. (1982). Gene **19**, 259-268.
16. Churchward, G., Belin, D. and Nagamine Y. (1984). Gene **31**, 165-171.
17. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984). Nucleic Acids Res. **12**, 7035-7056.
18. Casadaban, M.J., Chou, J. and Cohen, S.N. (1980). J. Bacteriol. **143**, 971-980.
19. Maniatis, T., Frisch, E.F. and Sambrook, J. (1982). Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
20. Anderson, S. (1981). Nucleic Acids Res. **9**, 3015-3027.
21. Chen, E.Y. and Seeburg, P.H. (1985). DNA **4**, 165-170.
22. Zubay, G. (1973). Ann. Rev. Genet. **7**, 267-287.
23. Wong, E.M., Muesing, M.A. and Polisky, B. (1982). Proc. Natl. Acad. Sci. USA **79**, 3570-3574.
24. Stocker, N.G., Pratt, J.M. and Holland, I.B. (1985). In James, B.D. and Higgins, J.J. (eds), Transcription and translation, a practical approach, IRL Press, pp 153-176.
25. Mulligan, M.E., Hawley, D.K., Entriken, R. and Mc Clure, W (1984). Nucleic Acids Res. **12**, 789-800.
26. Kanehisha, M. (1984). Nucleic Acids Res. **12**, 203-213.
27. Schümperli, D., Mc Kenney, K., Sobieski, D.A. and Rosenberg, M. (1982). Cell **30**, 865-871.
28. Fickett, J.W. (1982). Nucleic Acids Res. **10**, 5303-5318.
29. Grosjean, H. and Fiers, W (1982). Gene **18**, 199-209.
30. Zucker, M. and Stiegler, P. (1981). Nucleic Acids Res. **9**, 133-148.
31. King, T.C., Sirdeskmukh, R. and Schlessinger, D. (1986). Microbiol. Rev. **50**, 428-451.
32. Robertson, H.D. (1982). Cell **30**, 669-672.