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In vivo promoter activity of the synthetic Pribnow box

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The synthetic polydeoxyribonucleotides TGCA(TTATAATGCA)$_{1,2}$ containing the consensus Pribnow box, were inserted upstream of the promoterless *gal* operon in a specially constructed plasmid. Using the MacConkey agar indicator technique it has been shown that these inserts are able to induce an efficient in vivo expression of the *gal* operon.

Recombinant plasmid, *Gal* operon, Pribnow box, PCR, S1-mapping

1. INTRODUCTION

The comparative analysis of the *E. coli* promoter sequences has revealed two highly conserved regions, centered around the −35 and −10 positions with respect to the mRNA start point. The importance of these regions has been demonstrated by analysis of many promoter mutants and by experiments on contacts between RNA polymerase and promoters [1,2]. Nevertheless the specific contributions of −10 and −35 consensus sequences to RNA polymerase recognition, binding and initiation are not completely understood. To study the effect of −10 region (the so-called Pribnow box) on the promoter function we suggested as a model system the synthetic double-stranded polynucleotides containing the regularly repeated consensus Pribnow box TATAATG[1]:

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5'  TGCATTATAATGCATTATAATGCATTATAATGCATTATAA  3'   Duplexes I
3'.       AATATTACGTAATATTACGTAATATTACGTAATATTACGT ...5'
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These polynucleotides have been prepared by enzymatic polycondensation of the synthetic decanucleotide pTGCATTATAA [3]. Using the nitrocellulose filter binding technique we have shown previously that these polynucleotides could form heparin resistant complexes with *E. coli* RNA polymerase in vitro with a half-life of about 200 min [4]. In this paper we have studied the in vivo activity of the synthetic duplexes I, containing the Pribnow box as a plasmid constituent.

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[1]Prefix "d" – deoxy – in all formulae of polydeoxyribonucleotides is omitted for short.

2. MATERIALS AND METHODS

The oligonucleotides were synthesized by the solid phase methods [3,5]. The enzymes T4 polynucleotide kinase, T4 DNA ligase, DNA polymerase I (Klenow fragment), restriction endonucleases *Sma*I, *Bam*HI, *Hin*dIII, *Eco*RI were purchased from Boehringer, (Germany). Acrylamide, *N,N'* methylenebisacrylamide, ethidium bromide from BDH (England), ampicillin from Fluka (Switzerland), MacConkey agar, bacto triptone, yeast extract from Difco (USA), [γ-^{32}P]ATP (1000 Ci/mmol) from Amersham (England), the GeneAmp DNA Amplification reagent kit used for polymerase chain reactions (PCR) was obtained from Perkin-Elmer-Cetus (USA).

The plasmid pHD-001-14-11 [6] and *E. coli* F165' strain (*gal*$^-$), used as a recipient for this plasmid, were kindly gifted by Professor H.-J. Fritz (Göttingen, Germany).

Digestions of plasmid DNA with restriction endonucleases *Eco*RI, *Hin*dIII, *Bam*HI, *Sma*I, 5'-labelling of oligo- and polynucleotides, and plasmid fragments, filling in the protruding cohesive ends by DNA polymerase I (Klenow fragment), blunt-end ligation of synthetic polynucleotides and linearized plasmid pHD-001-14-11, isolation of plasmid DNA and total cellular RNA, S1-nuclease mapping were essentially performed as recommended in [7]. Preparation of liquid media and MacConkey agar indicator plates, selection and analysis of the clones were performed as in [8]. Transformation was carried out by the $CaCl_2$ technique [7]. Plasmids from selected clones for analytical purposes were isolated by the alkaline lysis method [7]. PCR (30 cycles: 94°, 1 min, 45°, 2 min, 70°, 1 min) was carried out with GeneAmp reagents in a 50 μl volume using *Taq* DNA polymerase in combination with a DNA Thermal Cycler (Perkin-Elmer-Cetus) [9]. DNA sequencing was carried out by the Maxam-Gilbert method [10].

3. RESULTS AND DISCUSSION

To study the in vivo promoter activity of the synthetic duplexes containing the repeats of the Pribnow box (duplexes I), the special plasmid pHD-001-14-11 has

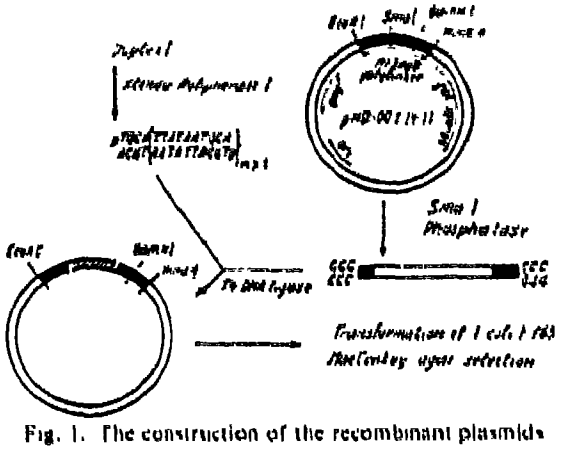

Fig. 1. The construction of the recombinant plasmids

been used. This plasmid is a pBR322 derivative in which the part of tetracycline resistance gene is replaced by the promoterless wild-type galactose operon of *E. coli* [6]. In this plasmid the *Eco*RI-*Hin*dIII fragment, carrying the *gal* promoter, was replaced by the *Eco*RI-*Hin*dIII polylinker fragment from M13mp8 DNA. The polylinker fragment contains no promoter signals and displays no promoter activity. Thus the insertion of some fragment, containing a promoter, is required for the expression of the *gal* operon. This expression can be easily indicated by the colour of colonies of *E. coli* (*gal*$^+$) cells, transformed by these plasmids, on MacConkey agar indicator plates [8].

The construction of the plasmids, carrying the syn-

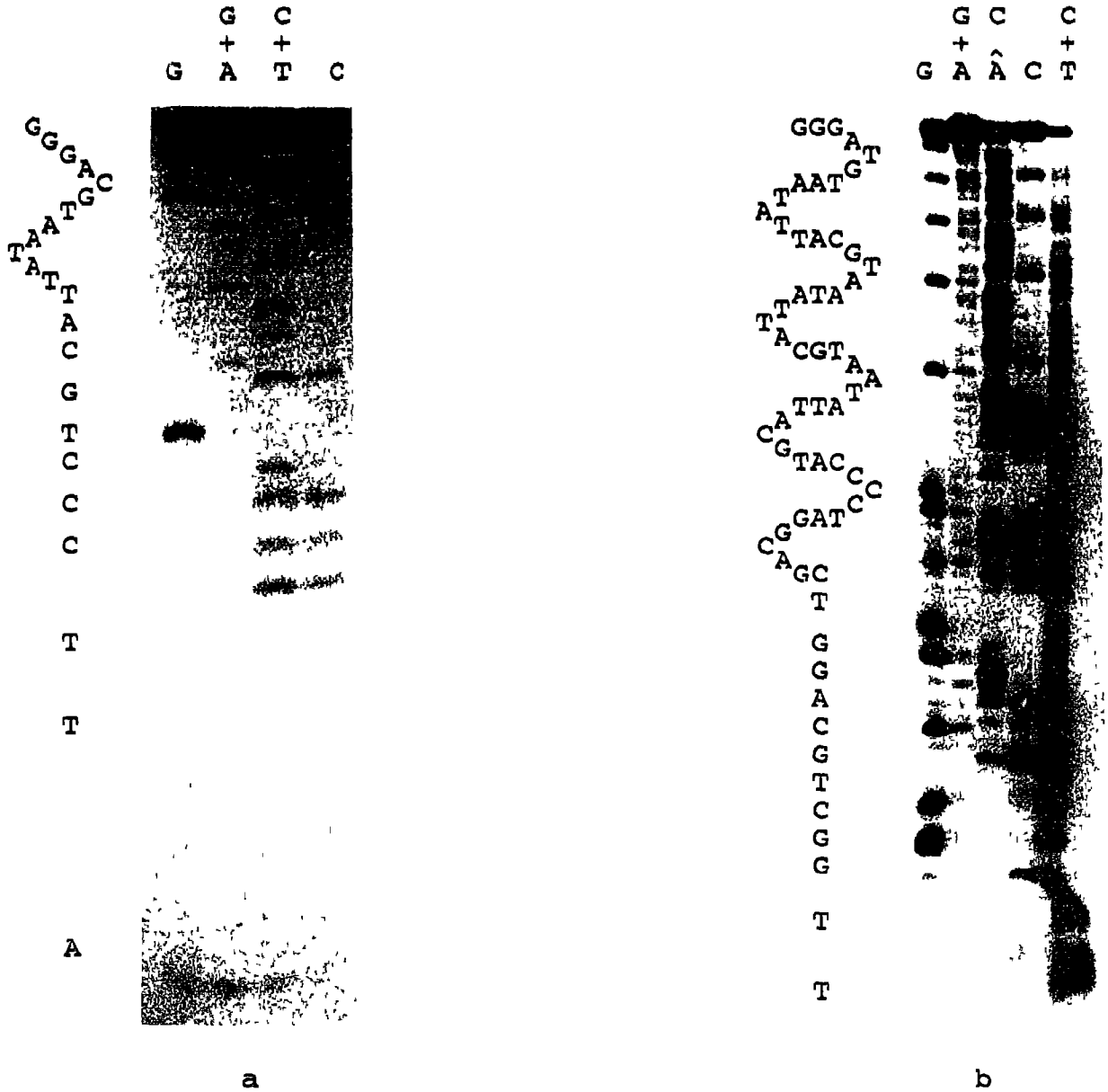

Fig 2 The sequence analysis (12% polyacrylamide gel electrophoresis) of the *Eco*RI-*Bam*HI (a) and *Hin*dIII-*Eco*RI (b) plasmid fragments, containing inserts pTGCATTATAATGCA and pTGCA $(TTATAATGCA)_4$, respectively

thetic duplexes I is schematically shown in Fig. 1. The *Sma*I linearized and dephosphorylated plasmid pHD-001-14-11 was blunt-end ligated with ~1000-fold molar excess of the synthetic duplexes I treated by DNA polymerase I (Klenow fragment) to generate blunt ends. In 16 h the ligation mixture was directly used to transform *E. coli* F165' cells. The transformants, harbouring the fusion plasmids, were classified according to the colour of their colonies on MacConkey agar plates supplemented with galactose (1 mg/ml) and ampicillin (50 μg/ml). Plasmids were isolated from randomly selected both red and white colonies, cleaved by *Eco*RI (or *Hin*dIII), dephosphorylated and 5'-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase. After subsequent *Bam*HI (or *Eco*RI) cleavage, a small labelled fragment was isolated and sequenced (Fig. 2a,b). The analysis revealed the presence of synthetic inserts only in plasmids isolated from red colonies. All synthetic inserts were derivatives of the initial synthetic duplexes I. To ensure that it was the synthetic inserts that caused the expression of the *gal* operon but not other mutations outside them, the small *Eco*RI-*Hin*dIII restriction fragments with inserts TGCATTATAATGCA and TGCA(TTATAATGCA)$_4$ were recloned in the pHD-001-14-11 vehicle. All clones, harbouring these newly constructed plasmids, revealed the expression of the *gal* operon (red colonies on MacConkey agar). The next step was to identify the in vivo transcription start site directed by the 'synthetic promoter'. We have slightly modified the well-known S1-mapping technique [7]. The 149-bp double-stranded DNA ^{32}P-fragment, carrying the 14 bp insert, was prepared by the PCR technique using AACCTATAAAAATAGGCG (1 μM, incorporated in the non-coding strand upstream of the insert) and [5'-^{32}P]*p*GACTTCCAATGTAACCGC (0.3 μM, 15 Ci/mmol, incorporated in the coding strand downstream of the insert) as primers and the plasmid under investigation as a template. After hybridization of this probe with total RNA (3 h, 42°C) from the cells harbouring plasmids with the 14 bp insert or w.t. plasmids (control) and S1-nuclease treatment (30 min, 37°C) the total digests were fractionated on an 8% polyacrylamide/8M urea gel next to a Maxam-Gilbert sequencing ladder of the same 149 bp ^{32}P-probe (Fig. 3). The size of the protected fragment (with 1.5 base correction [11]) allowed us to localize the unique transcription start site. The scheme below sums up the data obtained — the sequence of the 'artificial promoter' region of the plasmid with the 14 bp insert (underlined), containing the Pribnow box (marked by ~), and the transcription start point (marked by the arrow):

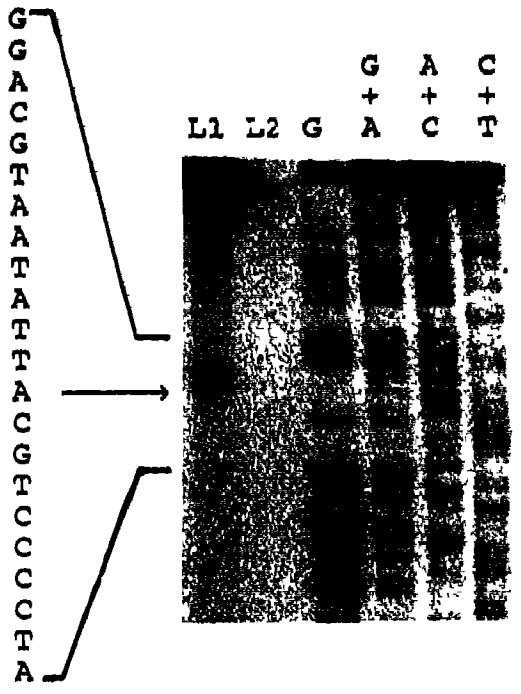

Fig. 3. The S1-mapping of the transcription start point using the 149-bp ^{32}P-fragment (see text) and RNA from cells harbouring plasmids with the 14 bp insert (Lane 1) or w.t. plasmid (Lane 2, control). The 4 lanes on the right compose the chemically digested sequence ladder of the same ^{32}P-probe. The sequence of the coding strand around the 5'-end of the protected region is shown on the left margin. The S1 protected DNA band is indicated by the arrow.

It is obvious from all experiments described that it is the synthetic insert that causes the high level of the *gal* operon expression. Moreover, we hypothesize that in the absence of the consensus −35-region (see the sequence above) the consensus Pribnow box TATAATG could be the only key structural determinant responsible for promoter function in our case (though the influence of other cryptic promoter elements, e.g. CATTATA as '−10' and CTTTCG or TCGTCT as '−35', cannot be completely excluded). Some recent publications [12,13] support this idea. The unconventional position of the transcription start point (the last T inside the Pribnow box) does not contradict this statement [14].

So the question arises: is the consensus Pribnow box sufficient for creating an active promoter in any other context or do we deal with a good instantaneous fitting of synthetic inserts and given flanking regions?

Just recently in a coupled transcription-translation cell-free system [6], we started to study the promoter strength of the new constructions described. The preliminary results show that the promoter strength of the plasmid region, carrying our 14-bp insert, is com-

5'- -3'
.CCCTTTCGTCTTCAAGAATTCCCTGCATTATAATGCAGGGGATCCGTCGA. *gal* operon .

parable with that of the w.t. type *gal* promoter and the synthetic tyrT ssu2 promoter.

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