Changes in DNA methylation during development in the B chromosome NOR of the grasshopper *Eyprepocnemis plorans*

M. D. LÓPEZ-LEÓN, J. CABRERO & J. P. M. CAMACHO*

Departamento de Genética, Facultad de Ciencias, Universidad de Granada, E-18071 Granada, Spain

Chromosomal patterns of DNA methylation were analysed by *in situ* digestion with restriction endonucleases in different stages of the life cycle in the grasshopper *Eyprepocnemis plorans*: 10-day-old embryos, last instar male nymphs and adults of both sexes. The results show that the nucleolus organizing region (NOR) located distally on the B chromosome is not methylated in embryos or in gastric caeca of adult males and females, but it is methylated in ovariole cells and spermatocytes in both last instar nymphs and adults. Demethylation of the B-NOR during early embryogenesis is proposed for the observed methylation pattern in embryos. Despite variation in methylation status through the life cycle, rRNA genes contained in the B-NOR were never observed to be active, which indicates that DNA methylation is not the direct cause of their inactivity. Other causes such as chromatin structure or repression by genes on A chromosomes are suggested.

Keywords: B chromosome, chromosome methylation pattern, *Eyprepocnemis plorans*, *in situ* digestion, *in situ* hybridization, rDNA.

Introduction

Increasing evidence has been accumulated in recent years for a role of methylation of cytosines within CpG dinucleotides in the regulation of gene expression in eukaryotes (Razin & Riggs, 1980; Bird, 1986; Cedar, 1988; Razin & Cedar, 1991; Yeivin & Razin, 1993). This has become apparent, for instance, from the usual correlation between expression and under-methylation (Chapman *et al.*, 1984; Ariel *et al.*, 1991) and from changes in methylation patterns during development (Frank *et al.*, 1991; Shemer *et al.*, 1991; Fronk *et al.*, 1992).

The differential sensitivity to methylation shown by certain restriction endonuclease isoschizomers (e.g. *MspI* and *HpaII*) has become a powerful tool for analysing methylation patterns not only at a molecular level but at a chromosomal level too. From the introduction of cytological techniques for *in situ* digestion of chromosomes with restriction endonucleases (Kaelbling *et al.*, 1984) extensive use has been made of this new approach to induce chromosome banding in eukaryotic chromosomes (see López-Fernández *et al.*, 1991, for review) and to analyse DNA methylation

patterns at the chromosome level (Bianchi *et al.*, 1986; López-Fernández *et al.*, 1989; Prantera & Ferraro, 1990; López-León *et al.*, 1991; Loebel & Johnston, 1993; Suja *et al.*, 1993).

From the first demonstration that DNA methylation may regulate the activity of the rRNA genes in a rat hepatoma cell line (Tantravahi *et al.*, 1981), the phenomenon has been extended to other organisms such as, for instance, peas (Watson *et al.*, 1987), wheat (Flavell *et al.*, 1988), frogs (Ruiz & Brison, 1989) and mice (Suzuki *et al.*, 1992). Interestingly, DNA methylation also seems to be involved in the inactivation of extra rRNA genes contained in a supernumerary chromosome segment that has arisen from amplification of a nucleolus organiser region (NOR) in the grasshopper *Pyrgomorpha conica* (Suja *et al.*, 1993).

The grasshopper *Eyprepocnemis plorans* shows an extensive polymorphism for B chromosomes in natural populations along the Spanish southern Atlantic and Mediterranean coasts (Camacho *et al.*, 1980; Henriques-Gil *et al.*, 1984) characterized by the existence of more than 40 different B variants (Henriques-Gil & Arana, 1990; López-León *et al.*, 1993) and the absence of drive for the three more abundant B-types (López-León *et al.*, 1992). The observation of a male carrying the B₂ chromosome fused to the largest auto-

*Correspondence.

some and showing the presence of an additional active NOR distally located on the B itself, suggested the presence of rRNA genes on this B chromosome that are usually inactive but had been reactivated by the chromosomal rearrangement (Cabrero *et al.*, 1987). Additional studies showed that the distal third of the B₂ chromosome (where the latent NOR is located) was methylated in normal nonfused Bs that lack NOR activity, but it was not in the fused B that showed NOR activity (López-León *et al.*, 1991), thus showing the typical methylation-inactivity correlation.

Since the results of Cabrero *et al.* (1987) and López-León *et al.* (1991) were observed in spermatocytes, and given the dependence of DNA methylation on cell type and developmental stage (see above), we planned the present work in order to analyse methylation patterns on the B₂ chromosome in embryos and different adult tissues. This is, to our knowledge, the first attempt at the chromosomal level to ascertain the developmental regulation of gene expression by means of DNA methylation.

Materials and methods

Adult males and females of *Eyprepocnemis plorans* were collected in Salobreña on the coast of Granada (Spain) and reared as heterosexual pairs in culture laboratory cages in order to obtain embryo offspring following the technique described in López-León *et al.* (1992). Females were injected with 0.01 mL 0.05 per cent colchicine in insect saline solution for 6 h prior to fixation of ovariole and gastric caecae in 3:1 ethanol:acetic acid. Testes were fixed before a colchicine pretreatment, similar to that in females, in order to fix male gastric caeca (Viseras *et al.*, 1990). Egg-pods were incubated for 10 days at 28°C and embryos were dissected out of the eggs and immersed in 1 mL of 0.05 per cent colchicine in insect saline solution for 1 h. One mL of distilled water was then added for hypotonic shock for 10 min and then the embryos were fixed in 3:1 ethanol:acetic acid. All fixed materials were stored in a refrigerator at about 4°C. In addition, several egg-pods were incubated to term in order to obtain adult offspring, of which several individuals were fixed at the last instar nymph stage. Chromosomal preparations of adult tissues (testes and gastric caeca in males, and ovarioles and gastric caeca in females) were made by squashing in 50 per cent acetic acid and subsequently removing coverslips after freezing the preparations in liquid N₂. Chromosomal preparations of embryos were obtained by the method described in Camacho *et al.* (1991). NOR activity was analysed by the silver impregnation technique of Rufas *et al.* (1982). During male meiosis, this technique reveals the nucleoli

attached to the active NORs during prophase I. In adult somatic and embryonic tissues, however, NOR activity has to be analysed in early mitotic prophase where chromosome identification is more difficult, because the nucleoli are not visible in later stages. For this reason, sequential silver impregnation and C-banding were performed to facilitate the identification of chromosomes showing active NORs, by means of the technique described in Pardo *et al.* (1993). Chromosomal methylation patterns were determined by *in situ* digestion with the *Msp*I and *Hpa*II restriction endonucleases, following the methods described in Sentís *et al.* (1989) and López-León *et al.* (1991). *In situ* hybridization with a ribosomal DNA probe was performed following the technique described in López-León *et al.* (1994).

Results

In situ hybridization showed that the B chromosome possesses, located in its distal third, the largest cluster of ribosomal RNA genes in the complement (Fig. 1), including those in chromosomes 9, 10, 11 and X, which are usually the only active NORs in testes (Cabrero *et al.*, 1987), and minor usually inactive rRNA gene clusters in centromeric regions of the remaining chromosomes.

Fig. 1 Mitotic metaphase cell from an *Eyprepocnemis plorans* embryo after fluorescence *in situ* hybridization with an rDNA probe. Note that the distal third of the B chromosome harbours the largest rRNA gene cluster in the complement.

The analysis of chromosome methylation patterns in embryos showed that the B-NOR possesses abundant CCGG targets since it is clearly digested by *Msp*I (Fig. 2a). The fact that this chromosome region was also digested by *Hpa*II (Fig. 2b) demonstrated that the B-NOR is not methylated in embryo cells. However, sequential silver impregnation and C-banding of early mitotic prophase cells showed that the B-NOR is inactive in 10-day-old embryos (Fig. 2c). Methylation and activity patterns of the B-NOR were the same in embryos that had inherited the B chromosome from the mother and those that had inherited it from the father.

A similar result was obtained in a somatic tissue (gastric caeca) in last instar nymph and adult individuals of both sexes: the B-NOR was digested by *Msp*I and *Hpa*II suggesting its under-methylation, and

sequential silver impregnation and C-banding showed that this NOR is not active in these cells (Fig. 3a).

In gonads, however, the result was different. In spermatocytes (Fig. 3b) and ovariole wall cells (Fig. 3c) (the first being germ cells but the second being somatic cells) the B-NOR was digested by *Msp*I but not by *Hpa*II thus indicating high levels of DNA methylation. However, the B-NOR was not active in either type of cell.

The B chromosome shows a relatively high frequency of spontaneous translocations (always centric fusions) with A chromosomes, so that several cases have already been described (Henriques-Gil *et al.*, 1983; Cabrero *et al.*, 1987). In fact, in populations with a high frequency of B chromosomes, e.g. Salobreña, in almost every year there appears one or more individuals carrying a new centric fusion (López-

Fig. 2 Mitotic metaphase (a and b) and prophase (c) cells from *Eyprepocnemis plorans* embryos submitted to *in situ* digestion with *Msp*I (a) and *Hpa*II (b) restriction endonucleases and to the double silver impregnation and C-banding technique (c). Observe in (a) and (b) that the distal third of the B chromosome is differentially digested by both enzymes thus suggesting that the B-rRNA genes are not methylated. Note in (c) that the two B chromosomes lack a nucleolus (nu) associated with their distal third, which demonstrates that their rRNA genes are not active in this life cycle stage.

Fig. 3 Selected B chromosomes from *Eyprepocnemis plorans* adults, treated with *Msp*I (1), *Hpa*II (2) and silver impregnation (3 and 4). In gastric caeca mitotic cells from both males and females, the B chromosome region harbouring rRNA genes (the distal third) is digested by both enzymes (1a and 2a) but does not show nucleolar activity (3a). In spermatocytes at diplotene and ovariole wall mitotic cells, this B chromosome region is digested by *Msp*I (1b and 1c) but not by *Hpa*II (2b and 2c) and does not show signs of nucleolar activity (3b and 3c). In diplotene spermatocytes from an exceptional male showing a spontaneous centric fusion of the B chromosome and the longest autosome (chromosome 1) (reported in Cabrero *et al.*, 1987), the distal third of the B chromosome was digested by both *Msp*I (1d) and *Hpa*II (2d), and showed a prominent nucleolus (nu) in about half of the primary spermatocytes at diplotene (3d) and the spermatogonial cells at mitotic prophase (4d). In a female carrying a spontaneous centric fusion between the B chromosome and the smallest autosome (chromosome 11), the distal region of the B in mitotic metaphase ovariole wall cells was digested by both enzymes (1e and 2e) thus suggesting absence of DNA methylation, but sequential silver impregnation and C-banding in prophase cells showed absence of nucleolar activity (3e).

León *et al.*, unpublished). The case of the B chromosome fused to the largest autosome described in Cabrero *et al.* (1987) was very interesting because this chromosome rearrangement seemed to cause the reactivation of the B-NOR in spermatocytes and it was paralleled by a low level of DNA methylation (López-León *et al.*, 1991). To illustrate this case, we have included here Fig. 3d which shows B-NOR digestion with both *Msp*I and *Hpa*II and a nucleolus attached to the distal part of the fused B.

We have recently had the opportunity to analyse a female that carried a B chromosome fused to the smallest autosome, the NOR-carrying chromosome 11. The results for this fused B were different from the former in that, although it was digested by both restriction endonucleases (thus showing low methylation level), the B-NOR was always inactive, which

contrasted with the activity of the standard NOR in chromosome 11 (Fig. 3e).

Discussion

Changes in DNA methylation level during embryogenesis have been observed for repetitive DNA (Chapman *et al.*, 1984) and for both tissue-specific and housekeeping genes (Cedar & Razin, 1990). Molecular techniques have also shown the involvement of DNA methylation in regulating the activity of rRNA genes in rats (Tantravahi *et al.*, 1981), mice (Bird *et al.*, 1981), peas (Watson *et al.*, 1987), wheat (Flavell *et al.*, 1988) and frogs (Ruiz & Brison, 1989). In two of these cases a relationship was observed between the level of methylation of rRNA genes and the developmental stage (Watson *et al.*, 1987) or age (Ruiz & Brison, 1989).

The present results demonstrate that the NOR distally located on the B₂ chromosome of *E. plorans* is not methylated in 10-day-old embryos (irrespective of whether it has been transmitted via male or female), at a level detectable by chromosome *in situ* digestion with *MspI*/*HpaII* restriction endonucleases. This indicates that the B-NOR is demethylated during early embryonic stages prior to the tenth day of development, a fact that has been reported in mice by means of molecular techniques (Frank *et al.*, 1991; Shemer *et al.*, 1991). Since methylation is clearly apparent in gonadal tissues in both sexes but not in gastric caeca, the B-NOR is methylated *de novo* during gonadal development.

Despite the observed changes in the level of methylation for the B-NOR in *E. plorans*, they were not strictly correlated with gene expression, because B-rRNA genes were always inactive irrespective of their methylation status. The only exception was observed in a B fused to the longest autosome that was not methylated and formed a nucleolus in about half of the diplotene cells (López-León *et al.*, 1991). In the cells analysed in the present work belonging to embryos and somatic tissues from adults, the B-NOR was not methylated but inactive. This indicates that methylation is not the direct cause of the repression of the rRNA genes carried by the B chromosome.

Similar hypomethylation in both active and non-active tissues has been reported for some genes in vertebrates (see Yeivin & Razin, 1993, for review). Likewise, no methylation causes the decrease in mRNA and protein synthesis occurring in chloroplasts during leaf growth and senescence (Tomás *et al.*, 1992). A direct correlation between methylation and expression has been reported even for the *MGMT* gene in humans (Pieper *et al.*, 1991; Wang *et al.*, 1992). The existence of other factors besides methylation for regulation of the level of expression and switching of the human globin genes has recently been suggested by Loo & Cauchi (1992). In this context, several recent results suggest that inhibition of transcription by DNA methylation is an indirect effect (Boyes & Bird, 1991) and that chromatin structure is a dominant determinant in maintaining the inactive state of genes (Weih *et al.*, 1991).

In the case of *E. plorans*, the existence of low levels of methylation in the promoter regions of rRNA genes in the B, that would be undetectable by the present chromosomal analysis, cannot be ruled out as a cause of the B-NOR inactivity. However, this does not explain why the apparently unmethylated fused B reported in López-León *et al.* (1991) showed NOR activity in certain diplotene cells but not in others, unless differential promoter methylation occurs between diplotene cells, and this is unlikely. Further-

more, the fact that the NORs on the standard chromosomes 9, 10, 11 and X were active in less than 100 per cent of diplotene cells (Cabrero *et al.*, 1987) indicates that the activity pattern was rather similar in standard and B-NORs of that individual, and suggests the possibility that rRNA genes in this fused B were submitted to the same control mechanisms operating on the remaining rRNA gene clusters.

The B chromosome when fused to other autosomes, e.g. the smallest one, also seems to escape from *de novo* methylation, but it never shows NOR activity in either spermatocytes (P. Arana, personal communication) or ovariole cells (this paper). Thus, DNA methylation is unlikely to be the only cause of the inactivity of the B-NOR.

Although it is necessary to study additional cases of Bs fused to other standard chromosomes before reaching a definitive conclusion, the present data suggest that the nonmethylation of the B-NOR in fused Bs and its reactivation are separate matters. Two possibilities to test in future work are (i) that different B fusions produce different changes in the chromatin structure of the B-NOR so that only some of them facilitate its expression, and (ii) that the longest autosome contains a gene(s) close to the centromere that is involved in the general regulation of NOR expression (presumably repressing the B-NOR) and that this gene is affected by the presence of the fused B, which is heterochromatic, by means of a position effect.

Acknowledgements

We thank Dr. P. Heslop-Harrison for facilities to perform *in situ* hybridization in his laboratory, Dr. M. Ruiz-Rejón for useful comments, and an anonymous referee for English corrections. This study was partially supported by grants from the Dirección General Científica y Técnica (no. PB90-0865) and the Plan Andaluz de Investigación, Grupo no. 3122 (Spain).

References

- ARIEL, M., MCCARREY, J. AND CEDAR, H. 1991. Methylation patterns of testis-specific genes. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 2317–2321.
- BIANCHI, N. O., VIDAL-RIOJA, E. L. AND CLEAVER, J. E. 1986. Direct visualization of the sites of DNA methylation in human and mosquito chromosomes. *Chromosoma*, **94**, 362–366.
- BIRD, A. P. 1986. CpG-rich islands and the function of DNA methylation. *Nature*, **321**, 209–213.
- BIRD, A. P., TAGGART, M. H. AND GEHRING, C. A. 1981. Methylated and unmethylated ribosomal RNA genes in the mouse. *J. Mol. Biol.*, **152**, 1–17.

- BOYES, J. AND BIRD, A. P. 1991. DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. *Cell*, **64**, 1123-1134.
- CABRERO, J., ALCHÉ, J. D. AND CAMACHO, J. P. M. 1987. Effects of B chromosomes on the activity of nucleolar organizer regions in the grasshopper *Eyprepocnemis plorans*: activation of a latent nuclear organizer region on a B chromosome fused to an autosome. *Genome*, **29**, 116-121.
- CAMACHO, J. P. M., CARBALLO, A. R. AND CABRERO, J. 1980. The B-chromosome system of the grasshopper *Eyprepocnemis plorans* subsp. *plorans* (Charpentier). *Chromosoma*, **80**, 163-176.
- CAMACHO, J. P. M., CABRERO, J., VISERAS, E., LÓPEZ-LEÓN, M. D., NAVAS-CASTILLO, J. AND ALCHÉ, J. D. 1991. G-banding in two species of grasshoppers and its relationship to C, N and fluorescence banding techniques. *Genome*, **34**, 638-643.
- CEDAR, H. 1988. DNA methylation and gene activity. *Cell*, **53**, 3-4.
- CEDAR, H. AND RAZIN, A. 1990. DNA methylation and development. *Biochim. Biophys. Acta*, **1049**, 1-8.
- CHAPMAN, V., FORRESTER, L., SANFORD, J., HASTIE, N. AND ROSSANT, J. 1984. Cell lineage-specific undermethylation of mouse repetitive DNA. *Nature*, **307**, 284-286.
- FLAVELL, R. B., O'DELL, M. AND THOMPSON, W. F. 1988. Regulation of cytosine methylation in ribosomal DNA and nucleolus organizer expression in wheat. *J. Mol. Biol.*, **204**, 523-534.
- FRANK, D., KESHET, I., SHANI, M., LEVINE, A., RAZIN, A. AND CEDAR, H. 1991. Demethylation of CpG islands in embryonic cells. *Nature*, **351**, 239-241.
- FRONK, J., TANK, G. A. AND LANGMORE, J. P. 1992. DNA methylation pattern changes during development of a sea urchin. *Biochem. J.*, **283**, 751-753.
- HENRIQUES-GIL, N. AND ARANA, P. 1990. Origin and substitution of B chromosomes in the grasshopper *Eyprepocnemis plorans*. *Evolution*, **44**, 747-753.
- HENRIQUES-GIL, N., ARANA, P. AND SANTOS, J. L. 1983. Spontaneous translocations between B chromosomes and the normal complement in the grasshopper *Eyprepocnemis plorans*. *Chromosoma*, **88**, 145-148.
- HENRIQUES-GIL, N., SANTOS, J. L. AND ARANA, P. 1984. Evolution of a complex B-chromosome polymorphism in the grasshopper *Eyprepocnemis plorans*. *Chromosoma*, **89**, 290-293.
- KAELBLING, M., MILLER, D. A. AND MILLER, O. J. 1984. Restriction enzyme banding of mouse metaphase chromosomes. *Chromosoma*, **90**, 128-132.
- LOEBEL, D. A. AND JOHNSTON, P. G. 1993. Analysis of DNase 1 sensitivity and methylation of active and inactive X chromosomes of kangaroos (*Macropus robustus*) by in situ nick translation. *Chromosoma*, **102**, 81-87.
- LOO, L. S. L. AND CAUCHI, M. N. 1992. DNA methylation patterns of the $\gamma\delta\beta$ -globin genes in human fetal and adult erythroid tissues. *Am. J. Hematol.*, **39**, 289-293.
- LÓPEZ-FERNÁNDEZ, C., GOSÁLVEZ, J. AND MEZZANOTTE, R. 1989. Heterochromatin heterogeneity in *Oedipoda germanica* (Orthoptera) detected by *in situ* digestion with restriction endonucleases. *Heredity*, **62**, 269-277.
- LÓPEZ-FERNÁNDEZ, C., GOSÁLVEZ, J., FERRUCCI, L. AND MEZZANOTTE, R. 1991a. Restriction endonucleases in the study of eukaryotic chromosomes. *Genetica*, **83**, 257-274.
- LÓPEZ-LEÓN, M. D., CABRERO, J. AND CAMACHO, J. P. M. 1991b. A nucleolus organizer region in a B chromosome inactivated by DNA methylation. *Chromosoma*, **100**, 134-138.
- LÓPEZ-LEÓN, M. D., CABRERO, J., CAMACHO, J. P. M., CANO, M. I. AND SANTOS, J. L. 1992. A widespread B chromosome polymorphism maintained without apparent drive. *Evolution*, **46**, 529-539.
- LÓPEZ-LEÓN, M. D., CABRERO, J., PARDO, M. C., VISERAS, E., CAMACHO, J. P. M. AND SANTOS, J. L. 1993. Generating high variability of B chromosomes in *Eyprepocnemis plorans* (grasshopper). *Heredity*, **71**, 352-362.
- LÓPEZ-LEÓN, M. D., NEVES, N., SCHWARZACHER, T., HESLOP-HARRISON, T. S., HEWITT, G. M., AND CAMACHO, J. P. M. 1994. Possible origin of a B chromosome deduced from its DNA composition using double FISH technique. *Chromosome Res.*, **2**, 87-92.
- PARDO, M. C., VISERAS, E., CABRERO, J. AND CAMACHO, J. P. M. 1993. A supernumerary chromosome segment in *Locusta migratoria*. *Genome*, **36**, 919-923.
- PIEPER, R. O., COSTELLO, J. F., KROES, R. A., FUTSCHER, B. W., MARATHI, U. AND ERICKSON, L. C. 1991. Direct correlation between methylation status and expression of the human O-6-Methylguanine DNA Methyltransferase gene. *Cancer Comm.*, **3**, 241-253.
- PRANTERA, G. AND FERRARO, M. 1990. Analysis of methylation and distribution of CpG sequences on human active and inactive X chromosomes by *in situ* nick translation. *Chromosoma*, **99**, 18-23.
- RAZIN, A. AND CEDAR, H. 1991. DNA methylation and gene expression. *Microbiol. Rev.*, **55**, 451-458.
- RAZIN, A. AND RIGGS, A. D. 1980. DNA methylation and gene function. *Science*, **210**, 604-616.
- RUFAS, J. S., ITURRA, P., DE SOUZA, W. AND ESPONDA, P. 1982. Simple silver staining procedure for the localization of nucleolus and nucleolar organizer under light and electron microscopy. *Arch. Biol.*, **93**, 267-274.
- RUIZ, I. R. G. AND BRISON, O. 1989. Methylation of ribosomal cistrons in diploid and tetraploid *Odontophrynus americanus* (Amphibia, Anura). *Chromosoma*, **98**, 86-92.
- SENTÍS, C., SANTOS, J. AND FERNÁNDEZ-PIQUERAS, J. 1989. In situ methylation of insect chromosomes with methylase HpaII. *Chromosoma*, **98**, 105-108.
- SHEMER, R., KAFRI, T., O'CONNELL, A., EISENBERG, S., BRESLOW, J. L. AND RAZIN, A. 1991. Methylation changes in the apolipoprotein AI gene during embryonic development of the mouse. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 11300-11304.
- SUJA, J. A., ANTONIO, C., GONZÁLEZ-GARCÍA, J. M. AND RUFAS, J. S. 1993. Supernumerary heterochromatic segments associated with the nucleolar chromosomes of *Pyrgomorpha conica* (Orthoptera) contain methylated rDNA sequences. *Chromosoma*, **102**, 491-499.
- SUZUKI, H., SAKURAI, S., NISHIMURA, M., KOMINAMI, R. AND MORIWAKI, K. 1992. Compensatory changes in silver-stainability of nucleolar organizer regions in mice. *Jap. J. Genet.*, **67**, 217-232.
- TANTRAHAHI, U., GUNTAKA, R. V., ERLANGER, B. F. AND MILLER, O. J. 1981. Amplified ribosomal RNA genes in a rat hepatoma cell line are enriched in 5-methylcytosine. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 489-493.

- TOMÁS, R., VERA, A., MARTÍN, M. AND SABATER, B. 1992. Changes in protein synthesis without evidence of DNA methylation in barley chloroplasts during leaf growth and development. *Plant Sci.*, **85**, 71–77.
- VISERAS, E., CAMACHO, J. P. M., CANO, M. I. AND SANTOS, J. L. 1990. Relationship between mitotic instability and accumulation of B chromosomes in males and females of *Locusta migratoria*. *Genome*, **33**, 23–29.
- WANG, Y., KATO, T., AYAKI, H., ISHIZAKI, K., TANO, K., MITRA, S. AND IKENAGA, M. 1992. Correlation between DNA methylation and expression of O⁶-methylguanine-DNA methyltransferase gene in cultured human tumor cells. *Mutat. Res.*, **273**, 221–230.
- WATSON, J. C., KAUFMAN, L. S. AND THOMPSON, W. F. 1987. Developmental regulation of cytosine methylation in the nuclear ribosomal RNA genes of *Pisum sativum*. *J. Mol. Biol.*, **193**, 15–26.
- WEIH, F., NITSCH, D., REIK, A., SCHÜTZ, G. AND BECKER, P. B. 1991. Analysis of CpG methylation and genomic footprinting at the tyrosine aminotransferase gene: DNA methylation alone is not sufficient to prevent protein binding *in vivo*. *EMBO J.*, **10**, 2559–2567.
- YEIVIN, A. AND RAZIN, A. 1993. Gene methylation patterns and expression. In: Jost, J. P. and Saluz, H. P. (eds) *DNA Methylation: Molecular Biology and Molecular Significance*, pp. 523–568. Birkhäuser Verlag, Basel.