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FEBS Letters 338 (1994) 157–160

FEBS 13583

FEBS LETTERS

The major cold shock protein of *Bacillus subtilis* CspB binds with high affinity to the ATTGG- and CCAAT sequences in single stranded oligonucleotides

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Received 25 November 1993; revised version received 13 December 1993

Abstract

We have characterized the nucleic acid binding properties of the major cold shock protein of *Bacillus subtilis*, CspB. CspB is a member of the cold shock domain (CSD) family, which is widespread among pro- and eukaryotes and shares the nucleic acid binding domain CSD. The CSD domain is highly conserved and binds with strong affinity to the Y-box motif, a cis-element that contains the CTGATTGG$^{C}/_{T}{}^{C}/_{T}$AA sequence. In a series of gel retardation experiments using oligonucleotides, which contain the Y-box motif and altered sequences, we show the preferential binding of CspB to single-stranded DNA that contains the ATTGG as well as the complementary CCAAT Y-box core sequence. In contrast CspB exhibits lower affinity to altered Y-box core sequences. Dependent on the length of the oligonucleotide and the degree of sequence deviation from the Y-box core sequence 3- to over 10-fold overexcess of CspB was needed for complete retardation.

Key words: Cold shock protein; CspB; Cold shock domain (CSD); Y-box sequence; *B. subtilis*

1. Introduction

Cold shock induced proteins, which may be involved in the regulation of adaptive processes required for cell viability at low temperature, have been identified recently in *Escherichia coli*, *Bacillus subtilis* and *Streptomyces clavuligerus* [1–3]. In the Gram-positive soil bacterium *B. subtilis*, we have cloned a gene encoding the major cold shock protein CspB and found that it shares about 62% identity to the major cold shock protein of *E. coli*, CS7.4 [2]. CspB and CS7.4 are small hydrophilic proteins consisting of 67 and 70 amino acids, respectively. Both proteins show striking similarity of 43% identity with the nucleic acid-binding domain of the Y-box factors, which is now referred to as the cold shock domain (CSD). The Y-box factors are a family of eukaryotic nucleic acid-binding proteins that preferentially bind to the Y-box, a *cis*-element found in the promoter region of mammalian major histocompatability complex class II genes, which has the consensus sequence CTGATTGG$^{C}/_{T}{}^{C}/_{T}$AA [4,5]. The underlined pentamer is especially conserved.

CspA has been shown to act as transcriptional enhancer of the synthesis of two other cold shock proteins: the nucleiod protein H-NS and the α subunit of DNA gyrase [6,7]. In the latter case it has been demonstrated that three ATTGG sequences in the proximal promoter region of the *gyrA* gene are required for specific binding of CspA.

The 3D-structure of CspB, recently determined by 2D-NMR and X-ray studies [8,9], revealed that the protein consists of an antiparallel five-stranded β-barrel: two consecutive β-strands contain the conserved RNA-binding motifs RNP-1 and RNP-2 and create a surface rich in aromatic and basic residues that could serve as a nucleic acid binding site. Remarkably, the basic folding pattern of CspB is similar to the recently characterized oligonucleotide and oligosaccaride binding (OB) fold [10], which may represent a universal nucleic acid binding domain that seems to be highly conserved throughout evolution.

We have characterized the nucleic acid binding properties of CspB and demonstrated the specific binding of CspB to a 54-mer single stranded oligonucleotide, which contains the Y-box sequence [8]. For further investigation of the specific binding sequence recognized by CspB we have performed gel retardation experiments with oligonucleotides homologous to the 54-mer that are altered in length and in the Y-box consensus sequence. The data presented here show that CspB binds to the pentamer sequences ATTGG and CCAAT with highest affinity in single stranded DNA, but also to other sequences. In addition, our results suggest that CspB could act as tran-

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SSDI 0014-5793(93)E1500-L

scriptional activator of cold shock genes by recognizing putative ATTGG-box elements shown to be present in promotor regions of genes induced under cold shock conditions.

2. Materials and methods

2.1. Overproduction and purification

CspB was overproduced and purified as described in Schindelin et al. [11].

2.2. Gel retardation experiments

Synthetic oligonucleotides shown in Table 1 (obtained from M. Krause, IMT, Marburg) were incubated with different amounts of purified CspB in binding buffer (50 mM NaCl, 20 mM Tris-HCl, pH 8.6, 5 mM $MgCl_2$, 6% glycerol) for 30 min at 25°C in volumes of 10 μl. After addition of 5 μl of probe buffer (10% glycerol, 0.1% Bromphelol blue) the samples were loaded on 12.5–22.5% non-denaturing PAGE and run at low voltage in TBE buffer (Tris-base 163.5 g/l, boric acid 29.86 g/l, EDTA 11.3 g/l). The gels were stained with ethidium bromide and subsequently with Coomassie brilliant blue. For double-stranded oligonucleotides, a mixture of the complementary single strands was heated to 65°C for 5 min and slowly cooled down to room temperature.

3. Results and discussion

Gel retardation experiments were carried out to investigate the nucleic acid binding properties of CspB. Tafuri et al. [12] described a 54-mer oligonucleotide, which contains the Y-box motif (CTGATTGGCCAA), that is specifically bound by CS7.4 and the Y-box factors FRG Y1 and FRG Y2. CspB binds to this oligonucleotide ($54YB^+$) with higher affinity than to the complementary strand ($54YB^-$); as shown in Fig. 1, complete retardation of $54YB^+$ is visible at 3 molar excess of CspB (lane 4), whereas 5 molar excess is needed for complete retardation of $54YB^-$ (lane 8). In contrast, the band of the double-stranded preparation of these oligonucleotides is not shifted quantitatively (lanes 10–13) compared to the free oligonucleotide (lane 9). The faint retarded band in lane 13 can be accounted to a small portion of single stranded oligonucleotides in the preparation that is bound by CspB. Thus, the double-stranded 54YB oligonucleotide like all other tested dsDNA is not bound by CspB, despite the ATTGG sequence.

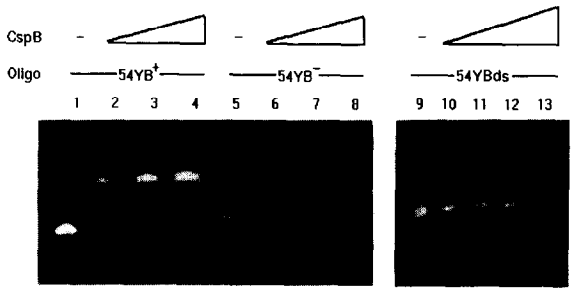

Fig. 1. Gel retention analysis of the 54YB-oligonucleotides. 15 pmol $54YB^+$ (1–4), 15 pmol $54YB^-$ (5–8) and 8 pmol of ds54YB oligonucleotides (9–13) were incubated with CspB (0 pmol: 1/5/9, 30 pmol: 2/6, 40 pmol: 10, 45 pmol: 3/7, 75 pmol: 4/8, 80 pmol: 11, 120 pmol: 12, 160 pmol: 13) for 30 min at 25°C and subjected to electrophoresis on 12.5% non-denaturing PAGE.

Previously we have shown that CspB binds specifically to the $54YB^+$ strand compared to an oligonucleotide of 54 bases without Y-box sequence [8]. Additional experiments have shown that the non-specific 54-mer oligonucleotide as well as other oligonucleotides are also bound by CspB, but complete retardation of these oligonucleotides required 3–6 times higher excess of CspB compared to the specific $54YB^+$ oligonucleotide (not shown). To identify the specific binding sequence of CspB we have employed 25-mer oligonucleotides that are identical to the central sequence of the 54 YB^+ oligonucleotide (in the case of $25YB^+$) but differ in the Y-box sequence (25YM-oligonucleotides) and the core sequence (25CM; Table 1). Compared to a 3-fold excess of CspB for the complete retardation of the oligonucleotide $54YB^+$ an 8-fold overexcess was needed in the experiment with the $25YB^+$ oligonucleotide (Fig. 2, lanes 1–4). This is in accordance with a general lower affinity of CspB to smaller fragments of ssDNA. Changing the outer bases apart from the ATTGG sequence in the Y-box has a minimal effect on the binding of CspB (oligonucleotide 25YMa, lanes 9–12), but changing the whole Y-box sequence reduces the affinity to CspB drastically, as shown with the ol-

Table 1
Oligonucleiotides used in CspB binding

Oligonucleotide	Sequence
$54YB^+$	GAATTCGCAGACGTGGGAATCCTACTG**ATTGG**CCAAGGTGCTGGTGGTGTGTGG
$54YB^-$	CCACACACCACCAGCACC**TTGGCCAAT**CAGTAGGATTCCCACGTCTGCGAATTC
54NS	CATGCTGCGTGAACAGGTTGCTCAGCTGAAACAGAAAGTTGGTGAACGTTGATA
$25YB^+$	ATCCTACTG**ATTGG**CCAAGGTGCTG
25YMi	ATCCTACTG**CCAAT**CCAAGGTGCTG
25YMa	ATCCTATAC**ATTGG**TGTGGGTGCTG
25YMk	ATCCTATACTAACCTGTGGGTGCTG
25CMA	ATCCTATACC**TTGG**TGTGGGTGCTG
25CMG	ATCCTATAC**ATTG**CTGTGGGTGCTG
25CMAG	ATCCTATACC**TTG**CTGTGGGTGCTG

Y-box sequences are underlined, core-sequences in bold letters.

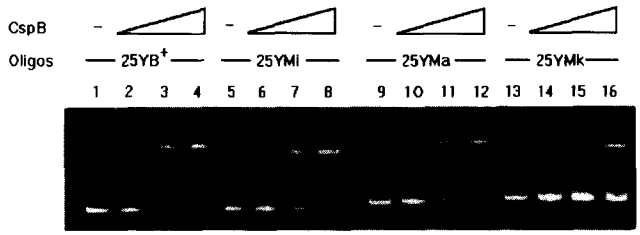

Fig. 2. Gel retention analysis of the 25YM-oligonucleotides. 30 pmol 25YB$^+$ (Y-box) (1–4), 30 pmol 25YMi ('CCAAT') (5–8), 30 pmol 25 YMa ('ATTGG') (9–12) and 30 pmol of 25 YMk oligonucleotides (no Y-box) (13–16) were incubated with CspB (0 pmol: 1/5/9/13, 90 pmol: 2/6/10/14, 150 pmol: 3/7/11/15, 240 pmol: 4/8/12/16) for 30 min at 25°C and subjected to electrophoresis on 22.5% non-denaturing PAGE.

igonucleotide 25YMk (lanes 13–16). The retardation of the oligonucleotide 25YMi (CCAAT instead of ATTGG) (lanes 5–8) is analogue to the complementary strand of 25YB$^+$, the complete retardation requires a 10-fold excess of CspB (data not shown).

In analogy with the 54YB$^+$ and 54YB$^-$ oligonucleotides the pattern of retardation of the 25YB$^+$ and 25YB$^-$ oligonucleotides reveals nearly equal efficient binding of the CCAAT and ATTGG containing oligonucleotide to CspB. In contrast, more efficient binding of both sequences is apparent in comparison with 25YMk (no Y-box). This indicates that CspB binds preferentially to both strands of an ATTGG-box.

If the adenine of the ATTGG-box is changed to cytosine (25CMA), the affinity to CspB is reduced markedly (Fig. 3, lanes 5–8) compared to the oligonucleotide 25YMa, which contains the complete ATTGG-box (lanes 1–4). At 8-fold excess of CspB about 30% of the 25CMA oligonucleotides are retarded (lane 8) in contrast to over 95% of the 25YMa oligonucleotide, which contains the unmodified pentanucleotide (lane 4). CspB affinity is reduced to a level that is comparable to the oligonucleotide 25YMk missing the binding motif (Fig. 2, lanes 13–16). Changes of the last guanine (25CMG) in the ATTGG-box to cytosine reduces the affinity to CspB (Fig. 3, lanes 9–12), but to a lesser degree than the exchange of the adenine (25CMA). The oligonucleotide

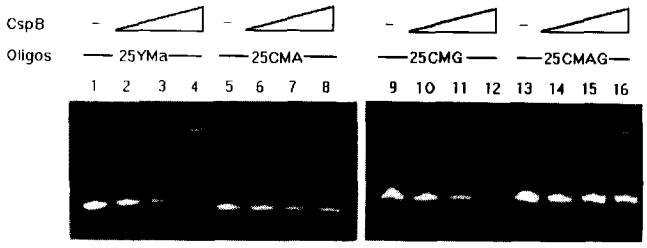

Fig. 3. Gel retention analysis of the 25CM-oligonucleotides. 30 pmol 25YMa ('ATTGG') (1–4), 30 pmol 25CMA ('TTGG') (5–8), 30 pmol 25 CMG ('ATTG') (9–12) (and 30 pmol of 25 CMAG oligonucleotides ('TTG') (13–16) were incubated with CspB (0 pmol: 1/5/9/13, 90 pmol: 2/6/10/14, 150 pmol: 3/7/11/15, 240 pmol: 4/8/12/16) for 30 min at 25°C and subjected to electrophoresis on 22.5% non-denaturing PAGE.

25CMG is retarded to approximately 80% at 8-fold molar excess of CspB (lane 12). The exchange of both adenine and guanine to cytosine (25CMAG) did not reduce CspB affinity further than the exchange of the adenine alone (Fig. 3, lanes 13–16). Thus, the ATTG-sequence of the conserved pentanucleotide confers the main specificity to the binding of CspB. The apparent high affinity of CspB to the sequence CCAAT cannot be explained by these experiments.

To our knowledge, these results are the first direct proof for the specificity of a CSD protein to the ATTGG and CCAAT sequence in single stranded DNA. The general nucleic acid binding character of CspB with different levels of affinities could explain, why CSD proteins have been found to interact with several unrelated oligonucleotides. Also, our results support the assumption from Wolffe and co-workers that many binding experiments of CSD proteins with double-stranded DNA could be interpreted as interaction with single stranded DNA present in the preparations of short double-stranded oligonucleotides [5].

Preliminary experiments employing antibodies to precipitate CspB from cellular extracts of *B. subtilis* and specific labelling of nucleic acids that were co-precipitated with CspB (radioimmuno-precipitation assay) have confirmed that CspB binds to nucleic acids in vivo.

The data presented in this paper show that CspB has the highest affinity to the sequence ATTGG (Table 2). The complementary sequence CCAAT is bound with little lower affinity, but still preferentially to other sequences. In vivo, CspB could bind to partial single-stranded DNA in promoters containing the pentanucleotide to stabilize the open complex of the RNA-polymerase. In analogy to the gene 5 binding protein of phage fd [13], a CspB dimer that was postulated from crystallography studies on CspB [9] could bind both strand of the DNA at the ATTGG and the CCAAT sequence.

Table 2
Retardation experiments of oligonucleotides.

Oligo-nucleotide	Binding motif	Complete retardation by excess of CspB				
		3×	5×	8×	9×	10×
54YB$^+$	CTG**ATTGG**CCAA	+				
54YB$^-$	TTGG**CCAAT**CAG		+			
54NS	none				+	
25YB$^+$	CTG**ATTGG**CCAA			+		
25YMi	CTG**CCAAT**CCAA					+
25YMa	**ATTGG**			+		
25YMk	none					−
25CMA	**TTGG**					−
25CMG	**ATTG**					+
25CMAG	**TTG**					−

Conserved Y-box sequence (binding motif) with conserved pentanucleotide in bold letter. + = complete (− = incomplete) retardation at *x*-fold molar overexcess of CspB.

NMR studies have hinted that CspB might form larger aggregates in solution [8]. Through cooperative binding of several CspB dimers the formation of an open complex could be stabilized, which may be a limiting step in the initiation of transcription at low temperatures. ATTGG and CCAAT sequences have been found in several promoters of cold shock genes in *E. coli*. Other possibilities are that CspB could stabilize mRNA or act on translation, which is assumed to be a limiting factor at low temperatures. This is consistent with findings that CspB plays an important role in the survival of *B. subtilis* cells at low temperatures [2].

Acknowledgements: This work was supported by the Deutsche Forschungs-Gemeinschaft and the Fonds der Chemischen Industrie.

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