# Molecular Cloning of the Restriction Fragments Derived from Double EcoRI/PstI Digestion of the Calf Satellite I DNA and Their Restriction Analysis

Jan Kłysik, Krystyna Furtak, Grażyna Szymczak, Ewa Bartnik*, Jacek Skowroński, and Henryk Panusz

Department of Biochemistry, Institute of Physiology and Biochemistry, Medical Academy, Lindleya 6, 90-131 Łódź, Poland

Z. Naturforsch. **34 c,** 1151 – 1155 (1979); received September 14, 1979

Calf Satellite I DNA, Restriction, Mapping, Cloning

Calf satellite I DNA digested with EcoRI and PstI gives three fragments 643, 621 and 83 bp long. Two of them, the 643 and 621 bp were cloned using pBR 322 vector and analyzed by means of the MspI and HaeIII restriction enzymes.

## Introduction

Calf satellite DNAs have recently become the subject of an extensive restriction analysis. Among them, the satellite I DNA ($\varrho = 1.715$ gcm$^{-3}$) belongs to the best analyzed ones. Restriction mapping gives information about the internal organization of the repetitive unit (RU). Furthermore, it is an inevitable preliminary step to direct sequence analysis. All the restriction maps of this satellite so far available, were obtained for enzymes having only few susceptible sites along the EcoRI basic repeat unit. Calf satellite I DNA, being a GC-rich chain fragment, should be preferentially hydrolyzed by nucleases, whose restriction sites are rich in G and C. Two restriction nucleases, the HaeIII (GGCC) and MspI (CCGG), among others, should theoretically be able to degrade the satellite I DNA into relatively short fragments. HaeIII, indeed, was proved to act this way [1]. The authors consider their results to prove that a part of the satellite I DNA contains several HaeIII susceptible sites, 12 bp apart from each other. It strongly suggests that a part of the EcoRI basic repeat unit of this satellite has an internal repetitive sequence. This observation adds a lot to the understanding of the internal organization of this satellite. One should stress however, that mapping by means of enzymes having multiple susceptible sites becames an extremly difficult task. We decided to apply the second above mentioned restriction nuclease, the MspI, hoping to obtain more information about the internal organization of the discussed RU.

In addition, MspI appeared to be the only one among several enzymes recognizing the specific sites CCGG, which exerts an equal activity on the methylated as well as on the unmethylated specific sequence [2]. This property allows us to assume the digestion pattern obtained on the whole satellite DNA to be identical with that obtained on cloned fragments. In order to minimize the difficulties in mapping experiments, we decided to subunit to MspI digestion fragments smaller than that obtained by means of EcoRI, namely those obtained by double digestion EcoRI/PstI [3]. Their isolation in sufficient quantities from the direct digest would be a very laborious procedure due to the preparative limitations in obtaining pure satellite I DNA. Therefore, we decided to apply for further digestion fragments cloned by means of the pBR 322 vector. We also digested the same cloned fragments by means of the HaeIII restriction nuclease in order to compare our patterns with those obtained by Kopecka *et al.* [1] by digestion of the whole satellite I DNA.

## Materials and Methods

*Calf satellite I DNA and its EcoRI basis repeat unit.* Both were isolated as described previously [4].

*Restriction enzymes.* The isolation of AluI and EcoRI was described previously [5]. PstI was prepared according to Smith *et al.* [6] with a slight modification in the bacteria disruption step: grinding with alumina was used instead of sonication. The enzyme

* Department of Genetics, Warsaw University, Al. Ujazdowskie 4, 00-478 Warszawa, Poland.

Reprint requests to J. Kłysik.

0341-0382/79/1200-1151 $ 01.00/0

was additionally purified on the heparose column [7]. HindII and MspI were prepared according to Bickle *et al.* HaeIII was the commercial preparation of Miles.

*Cloning of the restriction fragments.* The isolation of pBR 322 plasmid DNA, restriction, ligation and transformation were generally performed as described by Bartnik *et al.* [8]. For cloning of the restriction fragment deriving from the PstI digestion of the EcoRI basic repeat unit and limited by the two PstI sites, the whole EcoRI/PstI satellite I DNA digest was ligated with the pBR 322 plasmid DNA predigested by means of PstI. For cloning of the two remaining fragments limited by the EcoRI and PstI sites, the pBR 322 vector was digested with both those enzymes and ligated with the same EcoRI/PstI satellite I DNA digest.

*Large scale preparation of the restriction fragments.* The pBR 322 plasmid containing a given restriction fragment was isolated from 8 liters of the transformed *E. coli* HB 101 culture, digested with the proper restriction enzyme (or enzymes), and centrifuged in a preparative sucrose gradient (4 – 20%) in the SW 27 rotor of the L 5 – 65 Beckman ultracentrifuge at 23000 rpm for 12 h at 20 °C. Fractions forming the sharp peak of the cloned fragments were pooled, and DNA was precipitated by means of ethanol (1 : 1 by volume) and by cooling the solution in a dry ice/acetone bath. The precipitate was collected by centrifugation for 15 min at 15000 × *g* at 0 °C, dissolved in 0.1 SSC and used for restriction analysis.

*The analysis of the restriction fragments.* It was performed on acrylamide gels as previously described [5]. Gels were calibrated using fragments of λ dvh 93 DNA after HinfI or HpaII digestion. The length of the following fragments: 526, 213, 124, 106, 92 and 87 bp long, was derived from direct sequence analysis.

## Results and Discussion

*Cloning experiments.* The products of the complete PstI digestion of the EcoRI basic RU of the satellite I DNA revealed three electrophoretic bands in accordance with the results of Streeck and Zachau [3]. The bands correspond to: A -a fragment limited by the two PstI sites, 700 bp long, B -a shorter fragment limited by the EcoRI sensitive and by the PstI sensitive sequence, 620 bp long, and C – the shortest fragment, 80 bp long, also limited by EcoRI and PstI sensitive sites. The length values cited are those obtained by Streeck and Zachau [3]. This mixture of the digestion products was used for cloning experiments. In the cloning experiment concerning fragment A we selected 120 tetracycline resistant clones, of which 12 appeared to be ampicillin sensitive. Al-

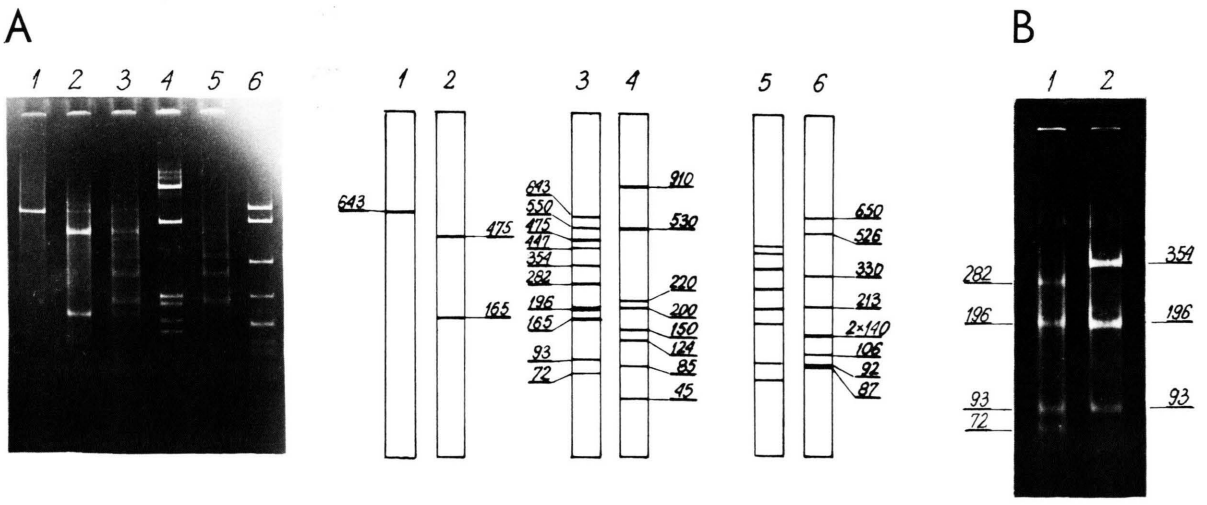


Fig. 1. 8% acrylamide slab gel electrophoresis of the cloned restriction fragment A digested with AluI, MspI or both. A) channel 1, the isolated cloned fragment A; channel 2, the cloned fragment A digested by AluI; channel 3, incomplete AluI/MspI digest of the fragment A; channel 4, DNA standards (λ dvh 93 digested by HpaII); channel 5, the same as 3 after more advanced digestion; channel 6, DNA standards (λ dvh 93 digested by HinfI); B) channel 1, complete AluI/MspI digestion products of the cloned fragment A; channel 2, complete MspI digestion products of the cloned fragment A.

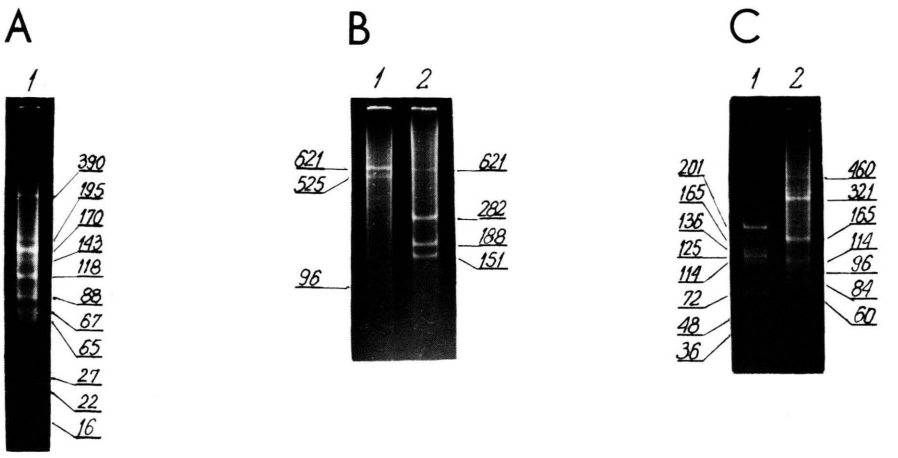


Fig. 2. 8% acrylamide slab gel electrophoresis of the digestion products of the cloned fragment A and B. A) channel 1, MspI digestion products of the cloned fragment B; B) channel 1, AluI digestion products of the cloned fragment B with the undigested fragment added; channel 2, HindII digestion products of the cloned fragment B mixed with the undigested fragment added; C) channel 1, the cloned fragment A digested with HaeIII; channel 2, the cloned fragment B digested with HaeIII.

ready the third investigated amplicillin sensitive clone appeared to contain the pBR 322 vector with the inserted satellite fragment A (Fig. 1 A channel 1). This fragment, as expected, had one AluI susceptible site, 165 bp apart from one of its ends (Fig. 1 A channel 2). In the cloning experiment concerning fragment B and C, we selected 125 tetracycline resistant clones. Most of them appeared ampicillin sensitive. About 50% of those contained the pBR 322 vector with the EcoRI/PstI limited insertions. The results of identification of the satellite fragment B by means of AluI are shown in Fig. 2 B channel 1. In this case the AluI susceptible site is localized 96 bp apart from the PstI-end of the fragment. The clone containing pBR 322 DNA with the inserted third small fragment C has not been found yet. Its screening, more difficult because of the small molecular size, is continued. This fragment lacks MspI susceptible sites (data not shown) and thus has no value for the investigation of the satellite I by means of this enzyme.

*MspI restriction analysis of the cloned fragment A.* The cloned fragment A contains two MspI susceptible sites and its complete digest separates on the acrylamide gel into three bands corresponding to 354, 196 and 93 bp long subfragments (Fig. 1 B channel 2). AluI, known to contain one susceptible site on the fragment A, cleaves the 354 bp MspI subfragment into a 72 and a 282 bp long one (Fig. 1 B, channel 1). This observation is sufficient for arranging the three MspI subfragments in an order 93-354-196 bp and is confirmed by the incomplete MspI/AluI hydrolysis (Fig. 1 A channel 3). The length of the fragment A, directly estimated by comparison with our molecular weight standards, amounts to 637 bp. If calculated by summarizing the lengths of the two AluI digestion products (475 and 165 bp) it gives 640 bp, whereas calculated by summarizing the size of the three MspI fragments (354 + 196 + 93 bp) it equals to 643 bp. We believe that the last value is the most precise estimated. This size is considerably lower than the 700 bp obtained by Streeck and Zachau [3].

*MspI restriction analysis of the cloned fragment B.* The cloned fragment B contains many MspI sensitive sequences and its digest, when submitted to gel electrophoresis, separates into many fragments giving a very complicated electrophoretic pattern (Fig. 2 A, channel 1). It seems to us that, despite using increased concentrations of MspI we did not succeed in obtaining complete hydrolysis of the investigated fragment. We assumed that the intensive bands 170, 118, 88, 67 and 65 bp, as well as the weak bands corresponding to lower molecular weights 27, 22 and 16 bp, represent the final digestion products, and the weak bands corresponding to 390, 195 and 143 bp represent incompletely digested fragments. The value

of the summarized sizes of the final digestion products (573 bp) does not fit with the size of the initial fragment B, having 621 bp in length. The last value was calculated in three ways by comparing with the molecular weight standards the sizes of: 1. the total fragment (620 bp), 2. the two fragments obtained from the AluI digestion (522 + 96 bp) and 3. the three fragments obtained from the HindII digestion (282 + 188 + 151 bp). Our size estimated in this case was in perfect agreement with 620 bp calculated by Streeck and Zachau [3]. The above discussed MspI digestion pattern of the fragment B can be explained only by assuming that some of its digestion products are amplified.

*HaeIII restriction analysis of the cloned fragments A and B.* Kopecka *et al.* [1] stated the existence of the following fragments resulting from the digestion of satellite I DNA by HaeIII restriction nuclease: 36, 48, 60, 72, 84 and 96 bp. Our electrophoretic patterns of HaeIII digests of fragments A and B (Fig. 2C channels 1, 2) revealed all of them. Some of the above sizes, however, were observed as degradation products of the fragment A, namely the 36, 48, 72 bp long ones. The remaining three, 60, 84 and 96 bp, resulted from the digested fragment B. None of them was found in both digestion mixtures at once. Our observation leads to the following conclusions: the 12 bp repeat units claimed by Kopecka *et al.* [1] to be present in the satellite I DNA are not aligned in form of a single block or at least this block is interrupted by one different sequence containing the PstI sensitive site. It is very difficult to explain, why there should appear a typical 12 bp RU in multiple blocks of 3, 4 and 6 in the fragment A and 5, 7 and 8 in the fragment B and how could it loose the sensivity of the intermediate sites. We consider the following possible explanations: 1) there are no real 12 bp long repetitive units of totally repeated sequence but there are structural requirements for that particular HaeIII sensitive one to appear in regular distances such as are observed, 2) the 12 bp long RU do exist in form of at least two blocks, but some structural hindrance (palindromes?, single strand stretches?, mutations?) protects a part of HaeIII sensitive sites from being digested by this enzyme.

*The compiled restriction map of the EcoRI basic repetitive unit.* Fig. 3 shows the correlated restriction map of the basic EcoRI RU, so far obtained in experiments performed in our laboratory. The application of some additional restriction nucleases acting on the cloned fragments of the EcoRI basic RU, leading to fragments of lower molecular weight, allowed us to define more precisely the lengths of the obtained fragments. The localization of the AluI sensitive sites, given in Fig. 3, is more reliable from those given in our previous papers [4, 5], and differs from the data given by Streeck and Zachau [3]. The lengths of the fragments produced by PstI digestion are similar to those obtained by the above cited authors with one exception: the fragment A, estimated by us to be 643 bp long, *i. e.* 57 bp shorter. The short fragment C was found to be 83 instead 80 bp long (data not shown).

HindII digestion products 282 and 188 bp long correspond to the 260 and 180 bp long fragments described by Philippsen *et al.* [9]. Our estimation of the total length of the basic EcoRI RU, obtained by summarizing the lengths of the individual smaller fragments, is 1347 bp, 53 bp less than the value estimated by Streeck and Zachau [3] and Gautier *et al.* [10, 11]. The EcoRI basic RU has been already cloned [10]. If replicated in bacteria, it revealed to gain new susceptibility for some restriction nucleases [11] due to the loss of methylation of cytosine residues in the recognized sequences. This fact caused an appearance of sites sensitive for HpaII. This enzyme is know to recognize the same sequence as MspI but the last one differs by being insensitive for methylation within CCGG. Gautier *et al.* [11] observed at least three CCGG sequences within the cloned EcoRI basic RU. Our results, obtained by means of MspI, revealed the existance of two sites within the fragment A and of at least 7 sites within the fragment B. The 83 bp long fragment C has no MspI susceptible sites.

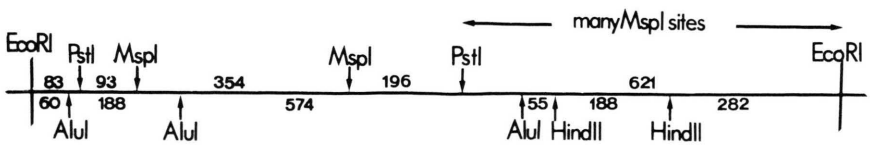


Fig. 3. Restriction map of the EcoRI basic repeat unit of calf satellite I DNA. For details see text.

*Acknowledgements*

The authors wish to express their thanks to Dr. R. J. Roberts for *Providentia stuarti* and *Moraxella sp.* strains, to Dr. N. J. Pieniążek for the heparose resin, to Professor H. Kössel for DNA standards, to Professor K. Taylor for $\lambda$ DNA preparation, to Mrs. S. Wiśniewska for her skillful technical assistance and to Dr. A. Płucienniczak for his interest in this work and for fruitful discussions. This work was supported by the Polish Academy of Sciences within the project 09.7.

[1] H. Kopecka, G. Macaya, J. Cortadas, J.-P. Thiery, and G. Bernardi, Eur. J. Biochem. **84,** 189 – 195 (1978).
[2] C. Waalwijk and R. A. Flavell, Nucleic Acids Res. **5,** 3231 – 3236 (1978).
[3] R. E. Streeck and H. G. Zachau, Eur. J. Biochem. **89,** 267 – 279 (1978).
[4] J. Skowroński, K. Furtak, J. Kłysik, H. Panusz, and A. Płucienniczak, Nucleic Acids Res. **5,** 4077 – 4085 (1978).
[5] J. Kłysik, K. Furtak, J. Skowroński, A. Płucienniczak, and H. Panusz, Bull. Polish Acad. Sci., in press (1978).
[6] D. I. Smith, F. R. Blettner, and J. Davies, Nucleic Acids Res. **3,** 343 – 353 (1976).
[7] T. A. Bickle, V. Pirrotta, and R. Imber, Nucleic Acids Res. **4,** 2561 – 2572 (1977).
[8] E. Bartnik, N. J. Pieniążek, and P. P. Stępień, Molec. Gen. Genet., in press (1979).
[9] P. Philippsen, R. E. Streeck, and H. G. Zachau, Eur. J. Biochem. **57,** 55 – 68 (1975).
[10] F. Gautier, H. Mayer, and W. Goebel, Molec. Gen. Genet. **149,** 23 – 31 (1976).
[11] F. Gautier, H. Bünemann, and L. Grotjahn, Eur. J. Biochem. **80,** 175 – 183 (1977).