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A possible role for the *pcnB* gene product of *Escherichia coli* in modulating RNA:RNA interactions

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Summary. The sequence of the PcnB protein of *Escherichia coli,* a protein required for copy number maintenance of ColE1-related plasmids, was compared with the PIR sequence database. Strong local similarities to the sequence of the *E. coli* protein tRNA nucleotidyltransferase were found. Since a substrate of the latter protein, tRNA, structurally resembles the RNAs that control ColE1 copy number we believe that we may have identified a region in PcnB that interacts with these RNAs. Consistent with this idea is our observation that PcnB is required for the replication of R1, a plasmid whose replication is also regulated by a small RNA.

Key words: Plasmid copy number – tRNA nucleotidyltransferase – PcnB – Replication control – ColE1

The replication of ColE1 and related plasmids is regulated by an interaction between two RNAs. RNA II is a 555 bp transcript which, after processing, serves as the initial primer for replication. RNA I, a small RNA transcribed from the DNA strand opposite to that encoding RNA II, is complementary to the 5′ end of RNA II and interacts with it to prevent this processing (for reviews see Simons and Kleckner 1988; Green et al. 1986). A plasmid-encoded protein, the product of the *rop/rom* gene, facilitates the inhibitory interaction between these two RNAs (Tomizawa and Som 1984), but no host protein which participates in the reaction has yet been identified.

A host gene, *pcnB,* whose product has been shown to be necessary for the maintenance of normal ColE1 copy number, has been identified in several laboratories (Lopilato et al. 1986; March et al. 1989; Liu and Parkinson 1989), and its sequence has recently been published (Liu and Parkinson 1989). In order to obtain information about the possible role of PcnB in plasmid replication we used the reported sequence to search the PIR protein database, Version 17 (George et al. 1986) for proteins showing sequence similarity to PcnB. A search was performed using an implementation of the inexact string matching algorithms of Smith and Waterman (1981) on the Active Memory Technology distributed array processor (Lyall et al. 1985). This program identifies the best local matches between portions of the query sequence and portions of proteins listed in the database. The two best matching regions of alignment to the PcnB sequence were within the same protein (see boxed sequences in Fig. 1). Matches of this quality are estimated to have a 0.002 and 0.03 chance, respectively, of occurring randomly in a database of the size analysed; that both should occur within the same protein is not likely to be a coincidence. The protein exhibiting this striking similarity of sequence is the tRNA nucleotidyl transferase of *E. coli,* the product of the *cca* gene (Cudny et al. 1986). The *cca* and *pcnB* gene products are distinct proteins; the genes encoding them are on opposite sides of the *E. coli* chromosome, at 67 (Foulds et al. 1974) and 4 min respectively (March et al. 1989).

More detailed comparisons of the two sequences have been made. PcnB and tRNA nucleotidyl transferase are basic proteins of about the same size (412 vs 410 amino acids) with 26% identical and 50% identical or conservatively substituted residues (Fig. 1). Their similarity is greatest in two regions (see boxes) both of which are in the amino-terminal half of each protein. This is shown graphically on the DOTPLOTS (Fig. 2). Liu and Parkinson (1989) have presented evidence that the amino-terminal portion of PcnB is required for its role in plasmid replication but that up to 20% of the carboxyl-terminal portion of the protein is dispensable. Functional similarities between the two proteins relating to the action of PcnB in plasmid replication are thus most likely to be found in the amino-terminal part of PcnB.

What is the function of tRNA nucleotidyl transferase and does this provide an indication of the way in which PcnB might function? tRNA nucleotidyl transferase attaches -CCA to the 3′ termini of tRNAs. In order to do this, it binds ATP, CTP and tRNA. Two nucleotide binding motifs of the form GXGXXG (see Fig. 1) occur in the Cca protein sequence and one of these is altered in a *cca* mutant with decreased AMP incorporating activity, suggesting that these sequences are indeed involved in nucleotide binding (Zhu et al. 1986). PcnB lacks consensus nucleotide binding sites and it is thus unlikely that it has nucleotide substrates. It seems more likely that the similarity between PcnB and tRNA nucleotidyl transferase indicates that PcnB binds a molecule which resembles a tRNA. tRNA nucleotidyl transferase can bind any tRNA. It must thus recognize either tRNA secondary structure or a sequence motif common to all tRNAs. RNA I and the 5′ portion of RNA II are each believed to adopt a stem-looped configuration which resembles that of tRNA and in addition the nonanuc-

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Cca     1 ........MKIYLVGGAVRDALLGLPVKDRDWVVVGSTP.Q.EMLDAGYQ 40
PcnB    1 MYRLNKAGYEAWLVGGGVRDLLLGKKPKDFD.VTTNATPEQVRKLFRNCR 49

       41 QVGRDF..P.VFLHPQTHE.E.Y.A.L.AR.TERKSg.sgytgFTCYAAP 80
       50 LVGRRFRLAHVMFGPEIIEVATFRGHHEGNVSDRTTSQRGQNGML.LRD. 97

       81 DV..TLEDDLKRRDLTINAL.AQDDNGEIIDPY.NGLGDLQNRLLRHV.S 125
       98 NIFGSIEEDAQRRDFTINSLYYSVADFTVRD.YVGGMKDLKDGVIRLIGN 146

      126 P.A.FGEDPLRVLRVARFAARYAHLGFRIADETLALMREMTHAGELEHLT 173
      147 PETRYREDPVRMLRAVRFAAK...LGMRISPETAEPIPRLA.T.LLNDIP 191

      174 PERVWKETESALTTRNP.QVFFQVLRDCGALRVLFPEIDALF..GVPAPA 220
      192 PARLFEESLKLLQAGYGYET.YKLLCEYHLFQPLFPTITRYFTENGDSPM 240

      221 KWHPE.I..DTG..IHTLMTLSMAAMLSPQVDVRFATLCHDLGKGLTP.P 264
      241 ERIIEQVLKNTDTRIHNDMRVN.PTFLFAAM.FWYPLL..ETAQNIAQES 286

      265 ELWPRHHghgpagVKLVEQLCQRLRVPNEIRDLARLVAEFHDLIHTFPML 314
      287 GL.TYHDAFALAMNDVLDEACRSLAIPKRLTTLTRDIWQLQ.L.RM.SRR 332

      315 NPKTIVKLFDSIDAWRKPQRVEQLALTSEADVRGRTGFESADYPQGRWLR 364
      333 QGKRAWKLLEH.PKFR.AA.YDLLAL.R.AEV.ERNA.E.LQ.RLVKW.W 372

      365 EAWEVAQSVPTKAVV.EAGFKGVEIREELTRRRIAAVASWKEQRCPKPE 412
      373 GEFQVSAPPDQKGIVNELD.EEPSPRQSYSSSTQTRTTS..... 410
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Fig. 1. Sequence comparison of tRNA nucleotidyltransferase (Cca) and PcnB proteins. Sequences were compared using the GAP program of the Wisconsin package (Devereux et al. 1984) with a Dayhoff scoring table. The *boxed* regions are those initially found as the best database matches to PcnB. The *bold lower case* sequences beginning at Cca residues 68 and 272 are putative nucleotide binding sites

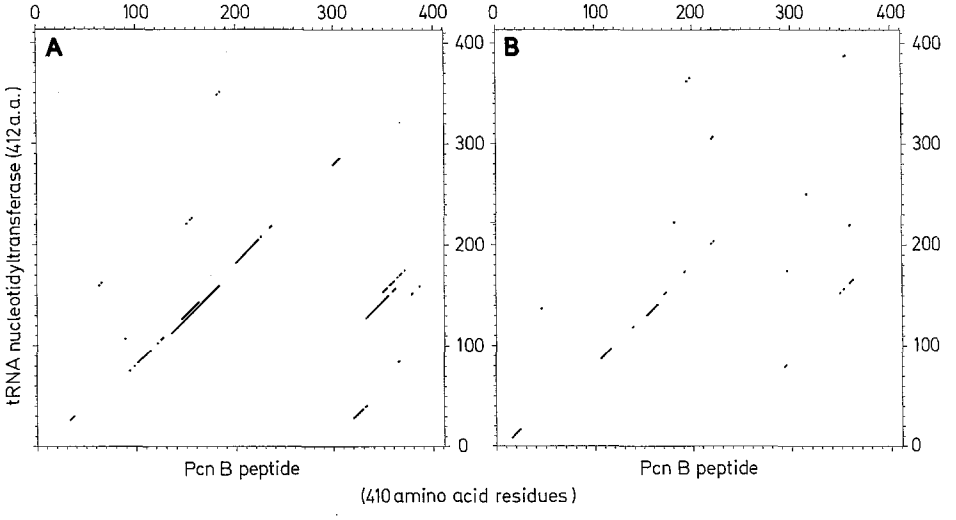

Fig. 2A and B. Sequence comparison of tRNA nucleotidyltransferase and PcnB protein. Sequences were compared using the Wisconsin package program DOTPLOT in the COMPARE mode, in which a dot is placed in the centre of each window which meets the required stringency criterion. **A** Window 50, Stringency 21. This comparison detects long regions of moderate homology best. **B** Window 15, Stringency 11. This comparison emphasizes short regions of strong homology

leotide 5′ AGUUGGUAG is shared by RNA I and many tRNAs (Yavachen and Ivanov 1988). We thus propose that PcnB acts in plasmid replication by binding to RNA I or to RNA II, thereby limiting the degree to which they can interact with one another.

ColE1-related plasmids are not unique in having an RNA:RNA interaction as an important element in copy number control. Most thoroughly documented in this respect is the mechanism of copy number control in IncFII plasmids (such as R1) (Nordstrom et al. 1984). In this plasmid group copy number is controlled by an interaction between two RNAs at the translational level. Initiation of replication of IncFII plasmids depends on a positively acting protein, RepA. RepA is translated, in part, from a transcript which includes several hundred bases upstream of the RepA coding sequence. This region includes a 91 bp sequence which is complementary to a small antisense RNA, CopA-RNA, which is transcribed from the DNA strand opposite to that encoding RepA. Binding of CopA-RNA to the RepA transcript inhibits its translation and, by limiting RepA synthesis, limits replication. CopA-RNA, like RNA I, is believed to adopt a stem-looped structure

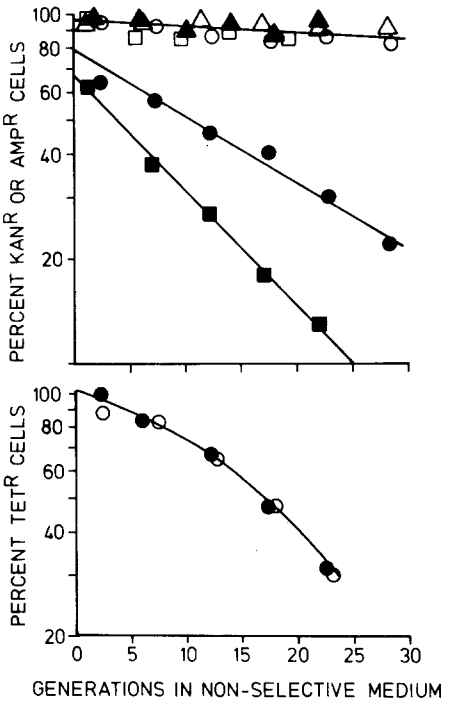

Fig. 3. Stability of plasmids in *pcnB21* and *pcnB*$^+$ strains. Derivatives of MM18 (*pcnB*$^+$; *open symbols*) and JM18 (*pcnB21; closed symbols*), each containing one of the test plasmids, were grown overnight in L broth supplemented with a selective antibiotic (100 μg/ml ampicillin for R1 and pGW71, 50 μg/ml kanamycin for pKN500, and 15 μg/ml tetracycline for pHP6). Cultures were diluted into fresh L broth without antibiotic and samples taken at intervals and plated on LB plates. Colonies were subsequently replicated onto plates containing the appropriate antibiotic and counted. Curves for R1 and its derivatives are shown in the upper portion: pKN500 -○-, R1 -△-, pGW71 -□-. Curves for pHP6 are shown in the lower part of the figure. pHP6 contains 0.9 kb of chromosomal DNA, including *oriC*, and the chloramphenicol and tetracycline resistance genes of pBR325

(Wagner and Nordström 1986). In addition it contains a 7/9 match to the nonanucleotide shared by RNA I and many tRNAs, although, in CopA-RNA, this sequence is located in a stem rather than, as is the case with RNA I, in a loop. If PcnB is a protein which modulates RNA:RNA interactions by binding to and sequestering one of the reactants we might expect that R1 replication would be limited in PcnB mutants. This, in fact, has proved to be the case.

R1*drd-19* and its derivatives pKN500 (Molin et al. 1979) and pGW71, derived from pOU71 (Larsen et al. 1984) by removal of the λ sequences, all obtained from K. Nordström's laboratory, were transferred by mating or transformation into MM18 (*pcn*$^+$) or JM18 (*pcnB21*) (March et al. 1989). Curing experiments (Fig. 3a) show clearly that the R1 mini-plasmids pKN500 and pGW71 are unstably maintained in JM18 as compared with MM18. Cells cured of the R1 plasmid were not observed in this experiment. However the fact that JM18 (R1*drd-19*) displays a reduced growth rate when compared with the other strains (g = 39 min as opposed to 27 min) suggests that plasmid free cells may have arisen but failed to survive. This could be anticipated as the R1 *parB* postsegregational killing system (Gerdes et al. 1986) is intact in R1*drd-19* but deleted from the mini-plasmids. To establish that R1*drd-19* is maintained at reduced copy number in JM18 the single cell ampicillin resistance of this strain was compared with that of MM18 (Uhlin and Nordström 1977). As expected, the ampicillin resistance (measured as the ampicillin concentration at which 50% of cells survive on plates) of the *pcnB21* host was reduced, from 375 to 200 μg/ml, suggesting that the copy number of R1 is approximately halved in the *pcnB* strain.

Although antisense RNAs are involved in controlling the copy number of ColE1-related and IncFII plasmids, no such interaction has been reported to control initiation of chromosome replication at *oriC*. As a control for our R1*drd-19* experiments, therefore, we measured the rate at which pHP6, an *oriC* plasmid carrying a gene for ampicillin resistance, is cured from MM18 and JM18 (Fig. 3b). As can be seen, the *oriC* plasmid, although unstable in JM18, is equally unstable in MM18. Thus no effect of the *pcnB21* mutation on initiation at *oriC* is apparent. This is consistent with our observation that chromosome replication is unaffected in JM18 (March et al. 1989).

PcnB is not required in an in vitro plasmid replication system reconstituted from purified proteins (Minden and Marians 1985), thereby excluding the possibility that it performs a unique and essential biochemical role in the replication process. This is consistent with our proposal that PcnB acts by limiting the reactions between complementary RNAs that normally limit the frequency with which plasmid replication can be initiated. According to this hypothesis PcnB can be considered to be a modulator of replication control circuits that acts by interfering with the repression of replication. We would thus expect that deletion of the *pcnB* gene would not totally prevent plasmid replication. Experiments are in progress to test this prediction.

Liu and Parkinson (1989) have reported that when the *pcnB* gene is inactivated, *E. coli* cell growth is impaired in consequence, suggesting that PcnB has a role in the host other than that of controlling plasmid replication. Antisense RNAs have been shown to have a role in controlling the expression of at least two *E. coli* genes, *crp* and *ompF*, as well as that of the transposase encoded by Tn*10* (for reviews see Simons and Kleckner 1988; Green et al. 1986). A protein which modulates RNA:RNA interactions could well have a role in these processes and it is there where the cellular role of PcnB is being sought.

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