Theor Appl Genet (1987) 75:46–50



# The biochemical and cytological characterization of *Vicia faba* DNA by means of MboI, AluI and Bam HI restriction endonucleases

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Received December 24, 1986; Accepted June 25, 1987
Communicated by G. Wenzel

**Summary.** Restriction endonucleases were employed to characterize both cytologically and electrophoretically the DNA of *Vicia faba*. The electrophoretic pattern of total DNA digested with AluI and MboI shows a continuous smear. Bam HI also shows a continuous smear for the bigger polynucleotide fragments and several bands in the lower part of the lane. Digestion of fixed chromosomal DNA produces metaphase longitudinal differentiation when MboI and AluI are used, while no appreciable banding pattern is present when Bam HI is employed. These results are discussed in relation to the organization of chromosomal DNA, to other data in the literature on chromosome banding and on the digestion of total DNA of other species.

**Key words:** *Vicia faba* – Restriction endonucleases – Electrophoretic pattern – Chromosome banding pattern – Cleavage of DNA

## Introduction

Restriction endonucleases, enzymes capable of cleaving DNA at specific base sequence targets, have stimulated molecular biology and particularly the development of genetic engineering during the last decade (for review see Maniatis et al. 1982). These nucleases have also recently been employed on cytological preparations to detect the localization of specific polynucleotide sequences (Mezzanotte et al. 1983a, b; Miller et al. 1983; Mezzanotte and Ferrucci 1984; Bianchi et al. 1985; Mezzanotte 1986; De Stefano et al. 1986; Babu and Verma 1986). Results obtained by digesting human and mouse chromosomes with enzymes such as AluI can be correlated with the localization of satellite DNAs, as can the cytological effect of the same enzyme on polytene chromosomes of *Drosophila melanogaster* with the localization of 5S RNA genes (Mezzanotte et al. 1983a; Mezzanotte and Ferrucci 1984; Mezzanotte 1986). However it has still to be demonstrated whether the cytological effect of restriction enzymes depends only on the presence or absence of specific DNA base sequences in specific chromosomal areas, or also on other parameters such as the chromatin compactness of specific chromosomal areas.

To answer this question, and to verify whether restriction enzymes act on plant chromosomes in the same way as on animal chromosomes, we carried out experiments by cytologically and electrophoretically characterizing the DNA of *Vicia faba* after digestion with either MboI, AluI or Bam HI. *Vicia faba* was chosen because its genome has been widely studied; specific DNA fractions have in fact been isolated in this species and the localization in situ of these DNAs has been correlated with the metaphase longitudinal differentiation produced by a number of banding techniques (Vosa and Marchi 1972; Pignone and Attolico 1980; Bassi et al. 1982; Cionini et al. 1984; Cionini et al. 1985; Natali et al. 1986).

The results we obtained have been discussed in the light of previously known data on *V. faba* and indicate that the DNA base composition of certain chromosomal areas is a necessary but not sufficient condition to explain some cytological effects of restriction endonucleases in this species.

## Materials and methods

### *DNA extraction*

DNA was isolated according to the method of Bendich et al. (1980) modified by Durante et al. (1985). That is, 5 mm long

roots were ground in a mortar and lysed with a solution containing 0.05 M Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.15 M NaCl and 1% sodium dodecylsarcosinate. The lysate was heated at 60 °C for 10 min and preincubated pronase (Sigma) was added to the lysate to a final concentration of 250 μg/ml. The mixture, incubated at 37 °C for 3 h, was centrifuged at 20,000 g for 15 min. Solid CsCl and ethidium bromide from a stock solution (10 mg/ml) were added to the supernatant up to a final concentration of 0.8 g/ml and 300 μg/ml, respectively.

The solution was centrifuged at 44,000 rpm at 20 °C for 20 h in a Beckman L5-65 ultracentrifuge, using a 50 Ti rotor and the DNA band, visualized under a long-wave UV light, was collected and recentrifuged. Ethidium bromide was then removed by gentle inversion of the solution with n-butanol.

*Restriction endonuclease digestion of total DNA*

Restriction endonucleases were purchased from Boehringer and New England Biolabs. Incubation buffers were those suggested by Maniatis et al. (1982). In detail: AluI and Bam HI were dissolved in buffer at medium ionic strength (M) (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, DTT), while MboI was dissolved in high ionic strength buffer (H) (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT). Digestion of total DNA was carried out at 37 °C for 4 h. DNA fragments were separated by electrophoresis at 30 V overnight on horizontal 1% agarose gel in 0.05 M Tris, 20 mM sodium acetate, 18 mM NaCl, 1 mM EDTA, pH 8.0. Gels were stained with 1 μg/ml ethidium bromide solution for 30 min and DNA was visualized by an UV transilluminator and photographed with Polaroid type 55 film. Densitometric tracings were made by a Joyce-Loebel densitometer. DNA fragment size estimation was made by comparing migration distances with those of λ DNA restricted by HindIII and Eco RI.

*Mitotic preparations*

*Vicia faba* seeds were germinated in dump peat moss at a constant temperature of 20 °C. When primary roots reached 1 cm in length, they were incubated for 24 h in distillated water in an ice bath to accumulate metaphases and subsequently excised. The meristematic part was then immersed in 45% acetic acid for 15 min and squashed under coverslips removed after immerging slides in liquid $N_2$. Cytological preparations were allowed to air dry in a desiccator at room temperature.

*Restriction endonucleases digestion on cytological preparations*

MboI, AluI and Bam HI digestion was performed according to Mezzanotte et al. (1983a). Enzyme working solutions were prepared by dissolving 10–20 units of each enzyme in 100 μl of the above-described incubation buffers. Treatment was carried out by placing enzyme solution on the cytological preparations and covering the solution with a clean coverslip to spread the enzyme evenly. Incubation was performed for 16 h at 37 °C.

Control chromosomes were those treated with incubation buffer without the relative enzyme, for the same time and at the same temperature. Standard chromosomes were those that did not undergo any post-fixation treatment. The chromosomes were then stained with Giemsa (2% in deionized water) or with ethidium bromide solution (1 μg/ml in 7 mM $Na_2HPO_4$; 2 mM $NaH_2PO_4$; 180 mM NaCl; 1 mM $Na_2EDTA$) for 10 min at room temperature.

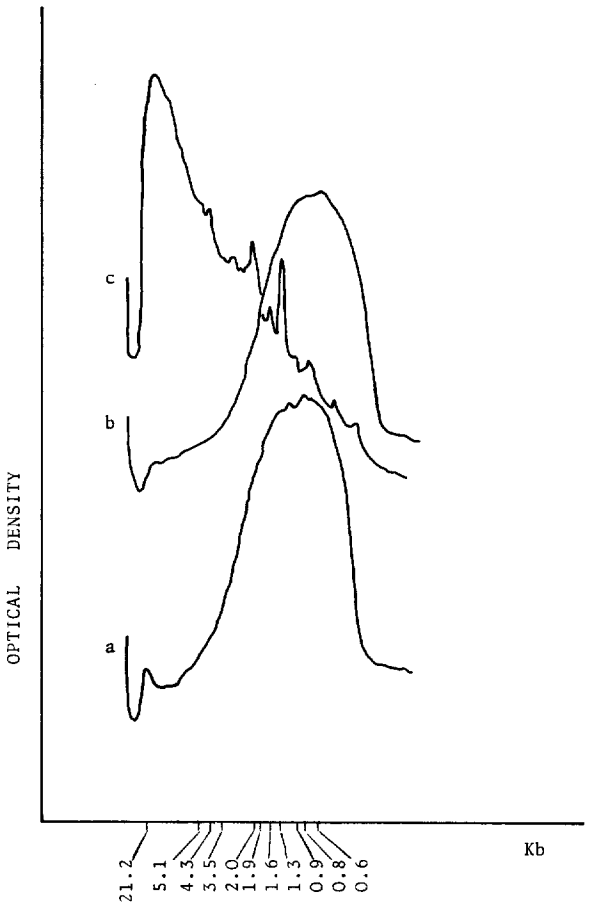


**Fig. 1.** Densitometric tracing of total *Vicia faba* DNA digested with AluI (*a*), MboI (*b*) and Bam HI (*c*)

## Results

*Restriction endonuclease digestion of purified DNA*

The electrophoretic pattern of total DNA digested with AluI showed a continuous smear produced by polynucleotide fragments whose length varies from 3,480 to less than 250 bp. No discrete DNA fraction produced the characteristic arithmetic series of bands that would imply digestion of tandemly linked polynucleotide sequences (see Southern 1975). The same result was obtained when electrophoresis was carried out on DNA digested with MboI. We stress that AluI and MboI are four base cutters whose restriction targets are AG ↓ CT and ↓ GATC, respectively. The six base cutter Bam HI (G ↓ GATCC) also showed continuous smear for the bigger polynucleotide fragments and a ladder made of an arithmetic series of bands in the lower part of the lane. Our results agree with the data previously reported by Kato et al. (1985) (Fig. 1).

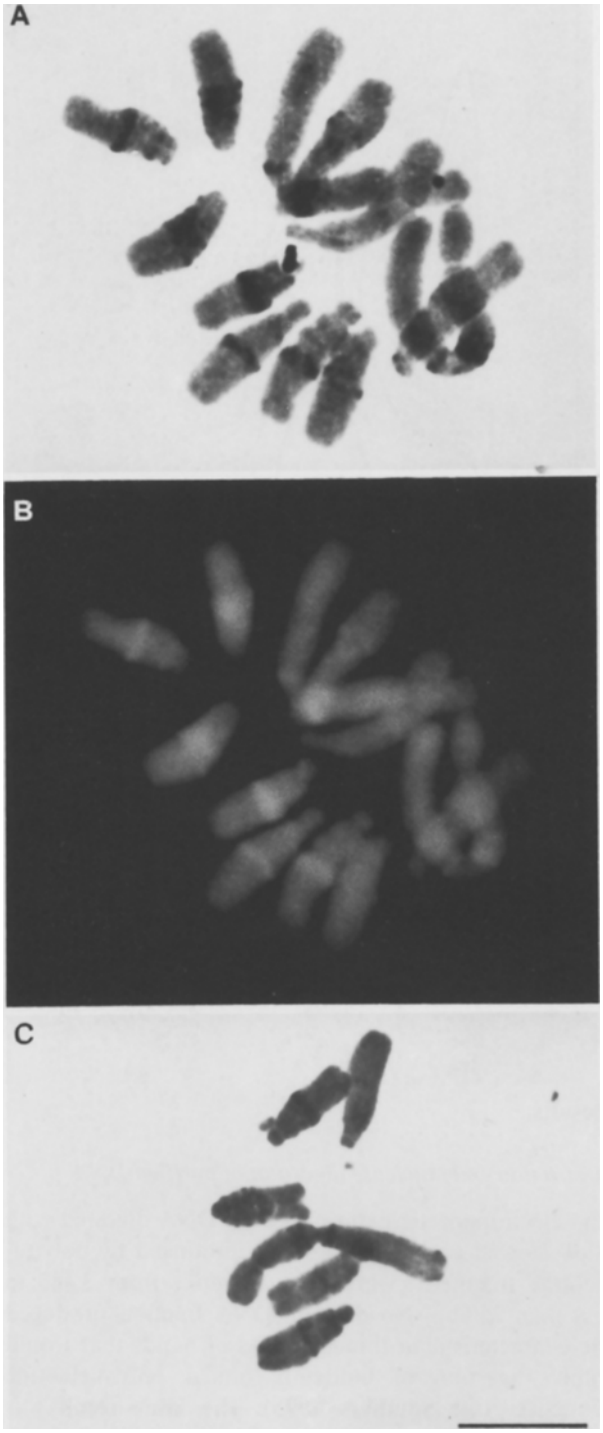


**Fig. 2A–C.** There cytological effect of MboI (**A, B**) and AluI (**C**) digestion of *V. faba* fixed metaphase chromosomes. The preparations were stained with Giemsa except in **C** where ethidium bromide was used after Giemsa staining. Bar = 10 μm

*Restriction endonuclease digestion of cytological preparations*

Digestion of fixed chromosome DNA produced metaphase longitudinal differentiation when MboI or AluI were employed, while no appreciable banding was present in chromosomes incubated with Bam HI. As we termed *V. faba* chromosomes according to Pignone and Attolico (1980), it is interesting that the banding pattern induced by MboI was about the same as that induced by AluI; that is, two paracentromeric bands were present on the satellited arm of chromosome 1, while the same chromosome showed one band on the long arm. Chromosome 2 revealed one thick band and one thin band at the proximal half and next to the centromere of the long arm. Two faint bands were present in the central area of the long arm of chromosome 3, while a thick band was shown on the long arm of chromosome 4. Two faint bands were also present in the central zone of the long arm of chromosome 5, while a very fine pattern of bands, at least four bands partially fused to each other, was present in the long arm of chromosome 6. These results were identical when the chromosomes were stained with either Giemsa or ethidium bromide (Fig. 2A–C).

## Discussion

The digestion of total DNA of a given species with a specific restriction endonuclease results in the cleavage of the DNA into fragments whose sizes depend on the distribution of the restriction enzyme target within the polynucleotide structure. Electrophoretic analysis shows a pool of DNA fragments of variable size which migrate in the gel, producing a characteristic smear or a particular banding pattern. Repeated DNAs may be an exception to this rule. The result of restriction nuclease digestion of a repetitive DNA depends on the periodical distribution of the restriction base sequence target spaced along the repeated polynucleotide structure. For instance, Eco RII digestion of mouse satellite DNA produces a characteristic electrophoretic pattern showing a ladder made of an arithmetic series of bands reflecting the periodic distribution of a repetitive structure formed by a 220 bp monomeric subunit (Southern 1975). A highly repeated DNA that does not contain the base sequence target of a specific endonuclease would result completely unattacked by such an enzyme.

If restriction endonucleases act coherently on the DNA of fixed chromosomes, as compared to naked DNA, one would expect that digestion in situ followed by staining with DNA-specific dyes would produce chromosome longitudinal differentiation whose alternating faint and intense staining reflects the cleavage

and extraction, or the failure to cleave and extract, chromosomal DNA. It is important to stress that removing DNA from fixed chromosomes depends strictly on the length of the fragments cleaved by a specific nuclease (Miller et al. 1983) and that 350 bp fragments should be extracted and 450 bp fragments not (Mezzanotte 1986).

Out biochemical data indicate that the DNA of *V. faba* is attacked to a limited extent by Bam HI and that the size of the fragments produced by digestion with this enzyme should result in little DNA loss from fixed chromosomes of this species. Unattacked chromosomes in Bam HI digested cytological preparations are thus coherent with biochemical data and confirm that the size of DNA fragments cleaved is a critical factor accounting for the effect of restriction enzymes on fixed chromosomes. On the other hand, the activity of MboI and AluI restriction endonucleases on purified DNA compared to the activity on the DNA of fixed chromosomes of *V. faba* merits special attention. In fact, one would expect some homogeneous class of MboI/AluI-resistant DNA to be localized in the chromosomal bands produced by these enzymes, while no homogeneous DNA fraction, resistant to MboI or AluI, is present in the electrophoretic pattern found after digestion of *V. faba* DNA by these enzymes.

It is still debatable whether DNA base composition is the unique parameter that accounts for the effect of restriction endonucleases on fixed chromosomes. Other factors, such as the peculiar structure of certain chromosome regions, specifically areas such as C-bands, believed to correspond to constitutive heterochromatin (Arrighi and Hsu 1971), may prevent enzyme activity. Thus, it seems noteworthy that a large amount of DNA is not attacked by certain endonucleases when it is part of fixed mitotic chromosomes, but is apparently digested when the treatment is performed on naked DNA (Mezzanotte et al. 1985 b).

MboI and AluI are enzymes known to act on fixed human and mouse chromosomes regardless of the organizational compactness of specific chromosome areas. For instance, mouse Y chromosome and certain C-band areas of human chromosomes are extensively digested by either MboI or AluI, in spite of being regions of constitutive heterochromatin (Mezzanotte et al. 1983 a, b; Miller et al. 1983; Mezzanotte and Ferrucci 1984; Mezzanotte et al. 1985 a). Moreover, the C-band, paracentromeric areas of human chromosomes 1, 9 and 16 are strongly attacked by HinfI, so as to appear as unstained gaps (Bianchi et al. 1985). If one considers that these areas represent the major sites of human satellite DNAs II, III and IV and that these are cleaved by HinfI into fragments of 10–250 bp, it follows that (1) certain restriction enzymes act coherently on purified DNA and on the DNA of fixed chromosomes and (2) at least some restriction enzymes act regardless of the structural characteristics of specific areas of fixed chromosomes. It is noteworthy that the secondary constriction of *V. faba* chromosome 1, where the nucleolar organizer is located (Matsui 1974) shows a C-band positive reaction but is digested by either MboI or AluI. This further suggests that chromatin compactness is not an insurmountable impediment to nuclease attack. It is important to stress that *V. faba* chromosomes show small C-bands corresponding to the bright quinacrine bands only in the paracentromeric regions and thick C-bands in the central areas of the long arm of most chromosomes where no bright quinacrine fluorescence is present. On the other hand, quinacrine bright areas correspond to the cytological labelling obtained by using nick-translated AT-rich satellite DNA of *V. faba* as a radioactive probe for in situ hybridization experiments (Cionini et al. 1985), according to the hypothesized role of AT-rich polynucleotides in determining quinacrine fluorescence enhancement (Weisblum and De Haseth 1972). On this basis, one could partially explain our findings by hypothesizing that MboI/AluI bands, coinciding with quinacrine bands, are due to the impossibility of cleaving satellite DNA that does not contain the base sequence targets of these two enzymes (MboI: ↓ GATC; AluI: AG ↓ CT). However, this contradicts the electrophoretic analysis of *V. faba* DNA showing that no discrete DNA fraction is resistant to MboI or AluI digestion, this making DNA extraction impossible. Our findings would be explained in terms of DNA base composition, by some highly repetitive DNA, previously not detected by ultracentrifugation due to its cryptic base sequence composition which is neither AT- nor GC-rich. Such a DNA would be localized in the MboI/AluI band regions and its structure would contain, within the highly repeated sequences, non-repetitive linker sequences which, randomly interspersed, would contain either MboI or AluI base sequence targets. This would account for the cleavage of a highly repetitive DNA into fragments of different sizes; it would also explain our failure to find by electrophoresis a discrete DNA fraction which is not attacked or is attacked only to a very small extent by either MboI or AluI.

On the other hand, it is known that constitutive heterochromatin, specifically the centromeric heterochromatin, is a particular type of chromatin and that different classes of chromatin may react differently to nuclease digestion. For instance, Sahasrabuddhe et al. (1978) have observed that "there may be a general correlation between the packaging order and the digestibility of chromatin" after using DNase I. Rattner et al. (1978) and Lica and Hamkalo (1983) have reported that the C-band, centromeric regions of mouse are specifically resistant to digestion with DNase I or

Eco RI or AluI. It is noteworthy that MboI/AluI incubation induces metaphase differentiation very similar to the C-banding pattern in *V. faba* chromosomes (for comparison, see Vosa and Marchi 1972; Pignone and Attolico 1980). It is thus possible that the structural organization of *V. faba* C-band regions, regardless of the presence of highly repetitive DNAs, represents an impediment to MboI/AluI activity. In fact, although Hadlaczky et al. (1982) have reported that "the higher order organization of plant and animal chromosomes is similar even if not the same", it is possible that the structure of specific chromosome regions (i.e. heterochromatic C-bands) is somehow different in the two cases, thus justifying our findings.

*Acknowledgement.* The present study was financially supported by the "Ministero della Pubblica Istruzione" Roma.

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