
DNA polymerase I and a protein complex bind specifically to *E.coli* palindromic unit highly repetitive DNA: implications for bacterial chromosome organization

Eric Gilson, David Perrin and Maurice Hofnung

Unité de Programmation Moléculaire et Toxicologie Génétique, CNRS UA271 INSERM U163, Institut Pasteur, 28 rue du Docteur Roux, Paris 75015, France

Received February 22, 1990; Revised and Accepted May 2, 1990

ABSTRACT

Starting from a crude *E. coli* extract, two activities which specifically protect highly repetitive bacterial DNA sequences (called PU for Palindromic Unit or REP for Repetitive Extragenic Palindromic sequence) against a digestion with Exonuclease III have been purified. We show that one of these activities is due to the DNA polymerase I (Pol I). This constitutes the first indication for a specific interaction between Pol I and a duplex DNA. This interaction requires the presence of PU. It was confirmed and analyzed by native gel electrophoresis and DNase I footprinting experiments. The other activity contained at least five polypeptides. Its binding to PU DNA sequences was confirmed by native gel electrophoresis. Implications for the possible origin and functions of PU are discussed.

INTRODUCTION

Palindromic Units or PU constitute a family of repetitive DNA sequences present in large numbers in the genome of several enterobacteria (about 1000 copies in *E. coli*) (1, 2). These sequences are also called REP for Repetitive Extragenic Palindromic sequence (4). The PU homogeneity (the number of bases identical to the consensus divided by the total number of bases) is extremely high, averaging 80% for 170 known *E. coli* PU sequences (3 and unpublished). The PU consensus DNA sequence is given in Figure 1. Part of the PU sequence (24 out of the 40 nucleotides of the consensus) exhibits a dyad symmetry. The nucleotides not included in this dyad symmetry constitute asymmetry elements which confer an orientation to the PU; for example, the sequence 5'-CTACPurine-3', which does not belong to the dyad symmetry, is particularly well conserved. PUs are arranged in clusters comprising from one to six occurrences. Within a cluster, successive PUs rigorously alternate in orientation. These clusters are always found outside structural genes.

Certain PU have been shown to play a functional role in gene expression such as mRNA stabilization or transcription termination (for recent reviews, see 3, 5). However, these functions do not require the high degree of sequence homogeneity observed for PU. In contrast, the observation that PU bind

nucleoid-associated protein (6) could provide a plausible cause for their sequence homogeneity. In addition, this property led us to hypothesize that PU could play a role in the folding of the bacterial nucleoid into independent supercoiled looped domains (3).

In order to find a clue to the presence, the organization and the possible functions of PU in the bacterial cells, we searched for specific PU DNA binding activities from a crude *E. coli* extract and we report here the purification and the characterization of two such activities.

MATERIALS AND METHODS

Bacterial strains and enzymes

The following *Escherichia coli* K12 strain has been used in our purification procedures: for *polA*⁺ cells: C600 (*thr*-1, *leu*-6, *thi*-1, *supE*44, *tonA*34, *lacY*1) (7); for *polA*1 cells: BW360 (*polA*1, *zig*-219::Tn10) (8).

DNA Polymerase I (endonuclease free) and DNA Polymerase I Large fragment (sequencing grade) were purchased from Boehringer and T7 DNA polymerase (both subunits: the T7 gene 5 protein (85 Kd) and the *E. coli* thioredoxin protein (12 Kd)) was purchased from Pharmacia.

Antibodies

Antisera against the Pol I protein (Pol I^B) and against the Klenow large fragment (Lf) were raised in rabbits with three injections. The first, was performed with 30 µg of protein in complete Freund's adjuvant; the second (at 3 weeks) and the third (at 4 months) were performed in incomplete Freund's adjuvant with, respectively 30 µg and 30 µg for Pol I^B and 50 µg and 60 µg for Lf. The antibodies were partially purified from serum samples by sodium sulfate precipitation and dialysis against PBS buffer. The specificity of these antibodies against the Pol I protein has been checked by enzyme-linked immunoadsorbant assay and by 'Western blotting' (data not shown).

DNA

DNA technologies (plasmid DNA preparation, restriction digestions, DNA end-labelling and gel analysis) were carried out as described (9). It has been shown that ethanol precipitation and subsequent drying of small DNA fragments can induce the

appearance of aberrant DNA conformations that can give rise to misleading interpretations of various DNA-protein binding assays (10). So, after elution of fragments from a polyacrylamide gel slice, DNA was concentrated by lyophilization in a Speed-Vac apparatus to a volume of about 20 μ l and desalted by spun-column chromatography with Sepharose CL6B.

Labelled DNA fragments

All the DNA fragments used are derived from the *malE-malF-malG* operon of *E. coli* (a partial restriction map of this region is shown in Figure 1).

The 222 bp *NcoI-BclI* fragment (F_C) was 5'-end-labelled at the *NcoI* sites (this fragment is described in 6). The 315 bp *BamHI-EcoRI* fragment (F_A), 5'-end-labelled at the *BamHI* sites, the 230 bp *BamHI-Sau3A* fragment (F_B), 5'-end-labelled at the *BamHI* sites, the 89 bp *NcoI-StuI* fragment (F_D), 5'-end-labelled at the *NcoI* sites, the 108 bp *MspI-Sau3A* fragment (F_F), 5'-end-labelled at the *MspI* sites and the 171 bp *NcoI-HinI*

fragment (F_E), 5'-end-labelled at the *HinI* sites were prepared from pPU3 (Figure 1). The DNA of this plasmid carries a filled *NcoI-NcoI* fragment containing the *malE-malF* intergenic region, inserted at the *SmaI* site of pUC18 (unpublished construction from our laboratory). The 539 bp *NcoI-NcoI* fragment (F_G) was prepared from pPU6 (unpublished construction from our laboratory) and contains a direct repeat of the *malE-malF* intergenic region i.e. 6 copies of PU DNA sequences (Figure 1).

The F_G fragment was dephosphorylated with alkaline phosphatase, purified from an agarose gel and labelled with $\gamma^{32}P$ ATP using T4 polynucleotide kinase. The labelled fragments were circularized at a concentration of 2 nM in Tris-HCl pH 8 50 mM, NaCl 50 mM, $MgCl_2$ 10 mM, 2 mM DTT and 1 mM ATP with 10 U T4 DNA ligase/ml for 12 hours at room temperature. Ethidium bromide (EtBr, 1 μ g/ml or 10 μ g/ml) was added to the incubation buffer to obtain the negatively supercoiled topoisomers. After ligation the mixture was adjusted to 1 M NaCl, 1% SDS and extracted by chloroform-isoamyl alcohol

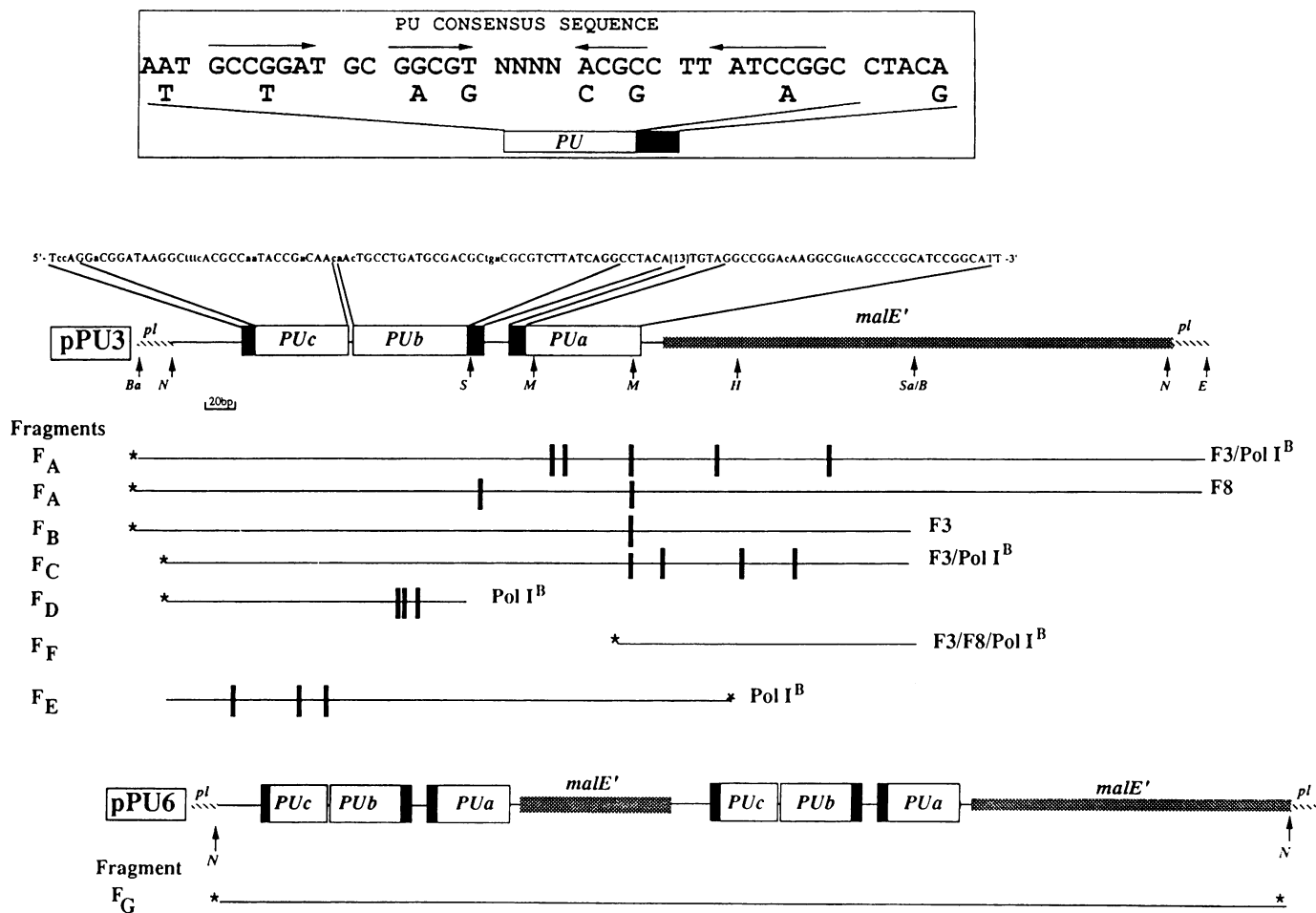


Figure 1. Partial restriction map of the *malE-malF* intergenic region and map of the protected DNA against ExoIII by F3, Pol IB and F8. *Top:* within a box, is indicated the PU consensus sequence (2). For simplicity, PU are represented by rectangles: the empty part corresponds to the dyad symmetry part of the PU and the black part corresponds to the CTAC Purine element which is not included in the dyad symmetry. The CTAC Purine element allows to orient the PU. N = A or T or G or C. *Middle part:* below the box is shown the DNA sequence of the PUs present in the *malE-malF* intergenic region (33) as well as a schematic representation and a partial restriction map of this region present in the pPU3 plasmid. The capital letters indicate bases which are present in the PU consensus sequence. The grey box represents the coding region of the *malE* gene. Below the map, for each DNA fragment, the border of the ExoIII resistant fragments (due to protection by fractions F3 and F8 and by Pol I^B, as indicated), which are visualized through the 5'-end label (star), are indicated below the map with a vertical bar. *Bottom:* the structure of the *malB* insert present in the pPU6 plasmid is shown. The two arrows indicate the *malB* DNA segment that is repeated in tandem. Below this structure is given the nature of the DNA fragment F_G : B: *BclI*, N: *NcoI*, Ba: *BamHI*, S: *StuI*, H: *HinI*, M: *MspI*, Sa: *Sau3A* and E: *EcoRI*, pl: polylinker region, *: 5'-end label.

(24/1; v/v) (11). The individual topoisomers were purified by PAGE on a gel (0.15×30 cm) containing 4% acrylamide-bisacrylamide (30/1; w/w), 45 mM Tris-borate and 1 mM Na₂EDTA, running at 150V for 12 hours (12).

The negatively supercoiled topoisomers (Ta and Tb) were identified by their consecutive appearance in a ligation reaction containing gradually increasing amounts of EtBr and by their ability to be completely relaxed by the action of the avian topoisomerase I (Amersham), i.e. comigrating with the topoisomer obtained from the ligation reaction without any EtBr (designated as R).

E. coli cellular extract

All procedures were carried out at 0–4°C. Frozen paste (50 g of packed cells) was diluted in 270 ml of buffer A (Tris-HCl pH 7.6 20 mM, NaCl 2.5 M, Na₂EDTA 1 mM and β-mercaptoethanol 1 mM). The suspension was passed through a French pressure cell once at 1200 bars, and the cell debris were removed by low speed centrifugation in a Kontron centrifuge using a A6.9 rotor run at 8000 rpm for 60 min. A half volume of PEG6000 30% (w/v) in buffer A was added to the supernatant. After over-night incubation, the precipitate was removed by centrifugation (8000 rpm for 20 min in A6.9 rotor). The supernatant was dialyzed against buffer B (Tris-HCl pH 7.6 20 mM, NaCl 50 mM, Na₂EDTA 1 mM, glycerol 10% and β-mercaptoethanol 1 mM) 48 hours with at least two buffer changes and centrifuged again (8000 rpm for 20 min). The final supernatant had a protein concentration of about 3 mg/ml.

Purification procedures

Step 1, Phosphocellulose column chromatography. The above supernatant was applied to a 100 ml phosphocellulose (Whatman P11) column which had been equilibrated with buffer B. After washing the column with 200 ml of buffer B, adsorbed proteins were eluted with a linear gradient of NaCl (from 50 mM to 500 mM in 400 ml) in buffer B, and 7 ml fractions were collected. In this and the following chromatography steps, protein elution profiles were monitored by measuring A_{280 nm} and the NaCl concentration gradient by measuring the conductivity. Fractions absorbing at 280 nm (50 fractions) were dialyzed against buffer C (75 mM NaCl, 5 mM MgCl₂, 0.1 mM Na₂EGTA, 15 mM Tris-HCl pH 7.4, 0.5 mM DTT, 2 mM Na phosphate pH 7.0 and 5% glycerol) and 10 μl from each fraction was subjected to an ExoIII protection assay (Figure 2I).

Step 2, Gel filtration chromatography. Fractions eluted between 100 mM and 200 mM NaCl from the phosphocellulose column were pooled (see results) and concentrated in a Diaflo cell to a final volume of 5 ml (at about 5 mg/ml of protein). A one ml sample was applied to a Superose 6 HR 10/30 column (attached to a Pharmacia FPLC system) equilibrated with buffer C. Proteins were eluted with the same buffer at a flow rate of 0.2 ml/min and 3 ml fractions were collected. 5 μl from each peak fraction was subjected to an ExoIII protection assay (Figure 2II). The calibration of this column has been determined, in the same experimental conditions, with Bio-Rad Gel Filtration standard proteins (670 Kd, 158 Kd, 44 Kd, 17 Kd and 1,350 Kd) and Catalase (230 Kd).

Step 3, Anion exchange chromatography. Fractions corresponding to a molecular weight of 200 Kd from 5 successive gel filtration column experiments were pooled (about 5 mg of proteins) see

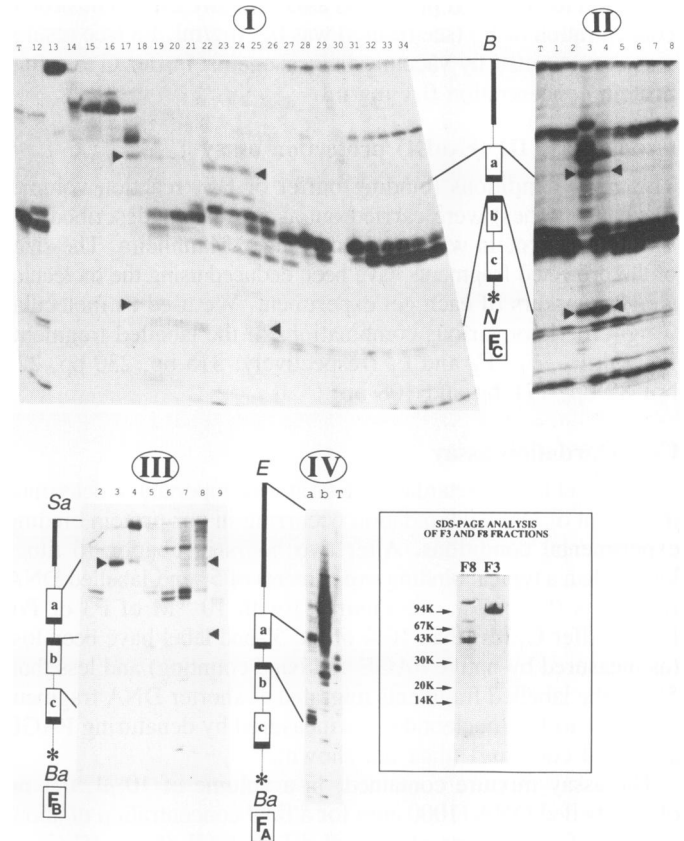


Figure 2. Fractions F3 and F8. Schematic representations of the DNA fragment used in each experiment as well as the corresponding position of the PU borders are shown for each gel. The positions corresponding, in the gel, to the full length fragment and to the PU border of the first, second and third PU met by ExoIII on the labelled strand are indicated by solid lines. These positions have been deduced using molecular weight markers for each gel. We used as molecular weight markers various combinations of the labelled fragments F_A, F_B, F_C, F_D, F_E and F_F (respectively: 315 bp, 230 bp, 222 bp, 89 bp, 171 bp and 108 bp). The black triangles indicate, within the gel, the position of the first and of the second PU met by ExoIII on the labelled strand. The other symbols are as in Figure 1. The standard assay was performed as described in Experimental procedures. (I) Phosphocellulose chromatography step. F_C was incubated with 10 μl from each fraction (12–34) and 0.1 μg of sonicated calf thymus DNA (or CT) before adding ExoIII. Lane T, ExoIII digestion with no added protein which exhibited the ‘natural stops’ (see text). (II) Gel filtration chromatography step. F_C was incubated with 5 μl from each fraction (1–8) and 0.3 μg of CT before adding ExoIII. Lane T, ExoIII digestion with no added protein. (III) Anion exchange chromatography step. F_B was incubated with 5 μl from each pooled fraction (2–9) and 1 μg of CT before adding ExoIII. (IV) Before adding ExoIII, F_A was incubated with 0.5 μg of CT and with: lane a, 30 μl of F8 concentrated to 0.1 mg/ml (see text); lane b, 10 μl of F3; lane T, no protein. The lane T exhibited the ‘natural stops’ (see text). Inside the box: SDS-PAGE analysis of fractions F3 and F8. A sample of each fraction was subjected to a SDS-PAGE analysis in a Phast system Pharmacia (8–25% polyacrylamide gradient gel and silver staining). The positions of molecular weight markers are indicated.

results) and applied to a MonoQ HR 5/5 column (attached to a Pharmacia FPLC system) with buffer C. After washing the column with 5 ml of buffer C, adsorbed proteins were eluted with a linear gradient of NaCl (50 mM to 1 M in 20 ml) in buffer C at a flow rate of 0.5 ml/min and 0.5 ml fractions were collected. Aliquot of each fraction was subjected to SDS-PAGE analysis in a Pharmacia Phast system (8–25% polyacrylamide gradient gel and silver staining). Fractions showing identical protein composition were pooled, dialyzed against buffer C and 5 μl was

subjected to an ExoIII protection assay (Figure 2III). The protein concentration of F3 (see results) was 0.3 mg/ml, F8 (see results) was concentrated by vacuum dialysis against buffer C to a final protein concentration 0.1 mg/ml.

Exonuclease III (ExoIII) protection assay

The assay conditions (binding buffer or BB, reaction volume, incubation times) were carried out as previously described (6). The ExoIII protein was from Boehringer-Mannheim. The sizes of the protected fragments have been deduced using the molecular weight markers of each gel experiment. We used as molecular weight markers various combinations of the labelled fragments F_A, F_B, F_C, F_D, F_E and F_F (respectively: 315 bp, 230 bp, 222 bp, 89 bp, 171 bp and 108 bp).

Gel retardation assay

To interpret the gel retardation data, it was important to determine the extent of DNA degradation occurring in our protein binding experimental conditions. After two hours incubation (6 times longer than a typical binding experiment) of 5'-end-labelled DNA fragments (F_A or F_E, see Figure 1) with 10⁻⁶M of F3 or Pol I^B, in buffer C, less than 10% of the 5'-end label have been lost (as measured by native PAGE and band counting) and less than 5% of the labelled fragments migrated as shorter DNA fragment from one to five nucleotides (as measured by denaturing PAGE and band counting) (data not shown).

The assay mixture contained, in a volume of 10 μ l, 0.1 ng of 5'-labelled DNA (1000 cpm for a final concentration of about 0.1 nM), 0–10000 ng of Poly(dI-dC).Poly(dI-dC) or IC (from Pharmacia) or of *Hind*III-digested lambda DNA (from Boehringer) and protein (as indicated) in buffer C. After incubation (20 min at 20°C), the mixture was directly loaded on a 4% polyacrylamide gel (acrylamide/bisacrylamide weight ratio of 30/1) containing 90 mM Tris-borate and 3 mM MgCl₂ (TBM buffer) running at 30 V. The gel (0.1×16 cm) was preelectrophoresed for 4 hours at 100V with recircularization of the TBM buffer. As soon as all the samples were loaded, the voltage was increased to 500 V for 2 min and then reduced to 100V for 4 hours at room temperature. Gels were dried and autoradiographed.

For experiments with circular plasmid DNA, a polyacrylamide gel was not suitable because the DNA-protein complexes migrate too slowly. Therefore, the assay mixture was separated on a horizontal 0.7% agarose gel (agarose NA from Pharmacia) containing TBM buffer. Electrophoresis was carried out in the same buffer system at 4V/cm for 6 hours at room temperature. After electrophoresis, the gel was stained in an ethidium bromide solution (0.1 μ g/ml) and photographed.

Quantification was done by excising bands of DNA for scintillation counting in ACSTM (Amersham). Each measure corresponds to the average of three gels.

DNase I footprinting assay

Ten ng of 5'-labelled DNA fragment and 100 ng of IC were preincubated in a 20 μ l reaction volume with various quantities of Pol I in binding buffer (BB) for 20 min at 20°C. 2 μ l of 5 μ l/ml DNase I (freshly diluted into ice-cold water from a 5 mg/ml stock solution) was added and the incubation was continued for 30 sec. The reaction was stopped by the addition of 5 μ l 3M ammonium acetate, 250 mM Na₂EDTA, 1 mg/ml sonicated calf thymus DNA and three volumes of ethanol. After ethanol precipitation, the samples were analyzed by a 20% and a 6%

urea-PAGE. Chemical base-specific cleavage reactions were performed as described by Maxam and Gilbert (9).

Protein sequence analysis

The protein PUP1 was digested overnight in 200 μ l of 0.1 M ammonium bicarbonate, pH 7.8 and 3 μ l of trypsin (1 mg/ml in 1 mM HCl) at 37°C. Peptide separation was performed by reverse-phase HPLC on a LKB instrument at room temperature on an Aquapore RP300 column (200×2.1 mm) operated at 0.3 ml/min during 3 hours. Buffers were A, 0.1% trifluoroacetic acid in water and B, 80% acetonitrile in water. A linear gradient of buffer B was increased from 0 to 55%. Peptides were monitored between 200–360 nm with a photo diode array detector (model LKB 2140 Rapid Spectral Detector).

The sequence analysis was performed on a gas-phase sequenator (model 470A, Applied Biosystems). The purified peptides were subjected to automated Edman degradation (13).

RESULTS

In preliminary experiments, we had shown that degradation by Exonuclease III (ExoIII) of DNA fragments carrying PU was arrested at the PU site in the presence but not in the absence of a nucleoid extract from *E. coli* (6). Here, we have used this inhibition of ExoIII as an assay to purify specific PU DNA binding proteins from a crude *E. coli* extract.

Two specific PU DNA-binding activities: F3 and F8

ExoIII, a 3' to 5' exonuclease, degrades double-stranded DNA fragments from both 3' termini until the substrate becomes single-stranded and resistant to further digestion. In the presence of ExoIII alone, the labelled DNA fragments are not fully digested: the remaining fragments are called 'natural stops'. In the presence of a specific DNA-binding protein, the progression of ExoIII is blocked at or near the binding sites and the 'natural stops' are replaced by new discrete fragment(s) (14).

As a probe for the PU DNA binding, we used various DNA fragments encompassing the three PUs located in the intergenic *malE-malF* region of the *malB* locus. These fragments were named F_A, F_B and F_C; their exact definition is given in 'Experimental procedures' and in Figure 1. To reduce the effect of non-specific binding, sonicated calf thymus DNA was included as competitor in each assay.

Using these fragments in the protection assay against ExoIII, we started from a crude high salt extract from *E. coli* and performed three purification steps as follows (a more detailed description of the extract preparation and of the chromatographic steps is given in 'Experimental procedures').

The first step was a phosphocellulose column. The results of the ExoIII protection assays with F_C are shown in Figure 2I. A number of fractions eluted from the column yielded protected fragments different from 'natural stops'. From our preliminary results (6), we expected that at least some of the PU binding proteins would give protected fragments corresponding to the border of the PU sequence. Indeed, protected fragments corresponding to the border of the first and of the second PU met by ExoIII were detected in the fractions eluted with approximately 100 mM NaCl (fractions 17–18); protected fragments corresponding to the border of the first PU were detected with fractions eluted at approximately 200 mM NaCl (fractions 21–23). In both cases, other bands were also visible: they corresponded mainly to sites upstream of the first PU

(fractions 17–18) and sites inside PU (fractions 21–23). We decided to pool the fractions 17 to 23 because they all exhibited protected bands at or near the border of a PU sequence.

As a second purification step, phosphocellulose fractions 17–23 were pooled and subjected to gel filtration chromatography. Protected fragments corresponding to the border of the first PU, as well as at upstream sites and at the border of the second PU, were detected in ExoIII assays with F_C in presence of fractions 3–4 (Figure 2II). These fractions correspond to proteins with an apparent native molecular weight of 200000 daltons (200 Kd).

In the third and last chromatography step, the pooled 3–4 fractions from the gel filtration column were loaded on an anion exchange column. A major protected fragment corresponding to the border of the first PU was detected in ExoIII assay with F_B in presence of fraction 3 (called F3; F3 eluted at approximately 150 mM NaCl) (Figure 2III). An ExoIII assay performed in presence of the same fraction but with F_A (Figure 2IV, lane b) or with F_C (Figure 3I, lanes b and c) revealed also in both bases protection at the PU border. Several other bands are also apparent. Their localization and intensities depended on the experimental conditions (nature of the fragment, concentration of carrier DNA...). These others bands could be due to various causes such as additional protein binding sites or alteration in the rate of the ExoIII digestion. The important observation is the presence of the band corresponding to the PU border in all cases (fragments F_A , F_B and F_C in Figure 1).

F3 contains an apparently homogeneous polypeptide of molecular weight 100 Kd as determined by SDS-PAGE analysis (Figure 2). This polypeptide was called the PUP1 protein.

A very slight amount of protected fragment corresponding to the border of the first and of the second PU could be detected in ExoIII assays with F_B in the presence of fraction 8 (called F8) which eluted approximately at 350 mM NaCl (Figure 2III). In order to check that this was due to specific protection, the assay was repeated after concentration of F8. The results revealed clearly protection at the beginning of the first PU (Figure 2IV, lane a). Interestingly, concentrated F8 was also able to protect specifically a region corresponding to the beginning of the second PU. By SDS-PAGE analysis, F8 was shown to contain three major polypeptides of 130 Kd, 51 Kd and 50 Kd and two minor polypeptides of 66 Kd and 80 Kd (Figure 2).

In the following, we will examine in more detail the properties of F3 and F8.

PUP1 is identical to Pol I

To identify the PUP1 protein (the 100 Kd polypeptide found in F3), we performed a sequence analysis of 6 tryptic peptides obtained from this protein. The sequence of 5 of these peptides matched the sequence of tryptic peptides derived from the primary sequence of the *E. coli* DNA polymerase I (EC 2.7.7.7; gene name: *polA*), i.e. peptides 287–295, 296–300, 301–321, 594–597 and 603–621 as numbered in (15). Commercially available Pol I (purchased from Boehringer and called Pol I^B) and F3 presented the same apparent molecular weights as PUP1 on SDS PAGE (100 Kd) and on gel filtration chromatography (step two of our purification procedure) (200 Kd) (data not shown).

Does commercially available DNA polymerase I exhibit the same ExoIII protection pattern as F3? We performed a series of ExoIII analyses with different types of DNA fragments by comparing F3 and Pol I^B (Figures 3I and 3II). In all the

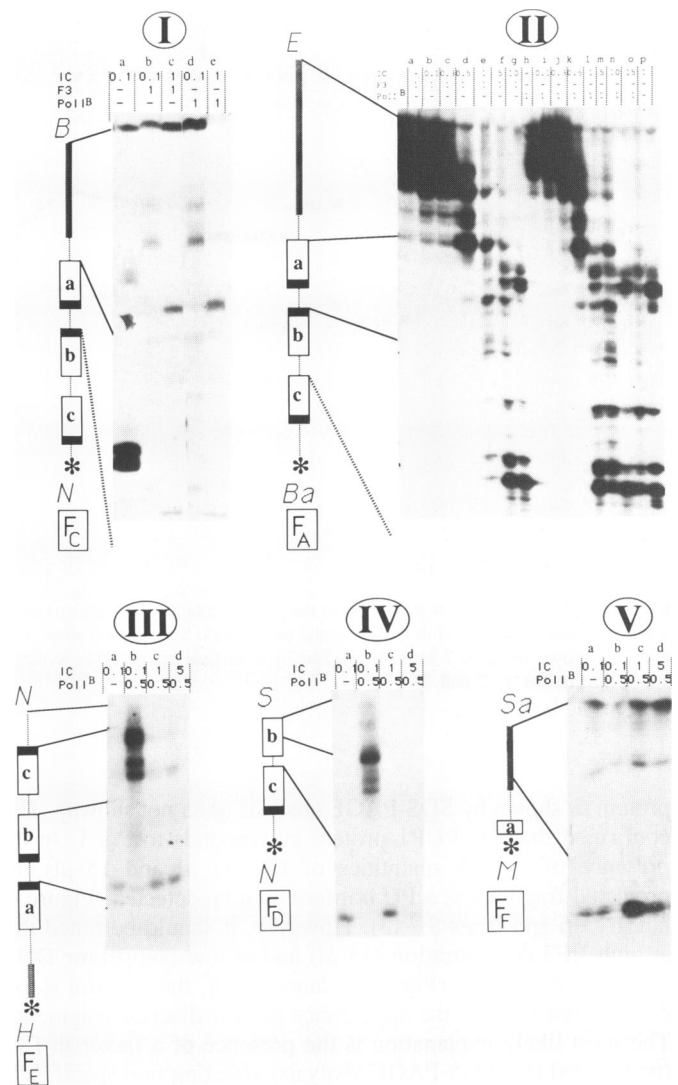


Figure 3. ExoIII protection by F3 and/or Pol I^B on different DNA fragment. The standard assay was performed as described in Experimental procedures. For each lane (indicated in lower case), the amount (μ g) of competitor DNA (IC) and of protein added before ExoIII is shown. The 'natural stops' (see text) are shown, for each experiment, in the following lanes: I, lane A; II, lane p; III, lane a; IV, lane a; V, lane A. The grey lines indicate that the corresponding position on the gel is not present on the photograph. Other symbols as in Figures 1 and 2.

experimental conditions used, F3 and Pol I^B exhibited identical ExoIII protection pattern. Compare, for example, in Figure 3I lanes b-c with lanes d-e or in Figure 3II lanes a-g with lanes h-o. The large C-terminal fragment of Pol I (the Klenow protein) did not protect the same PU DNA fragments from ExoIII digestion (data not shown).

To further confirm that the ExoIII protection activity of F3 was due to Pol I, we conducted the same purification experiment as above, but from *E. coli* cells carrying an amber mutation at residue 342 of the *polA* gene (*polA1*). The fractions obtained from the elution of the last chromatographic step were tested by the ExoIII protection assay. No fraction exhibiting the ExoIII protection pattern characteristic of F3 was found (data not shown). In addition, the fraction equivalent to F3 and called EF3 (i.e. eluted with the same NaCl concentration as F3 in the *polA* + strain) did not contain any detectable amount of the 100 Kd

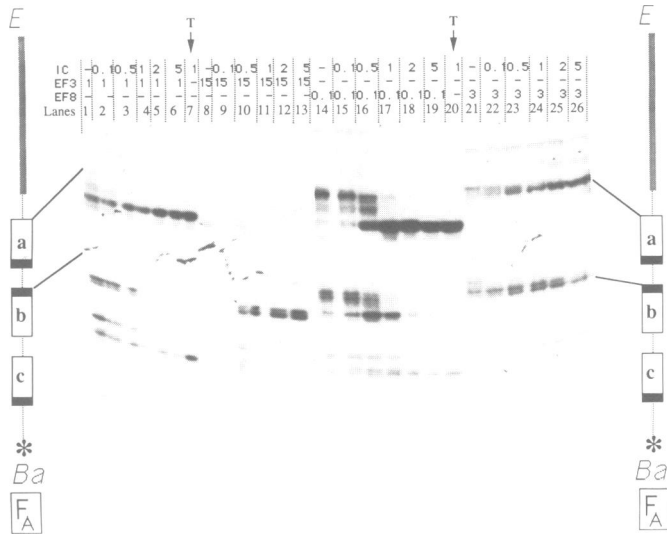


Figure 4. The F3 activity is not found in the *polA1* mutant. The standard assay was performed as described in Experimental procedures. The 'natural stops' (see text) are shown in lanes 7 and 20 (marked by a vertical arrow). The symbols are as in Figures 1, 2 and 3.

protein as shown by SDS-PAGE analysis (data not shown). This confirmed that the PUP1 protein corresponds to Pol I. In the presence of various quantities of EF3 (1 μ l and 15 μ l), no protected fragment at a PU border could be detected (Figure 4, lanes 1–6 and lanes 8–13). However, it should be noted that at high EF3 concentration (15 μ l) and at low competitor DNA quantity (< 0.5 μ g) (Figure 4, lanes 8–9), the 'natural stops' disappeared without the appearance of new discrete fragments. The most likely explanation is the presence of a factor in EF3 (undetected in a SDS-PAGE analysis) affecting non specifically the progression of ExoIII.

As a control in these experiments, we used a fraction equivalent to F8, purified from *polA1* cells and called EF8. This fraction displayed an ExoIII protection pattern similar to the F8 fraction obtained from *polA+* cells. Compare the protection pattern shown in lanes 14–16 and in lanes 21–26 of Figure 4 with the pattern shown in lane a of Figure 2IV.

In summary, these data show that F3 activity is due to Pol I and that the F8 activity is clearly distinct from Pol I.

To obtain information on the interaction properties between F3 (or Pol I^B) and PU DNA, we performed a series of ExoIII experiments with various DNA fragments of the *malE-malF* intergenic region and in presence of increasing amount of non-specific DNA competitor (a simple alternating copolymer duplex poly(dI-dC).poly(dI-dC), abbreviated as IC). Some of the results are shown in Figure 2IV and on Figure 3. A map of the protected fragments, summarizing all our experiments, is presented in Figure 1 (bottom part).

In presence of competitor DNA (IC) and of F3 or Pol I^B, all the DNA fragments containing PU DNA sequences exhibited a protected fragment corresponding to the exact border of the first PU met by ExoIII (Figure 3I, lanes b–e; Figure 3II, lanes b–f and j–m; Figure 3III, lane b). At low quantities of non-specific DNA, the fragments corresponding to the PU border were less abundant than the fragments corresponding to sites upstream and inside of PU (for example, see Figure 3II, lanes a–c and h–j).

F_D, which contains one truncated PU (PUB) and one intact PU (PUC), exhibited a protected fragment corresponding to the middle of the truncated PUB (Figure 3IV, lane b). This suggested that the remaining sequence present in the PUB, in particular an intact half PU, could be sufficient for the binding of at least one molecule of Pol I.

F_F, which does not contain intact PU DNA but only a PU flanking region, is not protected from the ExoIII digestion by F3 or Pol I^B (Figure 3V, lanes b–d).

In conclusion, the ExoIII protection patterns, obtained in presence of F3, can be interpreted by a preferential binding of the Pol I protein to PU DNA as reflected by the protected fragment corresponding to the PU border.

Pol I forms stable and specific complexes with a PU linear DNA fragment in a gel retardation assay

To confirm and understand better the PU DNA-Pol I interactions a series of gel retardation experiments were performed. The gel retardation experiment is based on the observation that DNA-protein complexes migrate more slowly than free DNA in a low ionic strength PAGE (16, 17).

We tested whether ³²P-labelled linear duplex DNA could be retarded by increasing concentrations of F3. We used two DNA fragments: F_E that contains the three PUs of the *malE-malF* intergenic region of *E. coli* and F_F that does not contain PU DNA (Figure 1). The results are shown in Figure 5I. For F_E and F_F, the amount of free DNA decreased while increasing levels of smearing (with a lower mobility than the free DNA) were detected. At high protein concentration (300–500 nM of Pol I), a discrete band of lower mobility appeared. They represent what we call here discrete complex or DC. At a Pol I concentration of 500 nM, almost all the F_E, that contains three PUs, was detected as DC while at the same protein concentration, less than 5% of the amount of F_F were detected as DC. Smearing probably indicates that some of the protein-DNA complexes are not stable, at least during the electrophoresis; however, DC are likely to represent stable complexes between PU DNA and F3. With T7 DNA polymerase, when protein concentration was increased in the same concentration range (up to 500 nM), smearing, similar to that obtained with F3, appeared but no discrete band, similar to DC, could be detected (Figure 5III). At much higher protein concentration (above 500 nM), DNA protein complexes of lower mobility were also detected.

DC were obtained with Pol I^B (Figure 5II) but not with Klenow large fragment of Pol I (Lf) (Figure 5IV). DC could be detected only if Mg²⁺ was present in the binding buffer and in the gel buffer and if the polyacrylamide gel was polymerized for at least 12 hours before electrophoresis (data not shown). Treatment of the binding reaction with 1% SDS just before loading converted DC or smears to a form that comigrated with the free DNA (data not shown). Thus, all the shifted DNA corresponds to DNA-Pol I complexes and not to modified forms of the DNA fragment.

For quantification, we determined the amount of free DNA. As discussed previously (18), the apparent dissociation constant (K_d (app)) is equal to the molecular concentration of the unbound form of Pol I (assumed to be a 100 Kd monomer) at which the free DNA amount is half maximal. Under the conditions of the assay shown in Figure 5I, the protein concentration required for half binding was about 5×10^{-8} M for both fragments.

These data can be interpreted by two types of binding complexes. One is not specific for PU DNA sequences and is

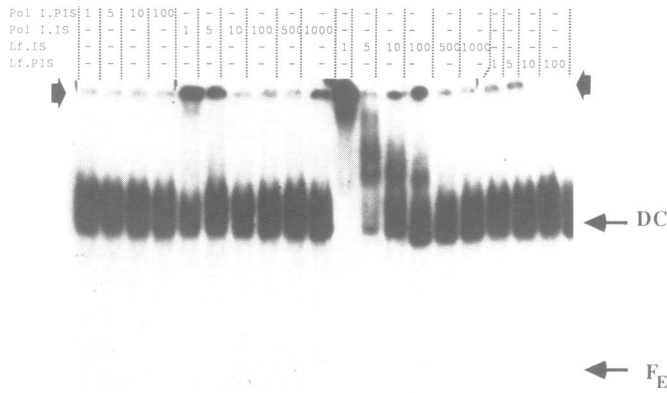


Figure 6. DC are recognized by antibodies against Pol I. The DNA fragments were incubated with 1 μ M Pol I^B and 0.1 μ g IC. Pol I^B and serum were incubated for 30 minutes before adding a mixture of labelled DNA and IC DNA. Sera were freshly diluted on ice into PBS buffer. For each reaction, the dilution at which the serum was used, is indicated. 1 μ l of undiluted or diluted serum was used in each reaction. Preimmune serum corresponding to the rabbit immunized with Pol I: Pol I.PIS; immune serum corresponding to the rabbit immunized with Pol I: Pol I.IS; immune serum corresponding to the rabbit immunized with Large Fragment of Pol I: Lf.IS; preimmune serum corresponding to the rabbit immunized with Large Fragment of Pol I: Lf.PIS. The thick arrows, on both sides of the gel, indicate the position of the wells.

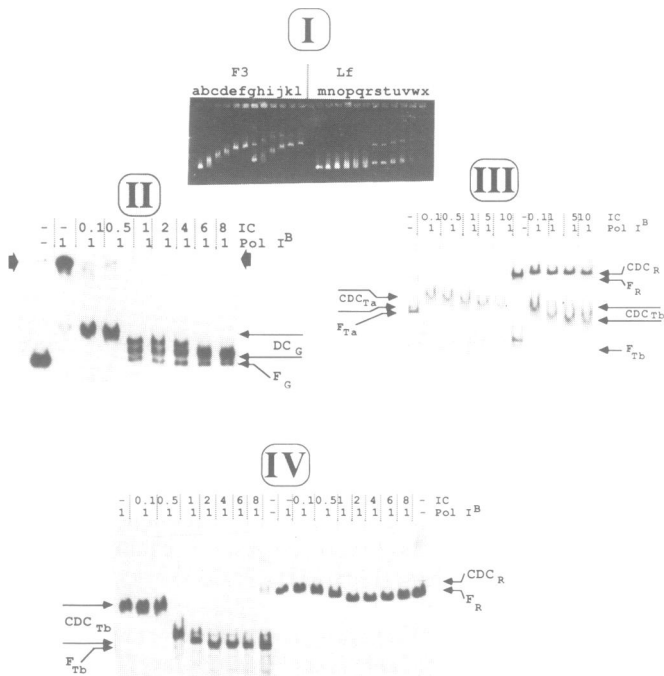


Figure 7. Pol I forms CDC with circular PU DNA. F_G indicates the gel position of free F_G; DC_G: DC with F_G; F_{Ta}: free negatively supercoiled minicircle, topoisomer a; CDC_{Ta}: CDC with topoisomer a; F_{Tb}: free negatively supercoiled minicircle, topoisomer b; CDC_{Tb}: CDC with topoisomer b; F_R: free relaxed minicircle; CDC_R: CDC with relaxed minicircle. (I) Lanes a–f, m–r: 100 μ g pPU3 supercoiled plasmid DNA; lanes g–l, s–x: 100 μ g pPU6 supercoiled plasmid DNA; lanes b–f and lanes h–l: increasing F3/plasmid molar ratio, i.e. successively 1, 5, 10, 50 and 100. ; lanes n–r and lanes t–x: increasing Lf/plasmid molar ratio, i.e. successively 1, 5, 10, 50 and 100 (II) (IV) The Pol I^B concentration is given in μ M and the IC content in μ g.

(0.5–1 μ M) (data not shown). By analogy, this discrete complex will be referred to with the term: circular discrete complexes (or CDC). In contrast with the linear substrate, minicircles did not exhibit smearing complexes at a low protein concentration (data not shown). These results support the hypothesis proposed for a linear DNA substrate, namely that the smearing corresponds to the binding to DNA ends and the DC to duplex DNA.

Effects of increasing amount of a DNA without any PU (IC) on the formation of DC and CDC are shown in Figure 7II, 7III, and 7IV. The competition for Pol I^B binding to PU DNA was roughly similar either for linear or for relaxed circular or for supercoiled circular form of the PU DNA (compare Figures 7II and 7III/7IV). Note that with the linear F_G, that contains 6 PUs, different types of DC are formed during the IC competition (called DC_G in Figure 7II). A similar competition, using increased amount of IC, with F_E, which contains 3 PUs, exhibits only one type of DC (Figure 5II and 5IV). This strongly suggests that the number of Pol I proteins bound to PU DNA increased as the number of PU sequences per DNA fragment increased. This constitutes another piece of evidence for the specific binding of Pol I to PU duplex DNA.

DNase I footprinting with Pol I at PU sites

Binding specificity between Pol I and PU DNA was further characterized by DNase I footprint analysis on F_E which contains the three PUs of the *malE-malF* intergenic region.

To visualize the footprint pattern at PU sequences, the reaction products were separated on a 6% polyacrylamide gel containing 7 M urea (Figure 8). With increasing quantities of Pol I, the DNA becomes increasingly sensitive or resistant to DNase I cleavage. These modifications begin at 100 ng of protein and are fully visible at 500 ng of protein (right part of Figure 8).

The major sites of increased sensitivity are flanking a central region of 1 to 3 nucleotides which are fully protected from the DNase cleavage. These patterns (called the ‘P’ patterns) are visible at DNA sites where at least one nick was produced by DNase I in the absence of Pol I or in the presence of low quantities of Pol I (< 80 nM). Not all these DNase I nicks, noted N1 to N10 in Figure 5, are converted to ‘P’ patterns: N6 to N9 are not converted while N1 to N5 and N10 are converted. This suggests strongly a sequence dependence in the interaction between Pol I and duplex DNA.

The ‘P’ patterns are all located at or very near a GCC triplet (Figure 9). Interestingly, the strongest patterns (at the N4 and the N5 nicks in Figure 8) are observed for regions in PU_a and in PU_b which overlap the dyad symmetry part and the asymmetry element CTAC_{Purine} of the PU sequence and which contains a GCC triplet on each strand. This region corresponds to the most conserved part of the PU (3).

The third PU (PU_c) is too far in the gel for good resolution, but the data is compatible with ‘P’ patterns also occurring in this region (examine the top of the photograph of the gel in Figure 8 at the level of the N10 nick).

To check if another type of DNA polymerase was able to exhibit the ‘P’ pattern at PU DNA sites, we performed a similar experiment with the T7 DNA polymerase (T7 DNA Pol). At 500 ng of T7 DNA Pol, it can be noted that the DNase I nicks were partially inhibited at all the nucleotide sites (left part of Figure 8). This pattern is quite different to the ‘P’ pattern of Pol I and probably reflected non-specific binding by the T7 DNA Pol along the DNA duplex. At 500 ng of Pol I protein, a decrease in DNase I sensitivity was not observed. These results suggest that T7 DNA

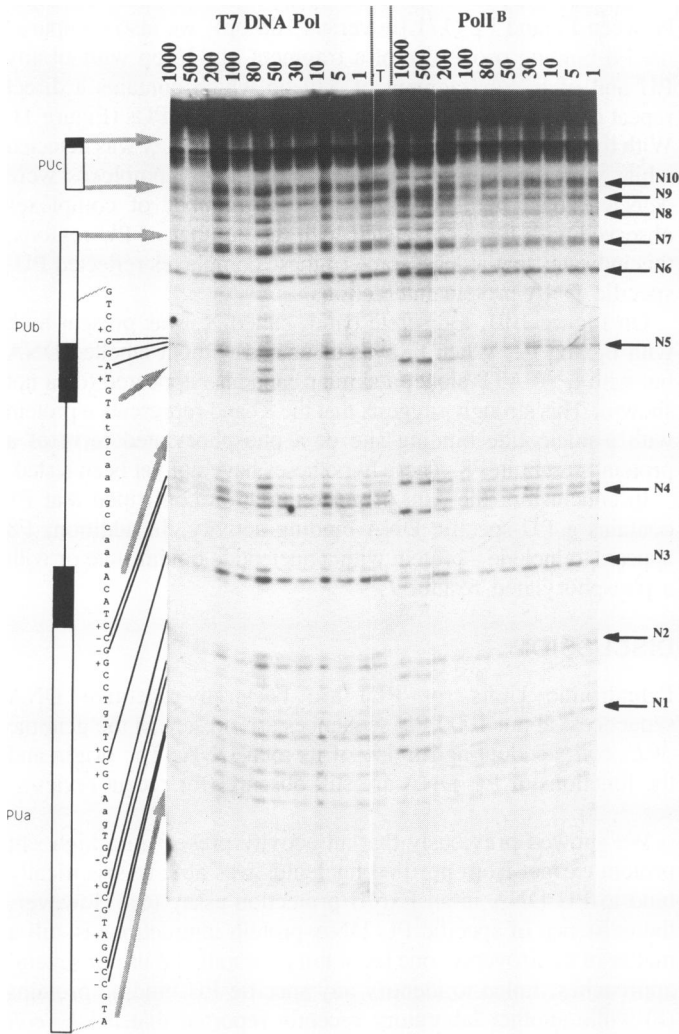


Figure 8. DNase I footprint analysis of PU DNA-Pol I interactions. The standard assay was performed as described in Experimental procedures. The reaction products were separated by using a 6% polyacrylamide gel with 7 M urea, alongside the base-specific reaction of the Maxam and Gilbert sequencing method (not shown on the figure). Lane T: no protein added before DNase I; right part of the gel: DNA pol I added before DNase I, the amount of protein (ng) is indicated above each lane; left part of the gel: T7 DNA pol added before DNase I, the amount of protein (ng) is indicated above each lane. The PU symbols are as in Figure 1. The sites protected against the DNase I cleavage, in presence of Pol I, are indicated on the DNA sequence by a ‘-’ and the correspondence of these positions between the gel and the sequence by a thin line. The sites enhanced for the DNase I cleavage, in presence of Pol I, are indicated on the sequence by ‘+’. The thick, grey arrows indicate the correspondence between the borders of the PU sequences on the schematic representation of the fragment and their position on the gel. N1 to N10 indicate the positions of the DNase I nicks (see text).

Pol does not bind PU DNA specifically, as shown by the absence of the ‘P’ pattern with T7 DNA Pol. Although the two polymerases shared significant protein sequence homologies (19), the capacity to specifically bind PU DNA seems Pol I specific.

To visualize the footprint pattern with Pol I at the end of the fragment, the reaction products were fractionated on a 20% polyacrylamide gel containing 7 M urea (data not shown). Nucleotides 10 to 23 on the template strand were protected by Pol I against DNase I. These nucleotides do not belong to the PU sequences present on the fragment. This result was consistent

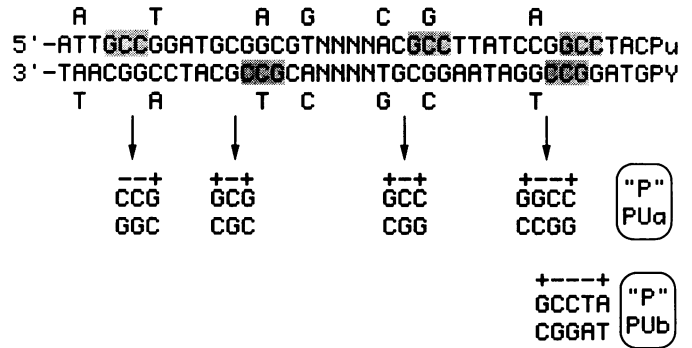


Figure 9. Localization of the ‘P’ patterns in PU sequences. At the bottom of the figure is indicated a PU consensus DNA sequence (Gilson *et al.*, 1984) and the grey boxes show the positions, on the two strands, of the GCC triplets. Below this sequence is indicated the positions of the P patterns (as deduced from the DNase I footprinting analysis, see text), both in PUa (‘P’PUa) and in PUB (‘P’PUB). ‘+’ and ‘-’ are as in Figure 5; N: A, T, G or C.

with the DNase I pattern obtained for the binding of Pol I to the end of a DNA fragment derived from pBR322 (20).

The footprinting technique was also used to obtain an estimate of the apparent affinity of Pol I for DNA. Under the conditions of the assay shown in Figure 8, the protein concentration required for half maximal footprinting intensity, as reflected by the ‘P’ patterns seen at the N4 and N5 DNase I nicks, is about 200 nM. This concentration represents an estimation of the apparent dissociation constant ($K_d(\text{app})$) (18). In the same experimental conditions, for the 10–23 protected nucleotides at the extremity of the template strand, the $K_d(\text{app})$ is about 10 times lower: 30 nM (data not shown).

All these data strongly suggest that in presence of PU DNA fragment, Pol I first binds DNA ends (at a $K_d(\text{app})$ of about 30 nM) and then binds duplex DNA, specifically at PU DNA sequences (at a $K_d(\text{app})$ of about 200 nM).

F8 exhibits specific PU DNA binding properties in gel retardation assay I

In order to confirm by another technique and to further analyze the interaction between F8 and PU DNA, a series of gel retardation experiments were performed.

The ^{32}P -labelled F_E (see ‘Experimental procedures’ and Figure 1), derived from the intergenic region *malE-malF* which contains three PUs, was incubated with F8. In the absence of competitor DNA, all the labelled fragment was retained as a single complex (Figure 10I, first lane). As competitor, we used three different DNA: i) DNA from plasmid pPU99 which carries, in tandem, 33 *malE-malF* intergenic regions (i.e. 99 PUs); ii) DNA from plasmid pMJM2 which has the same length as pPU99 and which does not contain any PU; iii) a simple alternating copolymer duplex: IC. With increasing quantities of the various competitor, two major protein-DNA complexes which migrated more slowly than the free labelled fragment were detected (C_{E1} and C_{E2}). The competition (especially for C_{E1}) was much more effective with the pPU99 plasmid DNA, which carries PU, than with the two others competitors which do not carry PU. For example, a full binding of the labelled DNA occurred at 500 ng of IC while, at 50 ng of pPU99, approximately 70% of the labelled DNA was already free and while, at 50 ng of pMJM2 DNA, no free labelled DNA was observed. This experiment clearly shows that the F8 activity binds preferentially PU DNA

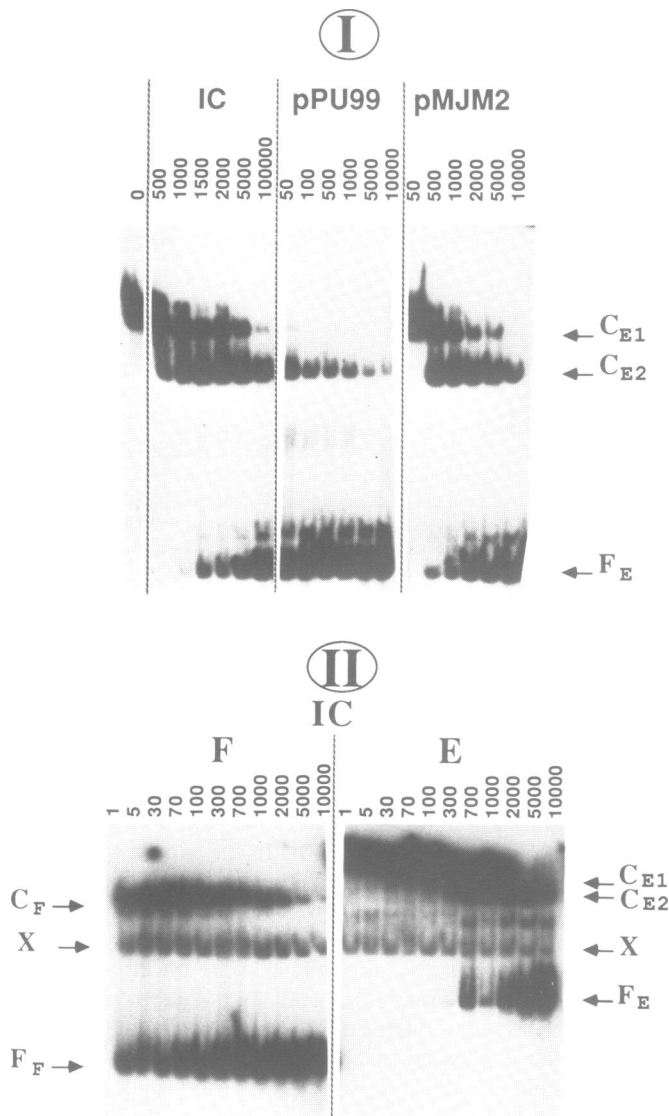


Figure 10. Gel retardation analysis of F8-PU DNA interaction. F_E : indicates the migration position of the free F_E , F_F : indicates the position of the free F_F , C_F : shifted F_F , C_{E1} - C_{E2} : different shifted F_E , X: see text. The standard assay was performed as described in Experimental procedures. The positions on the gel of the C_{E1} and C_{E2} complexes between different experiments have been estimated from their relative mobility according to the migration of the free DNA. (I) F_E is incubated with 0.9 μg of F8 and increasing amount of either IC or pPU99 plasmid DNA digested with *Hin*I or pMJM2 plasmid DNA digested with *Hin*I as indicated above each lane (ng). (II) F_E and F_F are incubated with 0.9 μg of F8 and increasing amount of IC as indicated above each lane (ng).

(pPU99) in comparison to DNA without any PU (pMJM2 or IC).

We also compared the F8 binding properties of the ^{32}P -labelled F_F without any PU (see 'Experimental procedures' and Figure 1) of the ^{32}P -labelled F_E with three PUs. Figure 10II shows the competition pattern obtained for the two fragments with a fixed quantity of F8 and with various amounts of IC as competitor DNA. Below 700 ng of IC, no free labelled DNA fragment could be detected with F_E while with F_F , even at 1 ng of IC, about 50% of the labelled fragment was free. This indicated that the affinity of F8 for F_E (three PUs) is much higher than for F_F (no PU) and thus confirms the specificity of the F8 binding properties for PU DNA.

To eliminate possible artefacts due to the difference in length between F_E and F_F (171 bp versus 108 bp), we also compared the F8 binding properties of a fragment of 436 bp without any PU and of F_G , a fragment of 539 bp which contains a direct repeat of the *malE-malF* intergenic region i.e. 6 PUs (Figure 1). With the 436 bp fragment, only one major complex was detected while with the 539 bp fragment, four major complexes were detected (data not shown). Thus, the number of complexes observed with F8 is correlated with the number of PU regions; this indicates that at least some of these complexes reflected PU-specific DNA-protein interactions.

On Figure 10II, a discrete band, called X, was present both with F_E and F_F . When F8 was incubated without labelled DNA but with $\alpha^{32}\text{P}$ ATP alone, a similar band also appeared (data not shown). This strongly suggests that the X band represents a protein with a nucleotide binding site or a phosphorylated form of a protein present in F8. These hypotheses have not yet been tested.

In conclusion, the data of gel retardation confirmed that F8 contains a PU-specific DNA-binding activity. In addition, F8 appears to include a protein with a nucleotide binding site or with a phosphorylated residue.

DISCUSSION

Palindromic Units (or PU) are bacterial repetitive DNA sequences: about 1000 PU sequences are present in the genome of *E. coli*, accounting for 1% of its total DNA. The origin and the functions of PU DNA are still obscure (for recent reviews, see 3, 5).

We showed previously that an activity present in a high salt protein extract from purified nucleoids was able to specifically bind to PU DNA in an ExoIII protection assay (6). However, the existence of specific PU DNA-protein interactions is still a matter of controversy: one laboratory, despite the use of several approaches, failed to identify any specific PU binding proteins (5) while another laboratory recently reported that the *E. coli* DNA gyrase bind specifically PU DNA (21).

In this paper, we present the purification of PU DNA binding activities, starting from a crude *E. coli* extract. Two such activities were isolated as measured by the ExoIII protection assay. One is the well known DNA polymerase I (Pol I); the other is still a mixture of at least 5 polypeptides (called F8).

Pol I binds PU duplex DNA

We provided strong arguments to show that the PU DNA binding properties of the fraction containing the Pol I protein (F3) were due to Pol I: i) in F3, SDS-PAGE analysis shows only one major polypeptide corresponding to Pol I, as revealed by protein sequence analysis; ii) similar binding properties were observed with a commercially available Pol I protein; iii) no binding occurred with an equivalent fraction purified from cells carrying a mutated *polA* gene.

The binding of Pol I to specific DNA sequences in a duplex DNA was unexpected. Indeed, previous work showed that unbroken duplex DNA was not bound by Pol I (22). These discrepancies may be due to the facts that both the sequence of the DNA substrates and the techniques used to examine binding were different. In particular, the DNA substrates used in the previous binding experiments (23) did not contain any PU: this is not only true for (dAT)₁₂ oligomers, ϕX174 DNA and T7 DNA for which the nucleotide sequence is known to be devoid of PU sequence (unpublished observations) but also very likely

for plasmid DNA isolated from *E. coli* 15T⁻ since PU sequences have never been detected in natural plasmid DNA (2 and unpublished observation).

Since DNA breaks, such as nicks and ends, are bound by Pol I (22), it can be asked whether the specific binding of Pol I to PU DNA was due to nicks introduced into the PU sequences of the labelled fragments. At least two arguments are against this interpretation. Firstly, when loaded on a denaturing polyacrylamide gel, the DNA fragments we used did not exhibit a band corresponding to a nicked molecule at the PU region (unpublished observations). Secondly, gel retardation assay experiments show that Pol I can form stable complexes with PU DNA, even with a circular unbroken duplex DNA template.

DNase I footprinting experiments of the PU DNA-Pol I interaction revealed an unusual protection pattern, called 'P'. The 'P' pattern is characterized by two DNase I hypersensitive sites flanking a central region of 1 to 3 nucleotides totally protected from cleavage. This pattern appears to occur at specific DNA sites. Whether the 'P' pattern reflects the binding of Pol I to an unbroken duplex DNA at a specific sequence or the binding of Pol I to a nick, introduced by the DNase I reaction, located within a specific DNA sequence can not be directly inferred from the footprinting experiment. However, we believe that the former possibility is the most plausible one because: i) the binding reflected by the 'P' patterns does not exhibit a dissociation constant similar to that for a DNA break (200 nM for 'P' pattern versus 30 nM for DNA ends); ii) in gel retardation assay, Pol I binds unbroken duplex DNA (see above).

What could be the DNA sequence specificity of the 'P' patterns? All the regions presenting such a pattern contain the triplet 5'-GCC-3'; in addition, the two regions which exhibit the more pronounced 'P' pattern contain two occurrences of the GCC triplet (Figure 9). Alternating regions of enhancement and inhibition of DNase I cleavage susceptibilities have already been observed for some DNA-protein interactions and have been explained by DNA wrapping or DNA looping (24). In the case of PU DNA-Pol I interaction, the interval between sites becoming sensitive and resistant to cleavage is different from that already observed; furthermore, this interval is not strictly constant. Thus, a different kind of DNA deformation has to be involved to explain this DNase I pattern. Whatever this local deformation is, it has to be either induced by the Pol I binding to the duplex DNA or to be recognized and stabilized by the Pol I molecule.

From all the above findings, we propose that Pol I is able to perform two types of DNA binding: the type A is its 'classical' capacity to bind DNA ends and nicks and the type B is its capacity to recognize duplex DNA in a sequence-dependent manner. In a DNase I footprint experiment with increasing amount of Pol I, the protein first binds DNA ends (type A), then, with a lower affinity, it binds PU duplex DNA (type B). We do not know whether type B binding can occur with DNA sequences different from that of PU. We measured a dissociation constant for the type B of 200 nM which is roughly equal to the Pol I concentration in the cell (assuming 500 Pol I molecules per cell); thus, we believe that this type of interaction could really occur *in vivo*.

The exact interactions involved in the type B binding are unknown, but they must be different from that present in the type A binding because: i) the two binding properties do not exhibit similar dissociation constant; ii) their DNase I footprinting pattern is very different; iii) the type B binding required Mg²⁺ while the type A binding does not; iv) Pol I but not the Klenow protein

is able to bind duplex DNA while DNA end binding can be performed both with the Klenow protein and with the Pol I protein.

One potential B-DNA binding site in the Pol I protein has been inferred from the structure of its large domain (the Klenow protein) as determined by crystallography (25). This site contains 'a deep crevice of the appropriate size and shape for binding double-stranded B-DNA' (25) with a finger constituted of two α helices which can be placed into a major groove. It is tempting to speculate that the PU DNA fits into this cleft, the specificity being conferred by interactions with the sides of the cleft and/or with some residues of the two α helices. But the Klenow protein alone is not sufficient to form specific PU DNA-Pol I complexes, as examined by Exonuclease III protection and by gel retardation assay. Thus, the other domain of Pol I (the small domain) could be necessary for the PU DNA-Pol I interaction.

Multiple protein-DNA interactions at DNA PU sites

Gel retardation experiments confirmed the existence of a specific binding of F8 to PU DNA. Since a specific binding of the *E. coli* DNA gyrase has been observed to PU DNA (21), we checked for the presence of such a protein in F8, both by comparing the migration pattern of F8 and DNA gyrase in SDS-PAGE analysis and by an oxolinic acid DNA cleavage assay (unpublished data). None of these assays revealed the presence of DNA gyrase in F8. The nature of the PU-binding proteins present in F8 is under investigation in our laboratory.

At least three different proteins have been shown to specifically bind PU DNA *in vitro*: DNA gyrase, DNA polymerase I and a yet uncharacterized protein fraction, called F8. All the three PU-binding proteins needed Mg²⁺ to interact with PU DNA and divalent cations are known to promote conformational changes in DNA (26, 27). Thus, these multiple DNA-protein interactions could be related to local non B-DNA structure at the PU site. It is clear that additional work is necessary to better understand the molecular nature of these multiple PU DNA-protein interactions.

What can be the physiological significance of this multiple DNA-protein interaction at the PU region? As already speculated (6, 21), PU DNA could serve as specific anchorage sites for supercoiled nucleoid domains. The eukaryotic Scaffold Associated Regions (SAR), which also contain topoisomerase II (homologous to bacterial DNA gyrase) binding sites, are believed to be rich in specific DNA binding sites for different proteins, thus facilitating the formation of various nucleoprotein complexes required for the control of chromatin organization and gene expression (topology, replication, transcription, folding...) (28). If some kind of similar functional organization of the genome exists in bacteria, PU regions are likely to play a role equivalent to SAR.

The PU DNA could serve as preferred entry sites for Pol I, playing a role in secondary replication initiation sites, in specific pausing sites during the polymerization reaction or in replication fidelity. A recent work on DNA replication errors due to Pol I is relevant to the latter possibility because it suggests that the major specificity determinants of certain errors involve sequence-specific polymerase-template interactions (29). For all of these processes, Pol I could interact with other proteins. For example, the *pcbA1* mutation allows a Pol I-dependent replication of *E. coli* cells and is likely to correspond to an allele of the *gyrB* gene (coding for the B subunit of DNA gyrase) (30, 31).

Another possible effect of the PU-Pol I interaction is that it

plays a role in the PU DNA region formation. Certain PU regions are associated with a direct duplication of a DNA segment containing either two PUs or a part of their flanking region (Gilson *et al.* in preparation). This type of genetic event can be favoured by a preferred entry or pausing site for Pol I in these regions.

PU DNA consensus and distribution is bacterial species specific, suggesting a possible mechanism of concerted evolution (3, 32). It has been postulated by us (3) and by others (5) that the PU concerted evolution could be achieved by a RNA-mediated gene conversion mechanism. If Pol I binds PU DNA, it is possible that it also recognizes the same site at the RNA level. Since 25% of all the 3' mRNA from *E. coli* cells are formed by PU sequences, thus adopting a complex RNA secondary structure (5) and since Pol I is able to use RNA template for polymerization reaction (22), a specific interaction between PU RNA and Pol I could favour the formation of RNA-DNA hybrids. These hybrids could then be converted to DNA-DNA hybrid fragments which can, in turn, be integrated into the chromosome, either by homologous recombination in a PU region, leading to PU gene conversion, or upon illegitimate recombination, leading to PU spreading.

ACKNOWLEDGEMENTS

This work was supported by grants from the Fondation pour la Recherche Médicale, the Ligue Nationale Contre le Cancer, and the Association pour la Recherche sur le Cancer. We want to express our sincere gratitude to David O'Callaghan for the critical reading of the manuscript.

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