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Regulatory C Protein of the *Eco*RV Modification–Restriction System

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Abstract—The *C* gene product of the modification–restriction system *Pvu*II binds to its own promoter (C box) and stimulates transcription of both the *C* gene and the endonuclease gene. According to our data the same regulatory mechanism is realized in the *Eco*RV system. It was found that upstream of the *Eco*RV endonuclease gene two ATG codons give rise to two open reading frames (ORF1 and ORF2) ending at the same point inside the endonuclease gene. Two DNA fragments corresponding to ORF1 and ORF2 were cloned, and the homogenous products of proteins encoded by them were found to be DNA-binding proteins. A specific DNA sequence (C box) recognized by the proteins was determined with DNAse I footprinting. The C box CCCATTTTGGGTTATCCCATTTTGGG is located inside ORF1 and, similar to the *Pvu*II C box consisting of tandem repeats of 11 nucleotides, is divided by four nucleotides. In its turn each of the repeats contains inverted repeats of four terminal nucleotides. The *Eco*RV C box sequence differs both from the *Pvu*II C box sequence and from the proposed consensus sequence of C boxes in other modification–restriction systems.

Key words: modification–restriction systems, C.*Eco*RV, DNA-binding proteins, gel-shift assay, footprinting

The existence of a third gene whose product, C protein (controller), stimulates expression of the endonuclease gene was shown for the *Pvu*II and *Bam*HI modification–restriction systems [1, 2]. Until the cell accumulates a sufficient amount of C protein, there occurs virtually no expression of the endonuclease gene. It was demonstrated for the *Pvu*II system that the product of *C* gene activates the endonuclease gene transcription [1]. Products of *C* genes of the *Pvu*II and *Bam*HI systems were also shown to be interchangeable [2]. On analyzing the nucleotide sequence preceding the *Eco*RV endonuclease gene, we found two ATG codons that give rise to two open reading frames (ORF1 and ORF2) ending at the same point inside the endonuclease gene. In this work we have cloned two DNA fragments that correspond to ORF1 and ORF2, prepared electrophoretically homogeneous preparations of the proteins encoded by them, and determined the DNA sequence (C box) to which the obtained proteins bind.

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MATERIALS AND METHODS

Escherichia coli BL21(DE3) strain (Novagen, USA), pET21d (Novagen) and pGL74 [3] plasmids, and restriction endonuclease *Nco*I (New England BioLabs, USA) were used in the experiments. The other enzymes (restriction endonucleases *Bsp*LU11I [4], *Bli*736I [5], and *Xho*I, DNA polymerases Taq and Pfu, polynucleotide kinase, and phage T4 DNA-ligase) were prepared by us. The plasmids were isolated by alkaline lysis [6].

Oligonucleotides were synthesized by Syntol (Russia). The following oligonucleotides were used:

(B10): 5′-ATGACCATGATTACG-3′;

(C1): 5′-ACCGTGCATCTGCCA-3′;

(3C2): 5′-GCGGTCTCGCATGCCAAAGAAAGAAACCC-3′;

(3C9): 5′-AATACATGTCTGTAAGAGAAA-3′;

(3C10): 5′-GCATTAATTCTCGAGGAACGAAGAC-3′;

T7-primer: 5′-TAATACGACTCACTATAGGG-3′.

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The oligonucleotides were labeled using [γ-^{32}P]ATP (Cluster Scientific Production Association, Russia) and phage T4 polynucleotide kinase as described by Maniatis et al. [6].

Polymerase chain reaction (PCR) was performed in two ways: using purified DNA as a template and directly from cell colonies. For this, part of the colony picked by the bacteriological loop was preliminary destroyed by boiling for 5 min in 50 μl water, then centrifuged for 1 min at 12,000*g* and 10 μl of the supernatant was used in the reaction. The reaction was performed in 100 μl of solution containing 1× ThermolPol-buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100), 200 μM deoxynucleoside triphosphate (each), 15 pmol oligonucleotides (each), 50 ng DNA template, and 2.5 units of the Taq- and Pfu-polymerases mixture (40 : 1 U/U). The reaction was performed in a Mastercycler (Eppendorf, Germany) with a temperature gradient on annealing. The temperature of annealing was chosen in a preliminary experiment.

Analysis of PCR products and other DNAs was made by electrophoresis using 1-1.5% agarose gels in 1× TBE-buffer (0.089 M Tris, 0.089 M boric acid, 1 mM EDTA, pH 8.3).

Purification of the PCR products from the reaction components was done using preparative electrophoresis. A cut-out gel band containing the required DNA fragment was dissolved in five volumes of 5 M guanidine thiocyanate, 0.1 M Tris-HCl, pH 7.0, the solution was transferred onto a QIAquick Spin column (Qiagen, USA) and further purification was carried out following the manufacturer's instructions.

DNA cleavage with restriction endonucleases was done under the conditions advised for the endonucleases.

Ligation was performed in a volume of 20 μl containing 7 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, and 1 mM ATP. The molar ratio of the vector 5′-ends to the insertion was 1 : 1. Phage T4 DNA-ligase was added in such an amount that 5 units of the activity fell on 1 pmol of DNA 5′-ends. The sample was incubated for 12 h at 16°C. When the reaction was terminated, DNA-ligase was inactivated by heating at 70°C for 15 min.

Cells competent for transformation of *E. coli* BL21 (DE3) were prepared as described in [7].

Transformation of competent cells with the ligase mixture was done using a BioRad electroporator (USA) at field intensity of 15 kV/cm and pulse length of 1 msec.

Gene expression was induced with an addition to the cell culture of isopropyl-β-D-thiogalactopyranoside (IPTG) to the final concentration of 1 mM. Prior to this, cells were grown in LB mixture (1% bactotryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) in the presence of ampicillin (100 μg/ml) with constant aeration at 37°C to the absorption (A_{600}) of 0.4. Following the induction the cells were incubated at 37°C for 4 h with vigorous stirring.

Cell disruption and Ni-NTA-agarose chromatography. After the induction the cells were collected by centrifugation, the pellet was suspended in a buffer for lysis (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM EDTA, 2 mM 2-mercaptoethanol), lysozyme was added to the final concentration of 1 mg/ml, and the mixture was incubated for 30 min on ice. Then the cells were disrupted using a UZDN-A ultrasonic disintegrator (Russia) for 2 min at 10°C with 10 sec sonications and 10 sec pauses. The lysate was centrifuged (30 min, 10,000*g*, 4°C) and the supernatant was transferred on the agarose column chelated with nickel-nitrilotriacetic acid—Ni-NTA-agarose (Qiagen). Such columns are designed to purify recombinant proteins with six histidine residues at their end that selectively bind to Ni ions. The protein was purified as recommended by the manufacturer. The protein concentration was determined using a spectrophotometer.

Electrophoresis of the proteins and lysates was done as described by Laemmli [8]. To analyze the total cell protein, the cell precipitate was also lysed according to Laemmli [8]. A mixture of Diaprot proteins (Dia M, Russia) were used as molecular mass markers.

Gel shift. To study the binding of C proteins with labeled DNA fragments, the gel-shift assay was employed [9]. Conditions of binding were chosen experimentally. Electrophoresis was done in 25 mM Tris-borate buffer, pH 7.6, at 10°C and 5 V/cm intensity.

Footprinting. The sequence recognized by C proteins was determined with footprinting using DNAse I [10]. DNAse I from calf pancreas (Koch Light, Germany) was used in the experiments. The solution of DNAse I (1 mg/ml) containing 150 mM NaCl and 50% glycerol was prepared from a dry preparation, stored at −20°C, and diluted before use to the required concentration in 1× MRB-buffer (10 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 50 mM NaCl). Labeled DNA fragments were hydrolyzed at room temperature in a solution (5 μl) containing 1× MRB-buffer, 0.5 pmol fragment, and 0.5 μl DNAse I. The DNAse I dilution and incubation time were chosen in preliminary experiments. The reaction was stopped by the addition of 3 μl of the stop-solution (deionized formamide containing 0.01% bromophenol blue, 0.01% xylene cyanole, 4 mM EDTA). Binding of the proteins with labeled DNA fragments was done under conditions similar to those in the gel-shift assay, and then the preparation was treated with DNAse I in the same way as the free fragment. The hydrolysis products were assayed using electrophoresis in 6% polyacrylamide gel containing 7 M urea and 1× TBE buffer at 50°C on a Macrophor instrument (LKB, Sweden). In parallel with the hydrolysis products the products of sequencing of the same fragment were also layered. The method of Sanger modified by Promega [11] with the use of the labeled oligonucleotide and Taq-polymerase was employed for sequencing.

RESULTS

Cloning and purification of C proteins. The aim of the work was to use PCR to prepare the DNA fragment located before the gene of the *Eco*RV restriction endonuclease, to clone the PCR product into an expression vector, to isolate the protein product of the cloned fragment, and to clarify whether the protein binds to DNA and, if so, in what sequence.

However, on analyzing the nucleotide sequence preceding the endonuclease gene [3] we have found in this region two open reading frames (ORF1 and ORF2) of 297 and 225 bp coding 99 and 75 amino acids, respectively (Fig. 1a). Hereinafter the 297-bp fragment will be called the "long" fragment, the 225-bp fragment will be the "short" fragment, and proteins coded by them will be called correspondingly C_{99} and C_{75}. It should be noted that ORF1 and ORF2 terminate at the same point within the endonuclease gene, and thus C_{75} differs from C_{99} only by the presence in the latter of an additional 24 amino acid residues at the N-terminus. Inasmuch as the only isolated product of the *C* gene of the *Pvu*II system has 84 amino acid residues and theoretical size of putative products of *C* gene in other systems varies from 103 to 78 amino acids, we decided to clone both fragments.

Fragments for cloning were prepared by PCR. Natural plasmid pLG74 containing genes of the *Eco*RV modification–restriction system was used as a template DNA. To prepare the long fragment, oligonucleotides 3C2 and 3C10 were used, and oligonucleotides 3C9 and 3C10 were used to prepare the short fragment (Fig. 1b). For subsequent cloning, a site for restriction endonuclease *Xho*I was inserted in oligonucleotide 3C10. Oligonucleotides 3C2 and 3C9 had sites of endonucleases *Bli*736I (isoschizomer *Eco*31I) and *Bsp*LU11I at their ends, respectively. Cleavage of the PCR products with these endonucleases produces ends that are complementary to the protruding ends formed on cleaving the expression vector pET21d by endonuclease *Nco*I. After purification the PCR products were cleaved with corresponding restriction endonucleases and then ligated with plasmid pET21d cleaved by endonucleases *Nco*I and *Xho*I. As a consequence, the PCR fragments were integrated in the vector so that reading frames of the cloned fragments coincided with the frame of six histidines from the vector molecule. Cells of the BL21(DE3) strain were transformed using a ligase mixture. The clones containing recombinant plasmids were selected from colonies by PCR. Finally, two clones were used: one with the long fragment and the other with the short fragment. The correctness of joining the fragments to the vector and the absence of errors during the PCR were confirmed by sequencing the isolated recombinant plasmids.

The selected clones were used for expression. The cell protein products after induction of expression were assayed using electrophoresis in 17% polyacrylamide gel in the presence of SDS. It was found that the clones express proteins with the molecular mass expected for each protein (Fig. 2, lanes *2* and *5*), the induced proteins constituting more than 10% of the cell protein.

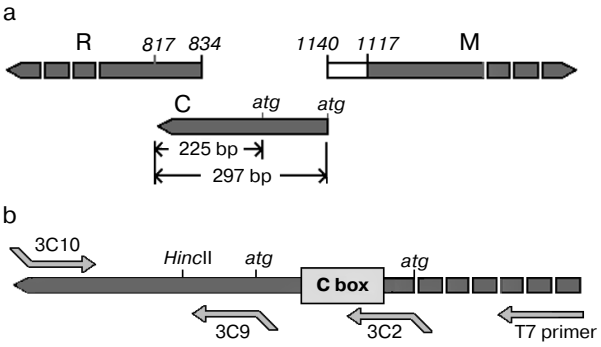

Fig. 1. a) Schematic representation of relative arrangement of genes of the *Eco*RV modification–restriction system according to the data of sequencing [3]: R, endonuclease; M, methylase; C, *C* gene. Numerals in italics, beginnings of the genes. b) Magnified image of *C* gene (solid line) and oligonucleotides used in PCR and sequencing. Dotted line, the sequence adjacent to the cloned *C* gene in recombinant plasmid pET21d. The scale is arbitrary.

Due to the presence at the C-ends of six histidine residues the cloned C proteins were purified to a homogeneous state using affinity chromatography on a Ni-NTA-agarose column. From 2 g of cell biomass containing recombinant plasmids after induction we obtained 10 mg

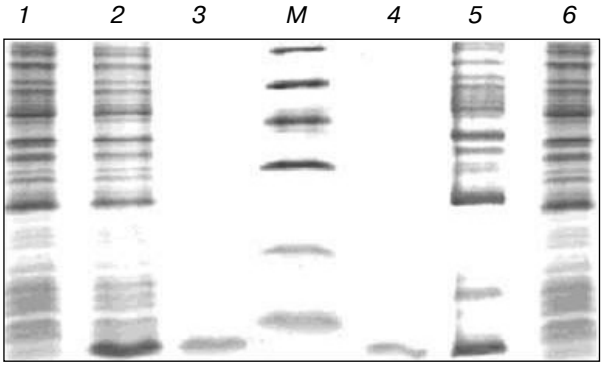

Fig. 2. Isolation of proteins C with Ni-NTA-agarose affinity chromatography. Electrophoresis in 17% polyacrylamide gel in the presence of SDS: *1*) lysate of non-induced cells carrying recombinant plasmid pET21d with an inserted long fragment; *2*) lysate of the same cells after induction; *3*) chromatographically purified protein C_{99}; *4*) chromatographically purified protein C_{75}; *5*) lysate of induced cells carrying recombinant plasmid pET21d with an inserted short fragment; *6*) lysate of the same cells before induction; M) molecular mass markers (95, 67, 43, 30, 20, and 14.5 kD).

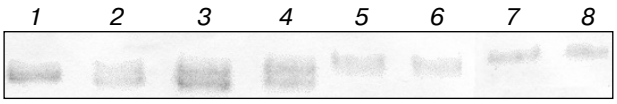

Fig. 3. Radioautograph demonstrating the binding of protein C_{99} to ^{32}P-labeled fragment 3C2–3C10. Electrophoresis in 6% polyacrylamide gel: *1*) initial fragment (0.15 pmol); *2-6*) fragment incubated with 0.08, 0.3, 1.25, 5.0, and 20.0 pmol protein; *7*) labeled fragment (0.15 pmol) that does not contain putative recognition site (control); *8*) the same fragment incubated with 20 pmol protein.

of electrophoretically pure products of each C protein (Fig. 2, lanes *3* and *4*).

Determination of the DNA fragment that binds to C proteins. In performing this part of the work we used the following considerations. First, Tao et al. [12] analyzed amino acid sequences of the products of putative *C* genes of some modification–restriction systems, including the *Eco*RV system, and demonstrated that they contain a helix-turn-helix motif specific for some DNA-binding proteins (e.g., phage λ cI-repressor). Second, it is known that the major part of DNA-binding proteins with this motif form homodimers and recognize on the DNA a sequence with dyad symmetry [13]. And third, it has been shown for C protein of the *Pvu*II system that it does bind to the sequence with such a feature [1] and that this sequence is located between the *C* and methylase genes. Taking the above into account, we analyzed the sequence between *C* and methylase genes. In this region we found a sequence with a dyad symmetry located within the long fragment at a distance of 9 nucleotides from the initial codon.

To confirm that the obtained C proteins bind to this sequence, we used a gel-shift assay: the mobility of the fragment bound to the protein decreases (is shifted) compared to the mobility of the free fragment. To this end, a long ^{32}P-labeled fragment 3C2–3C10 was prepared with PCR using labeled oligonucleotide 3C2 and unlabeled oligonucleotide 3C10. As a control fragment (containing no putative binding site) we used a 340-bp fragment prepared on the phage M13mp19 DNA template employing oligonucleotides C1 (labeled) and B10 (unlabeled) that flank the poly-linker sequence in the DNA. The purified fragments (0.15 pmol) were incubated with a varying amount of each C protein. A series of tests with different contents of $MgCl_2$, KCl, NaCl, and pH of Tris-HCl buffer in them were performed. Mixtures of 5 μl were placed in ice during their preparation till their insertion in 6% polyacrylamide gel.

The results of electrophoresis have shown that the mobility of the fragment is reduced in the presence of C proteins, i.e., the proteins really bind to the long fragment (Fig. 3). The following conditions were shown to be optimal for the binding: 10 mM Tris-HCl (pH 7.5), 10 mM KCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol. However, the difference in the mobilities of the free and bound fragments was very small. This can be explained by the fact that the used fragment has a rather large mass, while on the contrary C proteins have a small mass, as a result of which the masses of the fragment bound to the protein and of the free one differ little from each other. To enhance this difference, the initial labeled fragment was shortened to 200 bp by cleaving it with *Hin*cII restriction endonuclease (Fig. 1b).

The use of a shortened fragment lead to a greater shift in mobility (Fig. 4). It is seen from Fig. 4 that both C proteins bind to the fragment, but the binding of the whole fragment present in the reaction mixture occurs at a different ratio of proteins C_{99} and C_{75} to the fragment: C_{75} binds to the fragment more efficiently.

Thus, the results demonstrated that the isolated C proteins are DNA-binding proteins and the sequence recognized by them is really located in the long fragment.

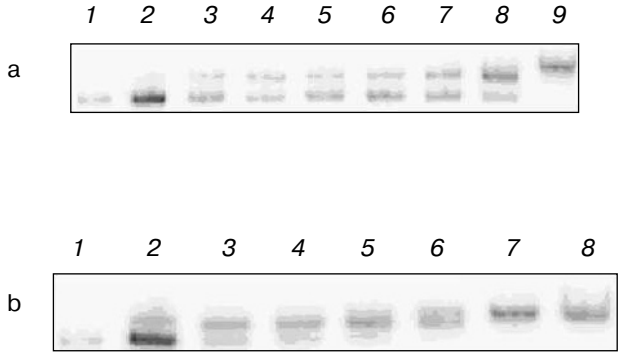

Fig. 4. Binding of proteins C with ^{32}P-labeled fragment 3C2–3C10/Hinc: *1*) initial fragment (0.15 pmol); *2-9*) fragment incubated with 0.001, 0.005, 0.02, 0.08, 0.3, 1.25, 2.5, and 5.0 pmol proteins C_{99} (a) and C_{75} (b).

Determination of the sequence recognized by C proteins. The next stage of our work was determination on the fragment of a certain sequence to which C proteins bind. To solve the task, footprinting was employed using DNAse I. We could not use fragment 3C10–3C2 because the putative recognition site overlaps in part oligonucleotide 3C2. Therefore we used T7-primer oligonucleotide that is complementary to the region located in the vector DNA pET21d instead of oligonucleotide 3C2 (Fig. 1b). Two labeled fragments were prepared by PCR. A labeled oligonucleotide 3C10 and unlabeled T7-primer (^{32}P-3C10–T7) were used for preparation of one fragment, whereas for preparation of the other fragment a labeled T7-primer and unlabeled 3C10 (^{32}P-T7–3C10) were used. Thus we obtained fragments ~400 bp long labeled in different chains. Preliminarily concentrations of DNAse I and the time of fragment incubation with it

were chosen so that each fragment present in the reaction mixture would have one disruption on average. This requirement was satisfied by incubation of 0.5 pmol fragment with 10 μg DNAse I for 1 min. The incubation of the fragments with C proteins was done in the same conditions as for the gel-shift assay. The incubated mixture was then treated with DNAse I under conditions similar to those for treating free fragments.

The results of footprinting are shown in Fig. 5. It is clearly seen that C proteins do bind to the predicted sequence. Regretfully, no good footprinting pattern could be achieved with fragment ^{32}P-3C10–T7 since in this case the recognized sequence is far from the beginning of the fragment (~300 nucleotides). Nevertheless, it is seen that the binding by the other DNA chain also occurs in the region containing the expected site of recognition. Figure 5 shows also that the recorded overlapping region exceeds the dimensions of the putative C box by ~10 nucleotides on each side. First of all this can be explained by the fact that, being a large protein, DNAse I cannot make disruptions in the immediate vicinity of the protein bound to the DNA. Moreover, in our case it is difficult to determine boundaries of the overlapping region due to uneven distribution of the bands along the "sequencing path", which is the result of varying DNAse I cleavage of different DNA sequences, and the fragment used in our experiments is enriched with polydA-blocks. However, the data demonstrate that both proteins bind to the DNA region that includes the CCCATTTTGGGTTATCCCATTTTGGG sequence.

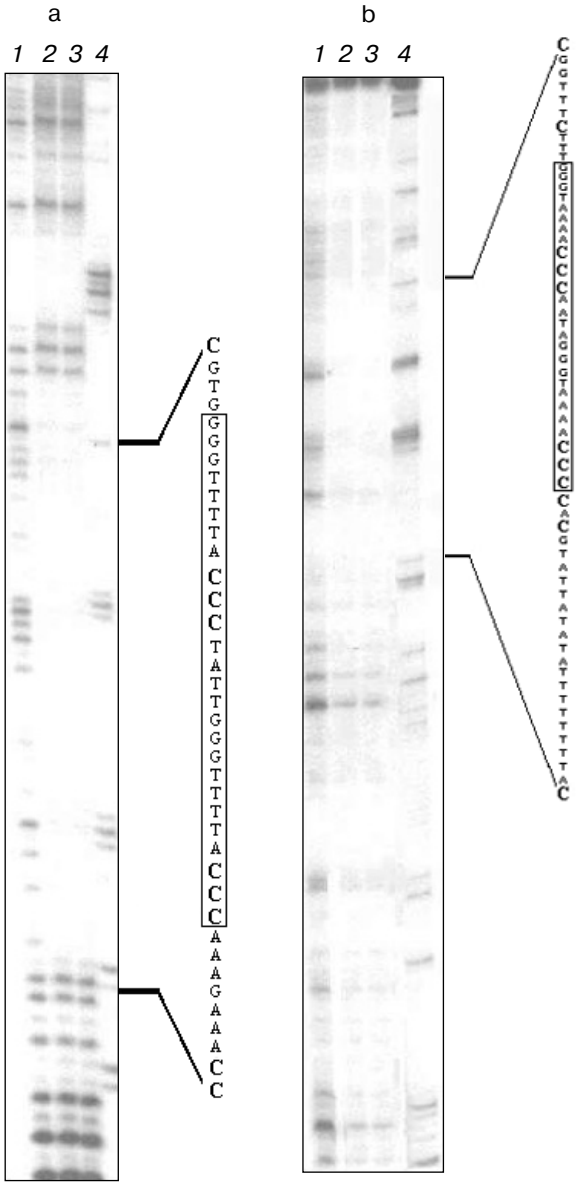

Fig. 5. Determination of the sequence recognized by C proteins with footprinting using labeled fragment ^{32}P-T7–3C10 (a) and labeled fragment ^{32}P-3C10–T7 (b): *1*) initial fragment treated with DNAse I; *2*) fragment incubated with protein C_{99} and then treated with DNAse I; *3*) fragment incubated with protein C_{75} and then treated with DNAse I; *4*) C sequencing of the unlabeled fragment with the use of labeled oligonucleotides—T7-primer (a) and 3C10 (b). Right, nucleotide sequence in the region of the C box; framed, C box.

DISCUSSION

The results as well as the literature data do not allow us to conclude unambiguously which of the two C proteins isolated is expressed in the cell. But some considerations lend support to protein C_{75} expression in the cell. First, the C box determined by us is away from the initial codon of protein C_{75} at the same distance as is experimentally found for the *Pvu*II system (minus 20 bp from the initial codon) [1] and as expected for other systems (approximately minus 40 bp). Second, comparative analysis of amino acid sequences of putative C proteins in the *Eco*RV, *Bam*HI, *Pvu*II, and *Sma*I systems with the amino acid sequence of phage λ cI repressor [12] reveals a quite good coincidence of the sequences in the regions having the helix-turn-helix motif. Protein C_{75} has all these regions, and the additional amino acid sequence in protein C_{99} does not contain this motif. And finally, the expression of protein C_{75} is supported by the fact that the C box is within the fragment coding protein C_{99}. However, mention should be made of the case when the protein-binding site is within the gene encoding this protein. For example, the binding site of protein O that is involved in initiation of phage λ DNA replication is within gene *O* [14].

Nakayama and Kobayashi [15] demonstrated the regulatory role of the *C* gene product in the *Eco*RV restriction–modification system. They introduced a mutation in the plasmid carrying genes of the *Eco*RV system, which lead to a shift of the reading frame in the

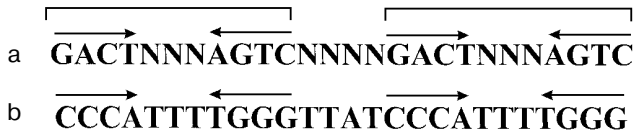

Fig. 6. Sequence of C boxes: a) consensus sequence obtained from comparative analysis of C boxes in *Pvu*II, *Bgl*I, *Bam*HI, *Mun*I, and *Sma*I [1]; b) C box of the *Eco*RV system. Arrows show inverse repeats.

region that presumably encodes C protein (between the endonuclease and methylase genes). It was shown that introduction of such a plasmid in the cells leads to a significant loss by the cells of their ability to restrict the growth of phage λ as compared with the cells containing the plasmid with the undisrupted reading frame. The loss of the ability to restrict the phage λ growth shows that no synthesis of the restriction endonuclease that cleaves the phage DNA occurs in the cells. The ability to restrict the phage growth is restored after incorporation into the cells of a compatible plasmid that carries an intact copy of the *C* gene. It was also shown that mutants in the *C* gene do not lose their ability to methylate DNA of phage λ introduced into the cells. On the basis of these data the authors concluded that the *C* gene is an active positive regulator of endonuclease transcription.

At present the most studied is the regulatory role of the *C* gene product of the *Pvu*II modification–restriction system [1]. For this system it was shown that C.*Pvu*II is a DNA-binding protein that binds to its gene promoter and stimulates transcription of its own gene and the gene of endonuclease as a polycystronic mRNA. The authors determined experimentally the sequence to which protein C.*Pvu*II binds (C box). Within the sequence the authors found symmetry elements and, by comparing the sequences adjacent to the beginnings of *C* genes in other four modification–restriction systems, proposed a consensus sequence of the C box (Fig. 6a).

A comparison of the sequence to which the proteins isolated by us bind with the consensus sequence described in [1] (Fig. 6b), reveals both common features and differences. The similarity of our and the consensus sequences is first of all in their identical length (26 nucleotides) and then in the same symmetry. It should be emphasized that the C box of the *Eco*RV system has practically ideal symmetry: it consists of a tandem repeat of 11-nucleotide sequences separated by four nucleotides; each of the repeated sequences in its turn contains an inverse repeat of the four extreme nucleotides. However, the nucleotide sequence of the C box of the *Eco*RV system differs greatly from the sequence of the consensus C box.

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REFERENCES

1. Vijesurier, R. M., Carlock, L., Blumental, R. M., and Dunbar, J. C. (2000) *J. Bacteriol.*, **182**, 477-487.
2. Ives, C. L., Sohail, A., and Brooks, J. E. (1995) *J. Bacteriol.*, **177**, 6313-6315.
3. Bougueleret, L., Schwarzstein, M., Tsugita, A., and Zabeau, M. (1984) *Nucleic Acids Res.*, **12**, 3659-3676.
4. Matvienko, N. N., Zheleznaya, L. A., and Matvienko, N. I. (1993) *Nucleic Acids Res.*, **21**, 1495-1496.
5. Matvienko, N. N., Kramarov, V. M., Zheleznaya, L. A., and Matvienko, N. I. (1993) *Biochemistry (Moscow)*, **58**, 803-814.
6. Maniatis, T., Fritsch, E., and Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, USA.
7. Dower, W. J., Miller, J. F., and Regsdale, C. W. (1988) *Nucleic Acids Res.*, **16**, 6127-6145.
8. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
9. Revzin, A. (1987) *BioTechniques*, **7**, 346-355.
10. Schmitz, A., and Galas, D. J. (1978) *Nucleic Acids Res.*, **5**, 3157-3170.
11. *fmol DNA Sequencing System Technical Manual* (1995) Promega.
12. Tao, T., Bourne, J. C., and Blumental, R. M. (1991) *J. Bacteriol.*, **173**, 1367-1375.
13. Ptashne, M. (1987) *A Genetic Switch*, Blackwell Scientific Publications and Cell Press.
14. Sanger, F., Coulson, A. R., Hong, G. F., and Petersen, G. B. (1982) *J. Mol. Biol.*, **162**, 729-773.
15. Nakayama, N., and Kobayashi, I. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 6442-6447.