In situ hybridization and chromosome banding in mammalian species

R. Chaves, a F. Adega, a S. Santos, a H. Guedes-Pinto a and J.S. Heslop-Harrison b

a Department of Genetics and Biotechnology, ICETA-UTAD, University of Trásos-Montes and Alto Douro, Vila Real (Portugal); b Department of Biology, University of Leicester, Leicester (UK)

This article is dedicated to Professor Karl Fredga to commemorate his retirement and lifelong cytogenetic research activities.

Abstract.

Chromosome banding is often required in conjunction with fluorescent in situ hybridization of labelled probes for chromosome painting, satellite DNA and low-copy sequences to allow identification of chromosomes and simultaneous probe localization. Here, we present a method that reveals both patterns with only one observation step. The band pattern is produced by restriction-enzyme digestion of chromosomes followed by fixation with paraformaldehyde in PBS, a short chromosome denaturation step in hybridization solution, and then standard in situ hybridization, washing and detection protocols. Using a range of different mammalian species, chromosome-banding patterns were immediately recognizable, although synchronization procedures normally required for high-resolution G-banding were not. Unlike other methods available, only one round of observation is required using a conventional fluorescence microscope, the method works without modification in many species, and in situ hybridization is not used for chromosome identification (allowing multiple targets and minimizing background). The banding pattern is preferably generated by a combination of DNA dissolution and heterochromatin reorganization after enzyme digestion. Followed by paraformaldehyde fixation of the new chromatin structure and incomplete denaturation. The method is of widespread utility in comparative genomics and genome organization programmes.

Introduction

G- or R-bandig of chromosomes is a key method for the identification and characterization of chromosomes in mammalian species, allowing analysis of chromosome polymorphism in large datasets (e.g. Fredga and Narain, 2000). To elucidate the molecular structure and evolution of chromosomes, in situ hybridization (ISH) techniques are used to localize particular sequences along chromosomes (e.g. Liu et al., 1998). With the exception of the sex chromosomes, it is often difficult to identify chromosomes by morphology alone following in situ hybridization, and R- or G-banding and fluorescent in situ hybridization methods are usually used sequentially (Liu and Fredga, 1999) or simultaneously (Richard and Dutrillaux, 1996), showing the value of combining the techniques. However, the in situ hybridization and R- or G-banding methods require different sets of treatments, hybridization of some probes has been difficult to detect, and considerable optimisation is required for each species.

Some of the first studies of mammalian genome organization used restriction endonuclease (RE) digestion (with Giemsa or fluorochrome stains) to band chromosomes (Gould et al., 1976; Miller, 1983; Schmid and Almeida, 1988), and these methods have been used to study chromatin structure and reveal heterochromatin banding patterns in various animal species (for review, see Gonsálvez et al., 1997; Verma and Babu, 1995; Pieczarka et al., 1998; Chaves et al., 2000). The RE-induced bands can be interpreted as due to faint staining implying extraction of material from a chromosome area and to strong staining, not necessarily related to lack of enzyme cleavage (Nieddu et al., 1999). The combination of these techniques with fluorescent in situ hybridization on human chromosomes showed that alpha-satellite DNA hybridisation was enhanced by previous digestion with Ali 1 and TaqI (Nieddu et al., 1999), perhaps due to chromatin reorganisation allowed by RE attack. However, the knowledge gained from RE digestion is hard to interpret in terms of genomic DNA sequences and genome structure or organization.

In this paper, we describe results showing strong fluorescent in situ hybridization signals (using either paint or satellite probes on chromosomes) simultaneously with restriction enzyme banding patterns, and we show the use of the method in species of different taxonomic orders including primates and Artiodactyla.
Materials and methods

Chromosome preparations

Human (46,XX) and sheep (54,XY) peripheral blood lymphocytes were cultured at 37 °C using standard protocols. Phytohaemagglutinin (Gibco, 1 %) and pokeweed (Gibco, 1 %)-stimulated lymphocyte cultures were grown in RPMI 1640 medium, supplemented with 15 % foetal calf serum (Myo- clone, Gibco), 1 % L-glutamine (200 mM) and 1 % penicillin/streptomycin (10,000 U/ml and 10 mg/ml respectively). For the sheep chromosome prepa- rations that were G-banded, a double synchronisation procedure was used to obtain high-resolution banding, adding thymidine (Tdr, Sigma, 25 μg/ml final concentration) overnight after 48 h of growth. The S-phase block was released 18 h later with two washes in fresh RPMI 1640. The cells were resus- pended in RPMI 1640 of the same composition as above, with the addition of 20 μg/ml final concentration) of bromodeoxyuridine (Brdu, Sigma) and after 1 h, Hoechst H33258 (Sigma) 20 μg/ml (final concentration) was added. The cells were allowed to grow for an additional 7 h, including 10 min with colcemid (0.02 μg/ml final concentration). Human chromosome prepara- tions were obtained only with late synchronisation using Brdu in the last 7 h of culture, at the same concentration as above. Non-synchronised cultures were grown for 72 h, as above, and treated with colcemid (0.1 μg/ml final concentration) for the last hour.

Cell suspension, swelling and fixation procedures were the same for the two culture types. A 75 mM KCl hypotonic solution at 37 °C was used for 20 min, and then the chromosomes were fixed three times with metha- nol:acetic acid solution (3:1) and left overnight at 20 °C.

Restriction enzyme digestion on fixed chromosomes

Air-dried slides were aged at 65 °C for 5 h and then submitted overnight to restriction digestion. The enzymes (Gibco) included Apal (sheep) and HaeIII (human) at 30 U/slide (see Results for complete list). Enzymes were used in the buffer specified by the supplier and 40 11 of solution was applied to the preparation before incubation in a moist chamber at 37 °C. Control slides were incubated without enzymes. After overnight incubation the slides were washed three times with water at room temperature and air-dried. Some preparations were stained with 5 % Giemsa (Karyomax Giemsa, Gibco) in Sörensen’s buffer for 10 min to verify the digestion effects on chromo- somes.

Fluorescent in situ hybridization (FISH)

General methods followed those of Schwarzacher and Heslop-Harrison (2000). Dry slides were placed in a 1x PBS solution (2 x 5 min) before fixa- tion (10 min) in freshly- prepared 4 % paraformaldehyde in 1x PBS (room temperature). Slides were dehydrated for 2 min each in 70 %, 90 % and 100 % chilled ethanol and air-dried. Some preparations (control slides) were not fixed in paraformaldehyde.

For hybridization on human chromosomes, a digoxigenin- labelled Coa-tasome X total chromosome probe (Oncor; not available by mid-2001) was used with 7II of probe plus 8II of Oncor hybridization buffer per slide; the probe mixture was denatured at 65 °C for 10 min and allowed to reanneal for 1h at 37 °C. For hybridization on sheep chromosomes, an - satellite I sheep clone (pOaKB9) (Chaves et al., 2000) labelled with biotin-16-dUTP (Sigma) was used: 1II of probe (corresponding to 100 ng per slide) was dissolved in 14-I hybridization buffer (50 % deionised formamide, 10 % dextran sulphate, 2x SSC, 0.5 M phosphate buffer, 1x Denhardt’s solution, pH 7.3). The probe mixture was denatured at 65 °C for 10 min and cooled on ice. The slide preparations were denatured for 30 sec in 70 % deionised formamide in 2x SSC at 65 °C, dehydrated through 70 %, 90 % and 100 % chilled ethanol (2 min each), and air-dried. After application of the probe, hybridization was carried out overnight at 37 °C in a moist chamber. The most stringent post- hybridization washes were at 42 °C in 2x SSC and 50 % (v/v) formamide, followed by washes in 0.1x SSC at room temperature. Biotin and digoxigenin labels were detected by FITC conjugated with avidin (Vector) and anti-digoxigenin (Oncor), respectively. Chromosomes were counterstained with DAPI and mounted in Vectorshield (Vector).

Chromosomes were observed under epifluorescence (Leica Dialux photomicroscope with a 50W mercury lamp). Metaphases were photographed for DAPI with Kodak 1635 Pan film or Kodak Ultra 400 ASA colour print film. We also used a Zeiss Axioplan 2 Imaging with a 100W mercury lamp, Axiocam digital camera and AxioVision software. Digitised photos were printed from Adobe Photoshop using only contrast, overlay and colour optimisation functions that affected the whole of the image.

Results

Figure 1a–c shows a metaphase from sheep digested with Apal before and after in situ hybridization. After restriction enzyme digestion, the metaphase was stained with Giemsa (Fig. 1a). Following in situ hybridization of an - satellite sheep probe and counterstaining with DAPI, major sites were seen along with a pattern similar to G-banding, particularly clear when the DAPI image was inverted, and allowing pairing and identification of all individual chromosome types.

Other restriction enzymes, including HaeIII, HinII, MspI (and, less strongly, Ddel, Drai, MboI and PstI) also revealed banding patterns, most of them G-like (results not shown), with some variability, and enough discrimination to allow pairing of individual chromosomes. The same technique has been applied to cattle, goat, some wild Bovidae species, elephant, dog and cat and found to be effective without modification as well as reproducible.

We also used a panel of eight enzymes on fixed human chro- mosomes. Figure 1d–f shows a metaphase restricted with HaeIII prior to in situ hybridization with an HSAX paint probe. After hybridization, a fine banding pattern was seen with simultaneous co-localization of the in situ hybridization signal of the X probe. Figure 1f shows some of the restricted HaeIII human chromosomes (1, 21 and 22) showing a G-like banding and alignment with 400-band level ideograms. In chromosome 1, the q arm is coincident with the ideogram except for one band, more resolved in the ideogram (line B). There are other bands (line A) where the resolution is increased with the restriction digestion. The bands, 33, 34.2, 35 and 36.2, on chromosome 1p are not evident in the restriction pattern, perhaps because the enzyme HaeIII digested more DNA. The restriction pattern of 21 and 22 chromosomes is substantially different, enabling the unequivocal identification of both chro- mosomes. The technique was successfully applied to fibroblast cultures (results not shown) with exactly the same protocol,
except for ageing slides overnight.

Discussion

Restriction enzyme banding is compatible with fluorescent in situ hybridization and the method presented here allows simultaneous banding and in situ hybridization in mammalian chromosome spreads from lymphocytes or fibroblasts with only one round of observation and minimal extra preparation steps. Banding patterns revealed by conventional staining methods such as high resolution G-banding are often lost during the in situ hybridization procedure even following re-fixation using relatively low temperatures and short times for denaturation. The restriction enzyme procedure reveals clear and characteristic bands on chromosomes, usually related to the G-banding pattern following in situ hybridization (Fig. 1).

Furthermore, the synchronisation procedures normally required for high-resolution G-banding do not seem to be required. Comparison of the ideograms and restriction band pattern (Fig. 1f) shows that the restriction pattern is less resolved than the best G-banding patterns but the G-band patterns are clearly related to restriction patterns, characteristic of each chromo- some, and reproducible.

The band pattern is produced by restriction enzyme digestion on chromosomes without the need for observation or photography at this stage, followed by a sequential fixation with paraformaldehyde and then a standard although short denaturation protocol. Restriction enzyme digestions without fixation with paraformaldehyde did not give well-defined patterns, perhaps because fixation is required for stabilization. The band pattern generated is the result of a complex number of events involving the enzyme digestion and then the reorganisation of the heterochromatin; the incomplete denaturation. of only 30 s, enables preservation without loss of further DNA and fluorescent signals. The method is certainly useful for identifying chromosomes and banding polymorphisms in combination with probes for large numbers of DNA copies, although it is possible that particular low-copy sequences may be lost in the banding procedure. Given the importance of both chromosome polymorphisms and DNA sequence organization, the rapid method of combining the two should be of value in both diversity and evolutionary studies, and in diagnostic applications for detecting DNA changes related to karyotypic abnormalities.

References


Fig. 1. (a–c) Metaphase chromosome preparation from a female sheep. (a) Metaphase restricted with Apal and Giemsa staining. (b) The same metaphase after in situ hybridisation with an \• satellite I clone pOaKB9 from sheep (seen in green) and counterstained with DAPI (blue). (c) The DAPI image as (b) with colour inversion and conversion to black and white to enhance the DAPI bands. (d–f) Metaphase chromosome preparation from human after (d) restriction digestion with HaeIII and in situ hybridization using a chromosome X painting probe (green) on DAPI counterstained chromosomes (blue). (e) DAPI-stained chromosomes from (a) after colour inversion and alignment as a karyotype. (f) Alignment of three chromosomes from karyotype (e), with the respective G-band ideograms (400-band level).