Palindromic units from *E. coli* as binding sites for a chromoid-associated protein

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Several hundred copies of a highly conserved extragenic palindromic sequence, 20–40 nucleotides long, exist along the chromosome of *E. coli* and *S. typhimurium*. These have been defined as palindromic units (PU) or repetitive extragenic palindromes (REP). No general function for PUs has been identified. In the present work, we provide data showing that a protein associated with a chromoid extract of *E. coli* protects PU DNA against exonuclease III digestion. This provides the first experimental evidence that PU constitutes binding sites for a chromoid-associated protein. This result supports the hypothesis that PUs could play a role in the structure of the bacterial chromoid.

(E.coli) *repetitive DNA sequence* *DNA-binding protein* *Chromoid structure*

1. INTRODUCTION

E. coli and *S. typhimurium* chromosomes contain DNA sequences of 20–40 bp long that are repeated several hundred times [1]. These repeated DNA sequences are called palindromic units (PUs) [2] or repetitive extragenic palindromes (REPs) [3]. They are arranged in clusters from one to 4 occurrences and are always found outside structural genes. They have the potential to form a cruciform structure within DNA. The stem is G-C rich and highly conserved while the loop is A-T rich and variable (fig.1).

No general function for PUs sequences has been identified yet ([2,3], Gilson et al., submitted). One hypothesis is that they play a role in the structure of the bacterial chromoid [2].

The remarkable conservation of the sequence of the stem of the PUs as well as the existence of a dyad symmetry suggest that they may constitute a protein binding site. To see whether such a protein existed, we used the exonuclease III protection assay that allows the detection of specific DNA-binding protein in a crude extract [4,5]. We anticipated that such a protein could be found associated with the chromoid. Thus, we used proteins extracted at 2 M NaCl from purified chromoids as a source of chromoid-associated protein extract. As a substrate for the assay, we chose two different regions which each contain three PU sequences; one region is located in the *E. coli* chromosome between genes *malE* and *malF* and here is called *interE*; the other one is located between genes *lamB* and *malM* and is called *interB* (fig.2).

2. MATERIALS AND METHODS

2.1. *Chromoid-associated protein extract (CHAP extract) preparation*

Chromoids from *E. coli* C600 were prepared by the method of Varshavsky et al. [9]. The band of chromoid from the sucrose gradient was removed by a syringe and adjusted to 2 M NaCl by adding

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5 M NaCl and incubated at 4°C overnight. The DNA was eliminated by centrifugation at 200000 × *g* for 2 h. The supernatant, containing the extracted proteins, was concentrated by pressure dialysis to a concentration of 1 mg/ml. This extract, when subjected to PAGE, showed a discrete pattern of less than 10 bands of proteins (not shown).

2.2. *DNA labelling strategy*

DNA technologies (restriction digestions, DNA end-labelling, sequencing reactions and gel analysis) were carried out as described in Maniatis [6].

An *Nco*I-*Nco*I DNA fragment containing the *interE* PUs (fig.2) was prepared from pUP1 plasmid DNA. This plasmid carries a *Bgl*II-*Pvu*II fragment of the *malE-malF-malG* region, inserted between the *Bam*HI and *Pvu*II sites of pBR322 (unpublished construction from our laboratory). Plasmid DNA was 5′-end-labelled at the *Nco*I sites with polynucleotide kinase and [γ-^{32}P]ATP according to the standard exchange reaction and cut at a secondary restriction site (*Bcl*I or *Stu*I site) to liberate fragments labelled at one extremity.

An *Eco*RV-*Dde*I fragment, containing the C-terminal part of gene *lamB* and the three *interB* PU sequences (fig.2), was isolated from the DNA of plasmid pMI1 [7] and 5′-end-labelled. The fragment was then cut at the appropriate secondary restriction site (*Stu*I or *Alu*I).

2.3. *Exonuclease III (ExoIII) protection assay*

The assay was carried out as described by Wu [5]. ExoIII was from Boehringer-Mannheim.

3. RESULTS

The exonuclease III enzyme, a 3′- to 5′-exonuclease, degrades a double-stranded DNA fragment progressively from both 3′-termini until the substrate becomes single-stranded and resistant to further digestion. Specific DNA-binding proteins block the passage of ExoIII and generate new fragment(s) [4].

3.1. *An activity in* E. coli *chromoid extract protects PU DNA from ExoIII digestion*

Purified chromoids from *E. coli* cells were extracted at 2 M NaCl and centrifuged. The supernatant was used as a chromoid-associated protein extract (called hereafter CHAP extract, see also section 2). When a 222 bp *Bcl*I-*Nco*I DNA fragment, containing the three *interE* PU sequences, was end-labelled at the *Nco*I site and digested with ExoIII, discrete bands were detected (fig.3, lane o). The mechanisms which govern the appearance of these 'exo-stops' in unprotected DNA are not well understood. In this case and in other cases studied, their locations do not correlate with obvious features of the DNA sequence [4].

In the presence of the CHAP extract, the first PU met (PUa) imposed a block on the progression of ExoIII (fig.4). Indeed, the major protection product is a 140 ± 3 nt band (fig.3, lane n) that corresponds to the size of a fragment ending at the first boundary of PUa (for the position of the PU boundaries, see fig.1).

In the presence of 5 or 10 μg (fig.3, lane m and l) of calf thymus DNA (CT DNA), the new band disappears. In these conditions (about 5000–10000-fold more CT DNA than PU DNA), the yield of the protected fragments is lowered most likely by significant competitive binding to CT DNA. It seems reasonable to explain this competition by non-specific binding to CT DNA and/or to the presence of rare PUs or PU-like sequences. Also a new band appears: it is probably due to competition for ExoIII activity by large excess of CT DNA, as suggested by the data on the *interB* region (see below).

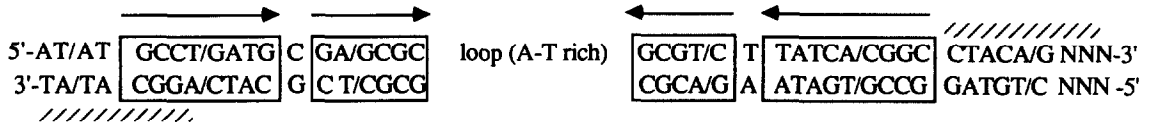

Fig.1. PU consensus sequence and ExoIII digestion boundaries. The boxed sequences, overlined by opposite arrows, could yield a cruciform structure in DNA and are called the stem of the PUs. The hatched areas indicate the two PU boundaries of the ExoIII digestion in the presence of the CHAP extract.

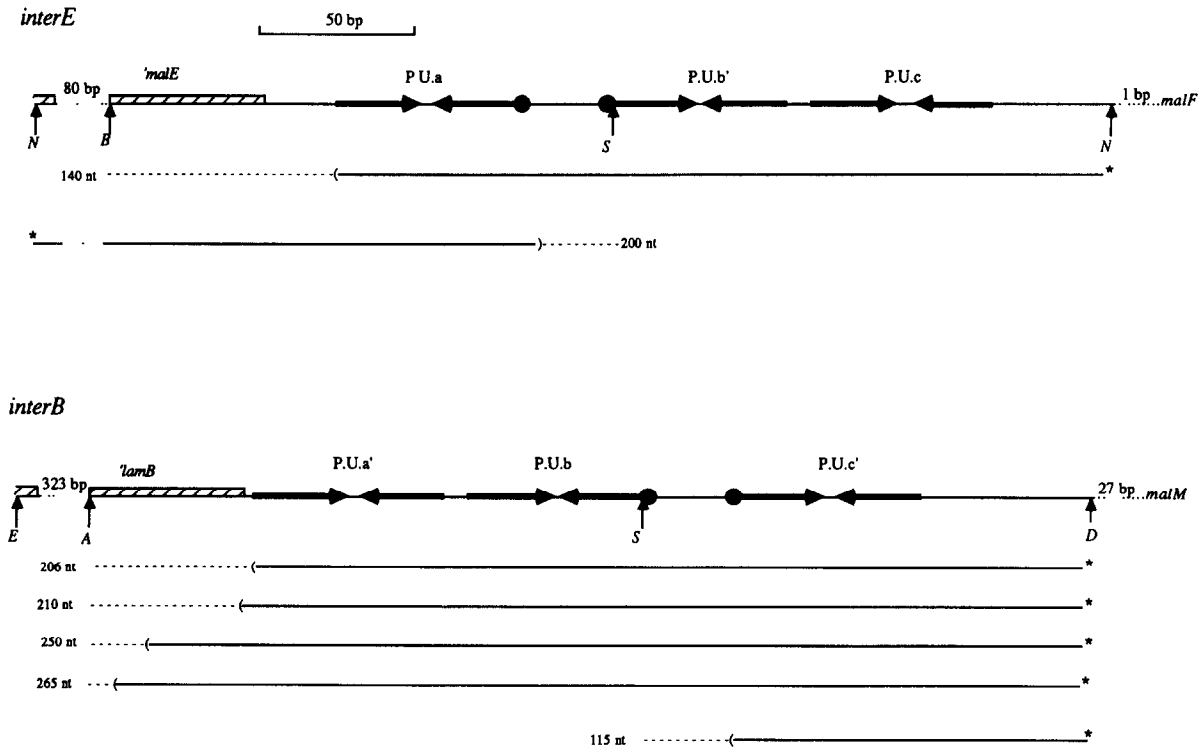

Fig.2. Map of the protected DNA of the *interE* and *interB* DNA region. The stems of the PU sequences are drawn as opposite arrows. (●) CTACA/G sequence, present at the 3′-end of the stem and loop structure and permitting orientation of the PU sequence (see fig.1); (a,b,c) PU sequence on coding strand; (a′,b′ and c′) on other strand; (hatched area) boundary position of each PU sequence. The ExoIII resistant fragments due to the CHAP extract which are visualized through the 5′-label (star), are aligned as solid lines below the map; the size of the protected fragments is indicated. The passage of ExoIII is shown by the dashes. *A*, *AluI*; *S*, *StuI*; *D*, *DdeI*; *B*, *BclI*; *S*, *StuI*; *N*, *NcoI*; nt, nucleotide; bp, base pair.

Similar results were obtained with the three *interB* PUs carried by a 276 bp *Dde*I-*Alu*I fragment, end-labelled at the *Dde*I site. In the presence of the CHAP extract, four fragments (206 ± 3, 210 ± 3, 250 ± 5 and 265 ± 5 nt in fig.4, lane d; see also fig.2) correspond to a block of the ExoIII digestion. The length of the smaller band corresponds to the first boundary of PUa′. The significance of the three other bands is not understood. The 210 nt band lies in the A-T rich region located between the stop codon of gene *lamB* and PUa′. The 250 and 265 nt bands have their 3′-end within the coding region of gene *lamB*. It should be kept in mind that the CHAP extract contains various DNA-binding proteins species that could protect some unknown sites on the DNA fragment used as a probe.

With an addition of 1 μg cold CT DNA (1000-fold excess), the four fragments were still apparent and smaller fragments appeared (fig.4, lane f). Also in the presence of CT DNA, but in the absence of extract, a new band appeared (cf. lanes c, e and g in fig.4). This can be interpreted as competition for ExoIII activity by the large excess of CT DNA. In the presence of 10 μg CT DNA, the protected DNA pattern looked like an unprotected DNA pattern (cf. lanes g and h in fig.4). This supports the idea of competitive binding by CT DNA.

3.2. *One PU is sufficient to stop ExoIII digestion in the presence of the CHAP extract*

In the above experiments, three PU sequences were present on the DNA fragment probe and protection always occurred at the first boundary of the

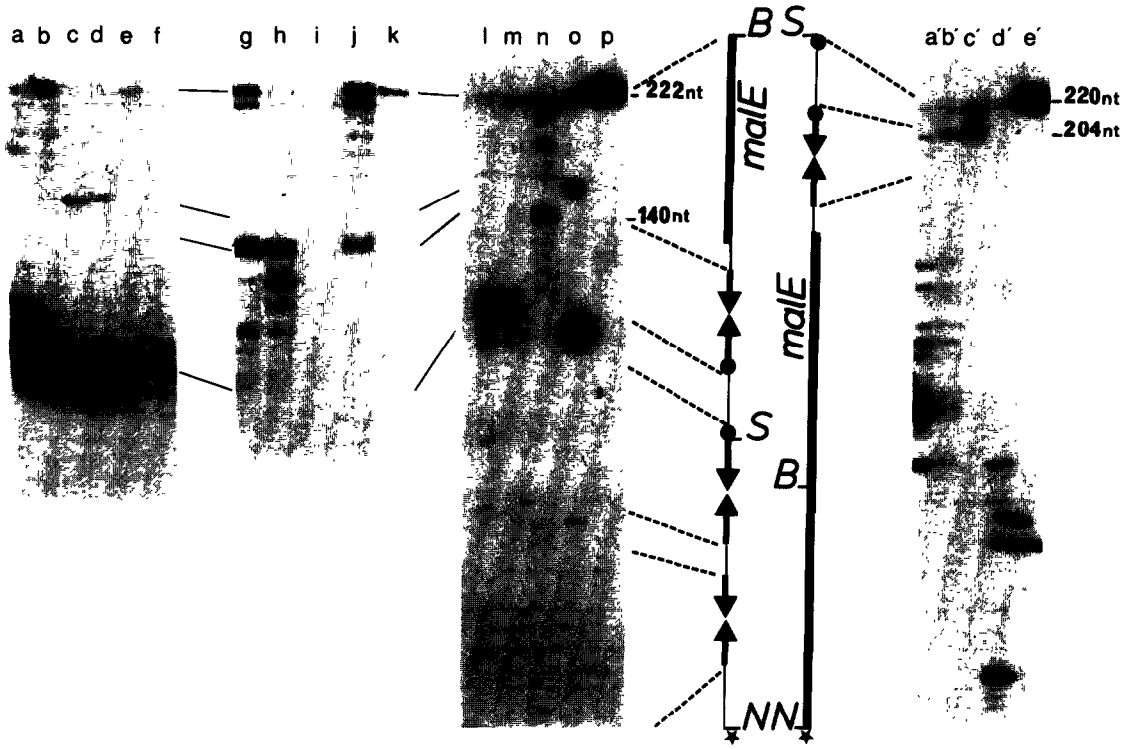

Fig.3. ExoIII protection of the *interE* region DNA conferred by chromoid-associated protein *E. coli* extract and purified DNA-binding proteins. A partial restriction map of the region is shown at the center of the figure. 5′-label (★) and remaining PU symbols are as in fig.2. The size of the fragments discussed in the text is indicated. The corresponding positions in independent gels are indicated by solid lines, using the molecular mass markers of each gel. Lanes a–p: *BclI-NcoI* fragment labelled at *NcoI* site. ExoIII digestion after a preincubation with (a) 1 μg HU protein, (b) 0.1 μg HU protein, (c) 1 μg 17 kDa protein, (d) 0.1 μg 17 kDa protein, (e) 1 μg SSB protein, (f) 0.1 μg SSB protein, (g) 15 μg CHAP extract preincubated for 30 min at 37°C, (h) 15 μg CHAP extract preincubated for 30 min at 37°C with 500 ng trypsin inhibitor (Sigma) and 250 ng trypsin, (i) 15 μg CHAP extract preincubated for 30 min at 37°C with 250 ng trypsin; the ExoIII digestion was then performed in the presence of 500 ng of trypsin inhibitor, (j) 15 μg CHAP extract preincubated for 30 min at 37°C with 500 ng trypsin inhibitor, (k) 15 μg CHAP extract preincubated for 30 min at 37°C with 250 ng trypsin, (l) 15 μg CHAP extract and 10 μg CT DNA, (m) 15 μg CHAP extract and 5 μg CT DNA, (n) 15 μg CHAP extract, (o) ExoIII digestion with no added CHAP extract, (p) undigested DNA. Lanes a′–e′: *StuI-NcoI* fragment labelled at *NcoI* site. Lanes a′–c′: ExoIII digestion after a preincubation with (a′) 15 μg CHAP extract and 10 μg CT DNA, (b′) 15 μg CHAP extract and 5 μg CT DNA, (c′) 15 μg CHAP extract. Lane d′, ExoIII digestion with no added CHAP extract. Lane e′, undigested DNA.

first PU encountered. We asked whether one PU was sufficient to stop ExoIII digestion.

A single PU (*interE* PUa) is carried by the 220 bp *NcoI-StuI* fragment, end-labelled at the *NcoI* site (fig.3, lanes a′–e′; see also fig.2). In the presence of the CHAP extract, a major resistant fragment of 204 ± 3 nt appeared during ExoIII digestion (fig.3, lane c′). This fragment corresponds to a stop of the digestion at the first boundary of PUa met by ExoIII (fig.2). Thus, both boundaries of the *interE* PUa can constitute ExoIII degradation barriers in the presence of extract.

The *interB* PUc′ is carried by a 134 bp *StuI-DdeI* fragment, end-labelled at the *DdeI* site. In the presence of the CHAP extract, a major resistant fragment of 115 ± 2 nt appeared during ExoIII digestion (fig.4, lanes a′ and b′). This fragment corresponds to a stop of ExoIII at the first boundary of PUc′ met by the enzyme (fig.2).

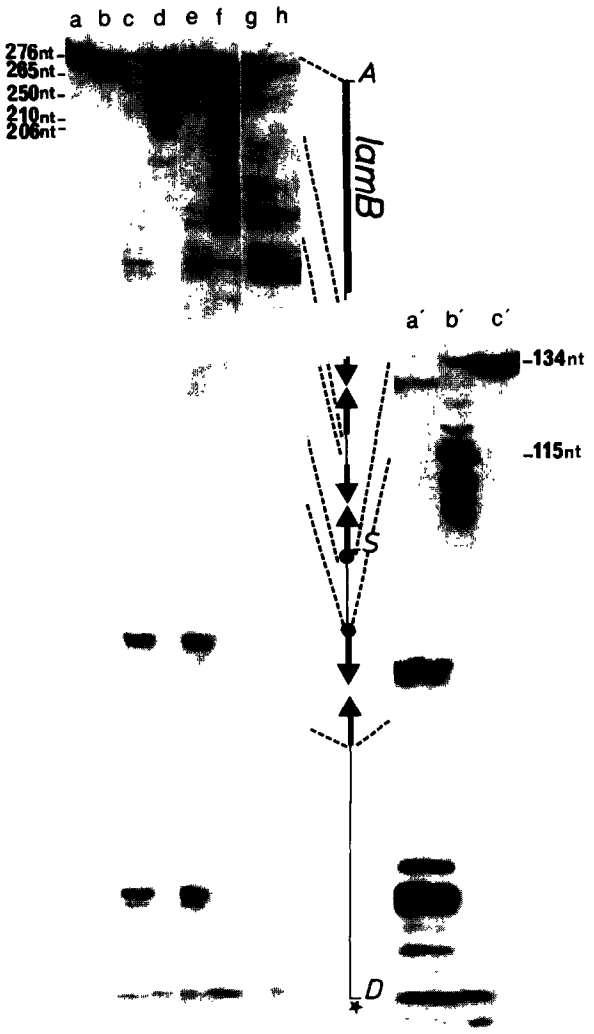

Fig.4. ExoIII protection of the *interB* region DNA conferred by chromoid-associated protein *E. coli* extract. A partial restriction map of the region is shown at the center of the figure. 5'-label (★) and remaining PU symbols are as in fig.2. The size of the fragments discussed in the text is indicated beside the photograph. Lanes a–h: *AluI-DdeI* fragment labelled at *DdeI* site. Lanes: a, undigested DNA; b, fragment incubated 10 min with 15 μg CHAP extract; c, ExoIII digestin with no added CHAP extract; d, ExoIII digestion after a preincubation with 15 μg CHAP extract; e, idem lane c with 1 μg sonicated CT DNA; f, idem lane d with 1 μg sonicated CT DNA; g, idem lane c with 10 μg sonicated CT DNA; h, idem lane d with 10 μg sonicated CT DNA. Lanes a'–c': *StuI-DdeI* fragment labelled at *DdeI* site. Lanes: a', ExoIII digestion with no added CHAP extract; b', ExoIII digestion after a preincubation with 15 μg CHAP extract; c', undigested DNA. In the incubations without ExoIII, mild endogenous nuclease activity can be observed in the CHAP extract (cf. lanes a and b). This activity has not been studied further.

3.3. *The CHAP extract activity is due to a protein*

Treatment of the CHAP extract with trypsin abolished the PU DNA specific resistance to ExoIII of the *interE* region (fig.3, lane i). In this experiment, the ExoIII assay was performed in the presence of trypsin inhibitor in order to keep the ExoIII enzyme functional. The same experiment without trypsin inhibitor yielded an intact DNA fragment, indicating that trypsin really destroyed ExoIII protein activities and that the trypsin inhibitor worked (fig.3, lane k). As further controls, ExoIII assay performed in the presence of the CHAP extract treated with trypsin inhibitor alone (fig.3, lane h) or with trypsin and trypsin inhibitor at the same time (fig.3, lane j) gave approximately the same pattern as in the presence of the CHAP extract (fig.3, lane g). These findings show that the activity responsible for the PU DNA protection from ExoIII is a protein.

We examined whether some known DNA-binding proteins were able to protect PU DNA from the action of ExoIII. Neither the HU protein [8] (fig.3, lanes a and b), histone-like 17 kDa protein [9] (fig.3, lanes c and d), SSB protein [10] (fig.3, lanes e and f), nor the *E. coli* RNA polymerase (not shown) allowed a specific ExoIII blockade at PU DNA.

4. DISCUSSION

We used the ExoIII protection assay in an attempt to detect PU binding activity in chromoid-associated protein *E. coli* extracts. Our results show that, only in the presence of the extract, PU sequences constitute a strong boundary to ExoIII digestion. A single PU, whatever its orientation, is enough to stop ExoIII digestion. A map of the protected PU DNA fragments is shown on fig.2. The stop occurs at the first boundary of the PU met by ExoIII. In addition, we show that no protection occurred when the extract was pretreated with trypsin. All these findings are consistent with the idea that a chromoid-associated protein is able to recognize and preferentially bind PU sequences. The ExoIII protection assay can thus be used to monitor the purification of the protein(s).

The biological significance of this PU DNA-protein interaction is not known. It would be consistent with our hypothesis that PUs play a role in the structure of the chromoid.

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