Unfixed and fixed human chromosomes show different staining patterns after restriction endonuclease digestion

D. PERETTI¹, R. MEZZANOTTE¹ and A. T. SUMNER²

¹ Istituto di Biologia Generale, Universita di Cagliari, Cagliari, Italy
² MRC Human Genetics Unit, Edinburgh, United Kingdom

Peretti, D., Mezzanotte, R. and Sumner, A. T. 1990. Unfixed and fixed human chromosomes show different staining patterns after restriction endonuclease digestion. — Hereditas 112: 187–192. Lund, Sweden. ISSN 0018-0661. Received January 15, 1990. Accepted February 7, 1990

Restriction endonucleases (REs) have been widely used to produce banding patterns on chromosomes, but it remains uncertain to what extent the patterns are due to the sequence specificity of the enzymes, and to what extent chromatin structure influences the pattern of digestion. To throw light on this question, we have digested with restriction endonucleases unfixed chromosomes prepared in two different ways (isolated, and whole metaphase cells spread with a cytocentrifuge) and compared the results with those obtained on conventionally fixed chromosomes. Unfixed isolated chromosomes are easily destroyed by REs; after fixation with cold methanol, which produced minimal alteration to the chromatin structure, the chromosomes are resistant to the action of REs, and conventional methanol-acetic acid fixation is required to permit the induction of banding patterns by REs. Unfixed cytocentrifuge preparations, in which the chromosomes are still surrounded by cytoplasm, are much more resistant to the action of REs, and again banding patterns were only induced after methanol-acetic acid fixation. We conclude that the action of restriction endonucleases on chromosomes is strongly influenced by chromatin organisation, and that methanol-acetic acid fixation is required to permit the induction of conventional banding patterns on chromosomes.

A. T. Sumner, MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU. United Kingdom

The possibility of using restriction endonucleases (REs) to induce banding patterns in metaphase chromosomes has resulted in the widespread use of these enzymes in cytogenetics in recent years (Mezzanotte et al. 1983a, b, 1985; Miller et al. 1983; Kaelbling et al. 1984; Bianchi et al. 1985; Babu and Verma 1986; Mezzanotte 1986; Ferrucci et al. 1987; Marchi and Mezzanotte 1988; Burkholder 1989). Certain REs produce bands whose location coincides with that of certain highly repetitive DNA fractions, while others induce linear differentiation generally resembling G-banding patterns (Mezzanotte et al. 1983a, b, 1985; Miller et al. 1983).

The mechanism and significance of RE-induced bands are still under investigation. Some authors claim that the DNA in all regions of chromosomes is available for digestion with REs (Miller et al. 1983) and that “chromatin structure is not the main cause underlying chromosome banding” produced by such enzymes (Bianchi et al. 1985). On the other hand, it is clear that although REs can act only by cleaving specific DNA sequences, their effects on chromosomes could potentially be limited, firstly by the accessibility of the chromosomal DNA to the enzymes, and secondly by the ability of the cleaved DNA to diffuse out of the chromosomes. Clearly chromatin structure could be an important factor in determining the patterns of banding produced by digesting chromosomes with restriction enzymes. On the one hand, regions such as heterochromatin are believed to have more compact organisation than euchromatin, perhaps dependent upon the presence of different proteins (Strauss and Varshavsky 1984). On the other hand the standard procedures for preparation of metaphase chromosomes, particularly methanol-acetic acid fixation, are known to extract many proteins from chromosomes (Hancock and Sumner 1982; Burkholder and Duczek 1980; Hutchison and Weintraub 1985).

In the present study we have investigated the action of restriction endonucleases on unfixed meta-
phase chromosomes prepared in two fundamentally different ways, and compared the results with those obtained on chromosomes fixed with minimal change in methanol, as well as with those from chromosomes fixed in methanol-acetic acid. We have found that the action of REs on chromosomes is greatly influenced by the method of preparation, and that methanol-acetic fixation is generally necessary for REs to induce banding on metaphase chromosomes. We conclude, in agreement with other observations (SUMNER et al., in preparation) that chromatin organisation is indeed an important determinant of RE-induced banding patterns on chromosomes.

Materials and methods

Cytocentrifuge preparations

Unfixed metaphase chromosome spreads were made as described by PERRY and THOMSON (1986). Briefly, human lymphocytes were cultured in the presence of phytohaemagglutinin, arrested at metaphase with colcemid, treated with hypotonic solution, and spun down on to slides using a Shandon Cytospin centrifuge. The preparations were allowed to dry thoroughly before further treatment.

Isolated chromosome preparations

Chromosomes were isolated from human lymphoblastoid cells arrested in metaphase with colcemid using the polyamine method of SILLAR and YOUNG (1981) or the KCM buffer method of GOODERHAM and JEPPESEN (1983). The isolated chromosomes were centrifuged on to 13 mm diameter coverslips in a multiwell plate using a Sorvall ST 60,000 refrigerated centrifuge at 1000 rpm. The chromosome preparations on the coverslips were kept in the appropriate isolation buffer at 4°C until required for further treatment.

Fixation

Both Cytospin preparations, and preparations of isolated chromosomes prepared by both methods, were examined unfixed, or fixed in cold 80 % methanol at −20°C for 20 min, or in methanol-acetic acid (3:1) for 20 min at room temperature.

Restriction enzyme digestion

Slides were incubated with the enzymes, obtained from Boehringer, dissolved in the appropriate buffers according to the manufacturer’s recommendation, as follows:

- **Alu I**: 35–40 units/slide in buffer A
- **Hae III**: 40 units/slide in buffer M
- **Hind III**: 30–40 units/slide in buffer B
- **Hinf I**: 36 units/slide in buffer H.

Control slides were incubated in the appropriate buffers in the absence of the enzyme. Unless otherwise stated, all incubations were for 3h at 37°C; in a few experiments the incubation time was reduced to 1h. After incubation the slides or coverslips were stained with Gurr’s Giemsa R66 (BDH Ltd) diluted 1:20 in distilled water, for 10 min.

Results

Cytospin preparations

Alu I largely destroyed unfixed chromosomes, although with a shorter, 1hr, digestion, some residual C-bands could be seen. Cytospin preparations fixed with cold methanol were apparently unaffected by the enzyme (Fig. 1a), while methanol-acetic fixed preparations were often C-banded (Fig. 1b). Control slides incubated in buffer showed uniformly stained chromosomes with good morphology.

Unfixed chromosomes digested with Hae III showed some C-bands, and occasionally some G-bands (Fig. 2). Poor quality G-banding was also seen on Cytospin preparations fixed with cold methanol or methanol-acetic acid. Unfixed control slides again showed uniformly stained chromosomes with good morphology.

After Hind III digestion, chromosomes of Cytospin preparations, whether unfixed or fixed, were essentially unbanded, or at best showed only indistinct banding, and thus did not differ significantly from control slides (Fig. 3).

Hinf I digestion proved to be very destructive to unfixed chromosomes (Fig. 4a), although control slides incubated only in buffer showed much better morphology (Fig. 4b). Chromosomes fixed in cold methanol or in methanol-acetic acid and digested with Hinf I often showed paler C-bands, however, (Fig. 4c), and patchy digestion (Fig. 4d).

Isolated chromosomes

No substantial differences were seen between the action of restriction enzymes on chromosomes isolated by the KCM method and those isolated by the polyamine method. In all cases unfixed chromo-
Fig. 1a and b. Cytospin preparations of metaphase chromosomes digested for 3h with Alu I. a Chromosomes fixed in cold methanol; no banding visible. b Chromosomes fixed in methanol-acetic acid; note the C-banding pattern (arrows).

Fig. 2. Unfixed cytospin preparation of metaphase chromosomes digested with Hae III and stained with Giemsa. Note the indistinct banding pattern (arrow).

Fig. 3. Unfixed cytospin preparation of metaphase chromosomes digested with Hind III and stained with Giemsa. No banding pattern.

Chromosomes were completely destroyed or dispersed as a result of 3h digestion, although unfixed controls, treated only with buffer, largely retained their morphology and stained uniformly (Fig. 5a), except for buffer H (control for Hinf I), which itself tended to destroy the chromosomes. After 1h digestion with Hae III, Hind III, or Hinf I recognisable, uniformly stained chromosomes were retained, while 1hr digestion with Alu I still destroyed chromosomes. Isolated chromosomes fixed with cold methanol were also largely destroyed or swollen by all the enzymes tested, but chromosomes fixed in metha-
Fig. 4a–d. Cytospin preparations of metaphase chromosomes digested with \textit{Hinf I} and stained with Giemsa. \textbf{a} Unfixed metaphase digested with \textit{Hinf I}; chromosomes badly damaged. \textbf{b} Unfixed metaphase incubated in Buffer H; chromosomal structure better preserved. \textbf{c} Metaphase chromosomes fixed with cold methanol and digested with \textit{Hinf I}. Note the pale centromeres (arrows). \textbf{d} Metaphase chromosomes fixed with methanol-acetic acid and digested with \textit{Hinf I}, showing patchy digestion and staining.

Discussion

Our results show clearly that the action of restriction endonucleases on metaphase chromosomes is strongly influenced by the method of preparation. In general, unfixed chromosomes are attacked most readily by REs, and are often largely destroyed. Fixation of either isolated chromosomes or cytopin-prepared metaphase cells increased their resist-
Fig. 5a and b. Isolated chromosomes. a Unfixed chromosomes incubated in Buffer A and stained with Giemsa. Morphology well preserved, and no banding. b Chromosomes fixed in methanol-acetic acid, digested with \( \text{Afe} \) and stained with Giemsa. Chromosomal material largely extracted, but residual C-bands remain.

ance to digestion. There is also a clear difference between the effect of REs on Cytospin preparations, which are more resistant, and isolated chromosomes, which are relatively labile even after fixation. Most importantly, it is only after methanol-acetic acid fixation that the banding patterns, known to be induced by REs in conventionally prepared metaphase chromosomes, can be seen, although results are generally less clear than on conventional preparations.

These results can be explained readily in the context of the available information about the different types of chromosome preparations. In Cytospin preparations, the chromosomes are still surrounded by a quantity of cytoplasm, which is clearly still present after fixation (see Figures) and which no doubt helps to stabilise the chromosome structure as well as inhibiting access of REs. In contrast, the enzymes have direct access to isolated chromosomes, and their greater lability is shown by their frequent loss or distortion during the procedures described in this paper. Indeed, it was the lability of completely unfixed chromosomes that led us to use cold methanol fixation, a procedure reported to result in least loss of chromatin organisation and of chromosomal components (HUTCHISON and WEINTRAUB 1985). Use of chromosomes fixed in cold methanol should therefore provide information on the effect of REs on stabilized chromosomes that, nevertheless, have an organisation close to that of native chromosomes. Our observations indicate that, in general, such chromosomes are not substantially affected by the restriction endonucleases tested. It requires fixation by methanol-acetic acid, a procedure known to alter the structure of chromatin, and to extract significant amounts of protein (HANCOCK and SUMNER 1982; BURKHOLDER and DUCZEK 1980; HUTCHISON and WEINTRAUB 1985) to permit sufficient access of the REs to produce clear banding patterns. Although our experiments do not address directly the question of whether, in conventional methanol-acetic acid fixed chromosomes, the banding pattern is influenced by chromatin organisation, rather than simply reflecting the distribution of recognition sites for the enzyme, they do indicate that chromatin organisation does affect restriction endonuclease digestion of chromosomes. It is important to note that the retention of a more nearly native organisation in methanol-fixed chromosomes could affect the results of RE digestion in two ways; firstly by preventing access of the enzymes to the chromosomal DNA, and secondly, by preventing loss of the DNA fragments resulting from nuclease digestion.

Our results differ from those of BURKHOLDER and SCHMIDT (1986) and of BURKHOLDER (1989), who obtained, on isolated unfixed mouse chromosomes, banding patterns induced by REs that resembled those obtained on conventionally prepared, methanol-acetic acid fixed chromosomes. These differences can probably be explained to a large extent by the use of different procedures for preparation.
of unfixed chromosomes, and by different protocols for RE digestion. Nevertheless, BurkhOLDER (1989) does present evidence that in some situations access of a restriction enzyme to chromosomal DNA can be inhibited.

Acknowledgements. — We are especially grateful to the Nuffield Foundation for a grant to enable Diletta Peretti to come to Edinburgh to carry out this work, and to Eric Thomson and Judith Fantes, who supplied us with Cytospin chromosome preparations and isolated chromosomes, respectively. Our thanks are also due to Ann Kenmure and Lesley Campbell for typing the manuscript, and to the Photographic Department, MRC Human Genetics Unit, for preparing the illustrations.

References

BABU, A. and VERMA, R. S. 1986. Expression of heterochromatin by restriction endonuclease treatment and distamycin A/DAPI staining of Indian muntjac (Muntiacus muntjak) chromosomes. — Cytogenet. Cell Genet. 41: 96–100

BIANCHI, M. S., BIANCHI, N. O., PAYTELIAS, G. E. and WOLFF, S. 1985. The mechanism and pattern of banding induced by restriction endonuclease in human chromosomes. — Chromosoma 91: 131–136

BURKHOLDER, G. D. 1989. Morphological and biochemical effects of endonucleases on isolated mammalian chromosomes in vitro. — Chromosoma 97: 347–355

BURKHOLDER, G. D. and DUCZEK, L. L. 1980. Proteins in chromosome banding. 1. Effect of G-banding treatments on the proteins of isolated nuclei. — Chromosoma 79: 29–41

BURKHOLDER, G. D. and SCHMIDT, G. J. 1986. Endonuclease banding of isolated mammalian metaphase chromosomes. — Exp. Cell Res. 164: 379–387

FERRUCCI, L., ROMANO, E., DE STEFANO, G. F. 1987. The Alu I-induced bands in great apes and man: implication for heterochromatin characterization and satellite DNA distribution. — Cytogenet. Cell Genet. 44: 53–57

GOODERHAM, K. and JEPPESEN, P. 1983. Chinese hamster metaphase chromosomes isolated under physiological conditions. — Exp. Cell Res. 144: 1–4

HANCOCK, J. M. and SUMNER, A. T. 1982. The role of proteins in the production of different types of chromosome bands. — Cytobios 35: 37–46

HUTCHISON, N. and WEINTRAUB, H. 1985. Localization of DNase I-sensitive sequences to specific regions of interphase nuclei. — Cell 43: 471–482

KAELBLING, M., MILLER, D. A. and MILLER, O. J. 1984. Restriction enzyme banding of mouse metaphase chromosomes. — Chromosoma 90: 128–132

MARCHI, A. and MEZZANOTTE, R. 1988. Restriction endonuclease digestion and chromosome banding in the mosquito, Culex longiareolata (Diptera: Culicidae) — Heredity 60: 21–26

MEZZANOTTE, R. 1986. The selective digestion of polytene and mitotic chromosomes of Drosophila melanogaster by the Alu I and Hae III restriction endonucleases. — Chromosoma 93: 249–255

MEZZANOTTE, R., FERRUCCI, L., VANNI, R. and BIANCHI, U. 1983a. Selective digestion of human metaphase chromosomes by Alu I restriction endonuclease. — J. Histochem. Cytochem. 31: 553–556

MEZZANOTTE, R., BIANCHI, U., VANNI, R. and FERRUCCI, L. 1983b. Chromatin organization and restriction endonuclease activity on human metaphase chromosomes. — Cytogenet. Cell Genet. 36: 562–566

MEZZANOTTE, R., FERRUCCI, L., VANNI, R. and SUMNER, A. T. 1985. Some factors affecting the action of restriction endonucleases on human metaphase chromosomes. — Exp. Cell Res. 161: 247–253

MILLER, D. A., CHOE, Y.-C. and MILLER, O. J. 1983. Chromosome localization of highly repetitive human DNAs and amplified ribosomal DNA with restriction enzymes. — Science 219: 395–397

PERRY, P. E. and THOMSON, E. J. 1986. Immunogold labelling of metaphase cells. — Cytogenet. Cell Genet. 41: 121–125

SILLAR, R. and YOUNG, B. D. 1981. A new method for the preparation of metaphase chromosomes for flow analysis. — J. Histochem. Cytochem. 29: 74–78

STRAUSS, F. and VARSHAVSKY, A. 1984. A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleosome. — Cell 37: 889–901