Elongated mouse chromosomes suitable for enhanced molecular cytogenetics

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Received 29 March 2004; accepted in revised form 7 September 2004

Key words: Elongated chromosomes, Intercalating agents, Mitotic block, Molecular cytogenetics

Abstract

Characterization of genetic disorders in humans and animal models requires identification of chromosomal aberrations. However, identifying fine deletions or insertion in metaphase chromosomes has been always a challenge due to limitations of resolution. In this study we developed a rapid method for chromosome elongation using two different intercalating agents: ethidium bromide and 5-bromo-2'-deoxyuridine (BrdU), together with a short-term mitotic block using colcemid. About 70% of the chromosomes from cells that underwent this elongation procedure reached three times longer than those prepared from control cells. FISH experiments using elongated chromosomes revealed a duplicated region of chromosome 11 that was not visible in cells prepared with conventional methods.

Introduction

The analysis of chromosomal aberrations, including translocations, deletions, insertions, duplications and comparative cytogenetics is essential for early diagnosis and the characterization of various genetic diseases including cancer (Hoo et al. 1983; Ushiki et al. 2002). Several techniques such as banding, *in situ* hybridizations, spectral karyotyping (SKY) (Liyanage et al. 1996; Schrock et al. 1996) and multicolor fluorescent *in situ* hybridization (mFISH) (Speicher et al. 1996) as well as multicolor banding (mBand) (Johannes et al. 1999) have been developed over the past years and have proven beneficial for studying chromosomal changes in humans, primates and other animal models (Ikeuchi 1984; Lemke et al. 2001; Liehr et al. 2002). However, due to the different degrees of chromosome condensation during metaphase, it

was always a problem identifying breakpoints, assigning small alterations in gene loci and detecting small insertions or deletions (Van Roy et al. 1993). This especially applies to mouse chromosomes that are smaller than human chromosomes and that are all acrocentric.

Over the past three decades, several approaches were developed to produce elongated 'high resolution' human chromosomes by using intercalating agents (Zhang et al. 1982). These agents included ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridium bromide) that interferes with chromatin structure (Paoletti 1979) due to its intercalation properties, and also by the electrostatic effects of its quaternary nitrogen in position 5 of the molecule (Paoletti 1979; Tomiyasu and Testa 1988; Lemke et al. 2001). The thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) has also been used. When incorporated into newly

synthesized DNA at S-phase, it can partially inhibit chromosome condensation (Schollmayer et al. 1981; Van Roy et al. 1993).

Due to the wide conservation of genetic information between humans and mammalian animal models, the animal models have become the most useful tool for *in vivo* genetic studies (Savill 1997; Wijnhoven and van Steeg 2003). Of these animal models, the mouse is a preferable model in many laboratories, due to the availability of different strains and sub-strains and a variety of transgenic and knock-out mice for a wide range of genetic disorders that are similar to humans (Rangarajan and Weinberg 2003). To take full advantage of this model system, it was necessary to develop a method for high-resolution mouse chromosomes. The only previously published high chromosome resolution studies in mice used actinomycin D and acridine orange to compare banding patterns between human and mouse chromosomes in mouse lymphoma cell lines (Yunis and Chandler 1978; Yunis et al. 1978; Sawyer and Hozier 1986; Sawyer et al. 1987, 1989).

In this study, we examined different protocols for the elongation of mouse chromosomes, focusing on near-diploid immortalized Pre-B mouse lymphocytes of BALB/c mouse origin (Mai et al. 1999). Due to its predominantly diploid nature, this cell line can serve as a model system for lymphoid mouse cells from both tissue culture and *in vivo* mouse preparations (Benedek et al. 2004). The protocol for elongated chromosomes that we developed is an improvement over existing protocols and applicable to any other mouse cell system whether lymphoid or non-lymphoid in nature. We used a combination of short-term chromosomal elongation concomitant with a short-term mitotic block to generate a high percentage of metaphases with elongated chromosomes. We compared the effects of ethidium bromide and BrdU on unsynchronized cells together with low concentration of colcemid. The new protocol we established using BrdU and colcemid achieves up to 70% of elongated chromosomes that are suitable for FISH and other molecular cytogenetic applications. Acridine orange was not included in this study because of the loss of sharpness of chromosomes accompanied with their rapid shrinking (Drouin and Richer 1989; Rangarajan and Weinberg 2003).

Materials

A. Cell culture

- Fetal bovine serum – GIBCO BRL cat. 10437-028 (GIBCO BRL Burlington, ON, Canada).
- L-glutamine – GIBCO BRL cat.25030-081.
- sodium pyruvate – GIBCO BRL cat. 11360-070.
- penicillin/streptomycin – GIBCO BRL cat. 15070-063.
- β -mercaptoethanol – GIBCO BRL cat. 21985-023.

B. Chromosomes elongation

- 5-bromo-2'-deoxyuridine (BrdU) – Sigma cat. B 9285 (Sigma, Oakville, ON, Canada).
- ethidium bromide (EtBr) – Sigma cat. E-8751.
- colcemid – GIBCO BRL cat. 21985-023.

C. Chromosomes preparation

- methanol – Fluka chemicals cat. 65542 (Fluka chemicals, Oakville, ON, Canada).
- Giemsa stain – Sigma cat. G-4507.

D. FISH

- anti-digoxigenin-antibody – Roche cat. 1207-741 (Roche, Laval, QC, Canada).
- Cy3- labeled chromosome 11 paint – Cambio cat.1200-11 M Cy3-02 (Cambio UK, CedarLane, Hornby, ON, Canada).

E. Equipments and softwares

- Axiophot microscope – Zeiss (Carl ZeissTM, Germany).
- SenSys camera – Applied Spectral Imaging (Applied Spectral ImagingTM, Santa Clara, CA, USA).
- MacProbe 4.4 software – Power Gene (Power GeneTM, League city, TX, USA).
- Leitz-Leica DMRB microscope (Leica, Heidelberg, Germany).
- Hamamatsu C 4800 cooled charge-coupled device camera (Hamamatsu, Herrsching, Germany).
- IP lab software (ScanalyticsTM, Fairfax, Virginia, USA).

Methods

Mouse PreB cells (Mai et al. 1999) were grown in RPMI medium supplemented with 10% heat inactivated fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, 1% penicillin/streptomycin and 0.1% β -mercaptoethanol. Cells were split 24 h

before the experiment. Time '0' was the time point of addition of either BrdU at a final concentration of 50 and 250 μM respectively or ethidium bromide at a final concentration of 2.5, 5 and 10 $\mu\text{g}/\text{ml}$ respectively together with 1 $\mu\text{g}/\text{ml}$ of colcemid. Untreated cells were considered as the controls for this study. Cells were then incubated at 37°C for 1, 2 and 16 h. At each time point, cells were harvested by centrifugation using an Eppendorf centrifuge 5180 R for 10 min at 800 rpm ($G = 129 \text{ rcf}$). Pellets were resuspended in hypotonic solution (0.075 M KCl) for 30 min, centrifuged as described above. Pellet-fixation of the chromosomes was then carried out according to Mai and Wiener (2002) and Beatty et al. (2002). In brief, fresh fixative solution 3:1 methanol/acetic acid was gently added in 2, 3, 4 and 5 drops respectively every 1 min and then 10, 15, 30 and 60 drops respectively every 2 min. Cells were then centrifuged and pellets were resuspended in 5 ml of fixative and left at room temperature for 10 min, then cells were centrifuged. The cycle of centrifugation, resuspension, and incubation at room temperature for 20 and 30 min respectively, was repeated twice. Final pellets were resuspended in an appropriate volume of fixative solution and kept at -20°C until further use. Slides were prepared as described by Mai and Wiener (2002) and Beatty et al. (2002). In brief, resuspended cells were dropped using a Pasteur pipette from a distance of approximately 50 cm onto a chilled slide, the slide was then warmed briefly on a slide warmer at 37°C, dipped in 50% acetic acid solution and dried on a slide warmer at 37°C. The slides were then stained with Giemsa stain and air-dried. Slides were examined using Axiophot microscope connected to SenSys camera. Selected metaphase spreads were imaged using MacProbe 4.4 software.

FISH experiments were carried out as described by Mai and Wiener (2002) and Beatty et al. (2002). Cells were hybridized with a digoxigenin-labeled mouse chromosome 11 cDNA probe mapping to sub-telomeric region E2 of chromosome 11. The Genbank accession number for the clone is BC024349.1 and was obtained from Openbiosystems (<http://www.openbiosystems.com>). The probe was visualized using an anti-digoxigenin-antibody. Nuclei and chromosomes were counterstained using 1 $\mu\text{g}/\text{ml}$ 4',6-diamidino-2-phenylindole (DAPI). The slides were

imaged on a Leica DMRB microscope equipped with a Hamamatsu C 4800 cooled charged-coupled device camera. Cy3-labeled chromosome 11 paint was used as described in Mai and Wiener (2002) and Beatty et al. (2002).

Chromosome spreads were first subjectively classified according to their apparent length into four groups: short, medium, long and very long as shown in Figures 1 and 2, and Table 1. Objectively, chromosomes-length was measured and assessed accordingly based on (1) the length measurements of chromosome 11 from different spreads using IP lab software, where the system is calibrated using a known measurement of 1 μm reference. Based on this calibration the distance between any two points can be measured. Additional information on the software has been previously reported (Mai et al. 1996). Chromosomes that were subjectively classified as short had the length of chromosome 11 in the range of $4.0 \pm 0.3 \mu\text{m}$, medium, long and very long were $6.5 \pm 0.4 \mu\text{m}$, $8.0 \pm 0.6 \mu\text{m}$ and $11.3 \pm 0.7 \mu\text{m}$ respectively (Figure 2). (2) FISH was performed using the subtelomeric probe BC024349.1 (Figure 3). The experiments were repeated six times to confirm reproducibility, and 235 metaphases were scored.

Results

For control cells, it was observed that no chromosomes fell in the 'very long' group, as well as 'long' chromosomes were only 8%. For cells treated with BrdU, no significant difference was observed when using 50 or 250 μM . However, longer metaphase chromosomes were observed depending on incubation time (Figure 2a, b). For cells treated with ethidium bromide, the length of chromosomes as well as percentages falling in 'long' or 'very long' was dependent on the concentration of the chemical rather than the time course (Table 1, Figures 1b and 2c, d). It was also observed that upon longer incubations with ethidium bromide the chromosomes tended to be extremely long producing metaphase plates with lots of overlaps (Figure 2d). A comparison between untreated cells (control) and cells treated with BrdU or ethidium bromide is shown in Table 1 and Figure 1a, b, respectively.

A third protocol variation involved the combined application of BrdU (50 μ M) and colcemid (1 μ g/ml) (1 h each). This method gave the best results, in that chromosomes were not too long so that we were not dealing with too many overlaps. Finally, 70% of all metaphases prepared by the simultaneous application of BrdU and colcemid fell into the category of 'long chromosomes' (Table 1). Therefore the remaining chromosomes plates were not classified into the short, medium or very long categories.

Thus, for FISH experiments, cells were split 24 h prior to the addition of BrdU and colcemid. To assess the quality of the elongated chromosomes, we chose mouse chromosome 11 and studied the telomeric region of the chromosome. To this end, PreB cell chromosomes were hybrid-

ized with both a chromosome 11 paint (red) and the subtelomeric chromosome 11 cDNA probe (BC024349.1) (green) (Figure 3). While a non-elongated chromosome 11 does not reliably show the duplication at the E2 end-region, the elongated chromosome 11 shows this clearly as documented by the co-hybridization of chromosome 11 paint and subtelomeric probe (Figure 3). Note the duplicated signals for the subtelomeric probe (green) on chromosome 11 and the elongated chromosome 11 paint (red) (Figure 3b).

Discussion

Elongated chromosomes, from an unsynchronized mouse PreB cell line, were generated using inter-

Figure 1. Length comparison of chromosomes prepared from PreB control cells or cells treated with BrdU or ethidium bromide (EtBr). For control cells (untreated cells), no differences in the classification was observed between 1 and 2 h. (a) Control cells (untreated cells) and cells treated with 50 μ M BrdU (data from cells treated with 250 μ M is not shown). (b) Control cells and cells treated with 2.5, 5 and 10 μ g/ml EtBr respectively for 1 and 2 h (data for the 16 h time point are not shown).

Figure 2. Representative bright field images (taken at $63 \times$ oil) of chromosomes with various lengths. The images were selected from either BrdU or EtBr treatments. (a) short ($4.0 \pm 0.3 \mu\text{m}$): $50 \mu\text{M}$ BrdU for 1 h, (b) medium ($6.5 \pm 0.4 \mu\text{m}$): $50 \mu\text{M}$ BrdU for 2 h, (c) long ($8.0 \pm 0.6 \mu\text{m}$): $5 \mu\text{g/ml}$ EtBr for 1 h and (d) very long ($11.3 \pm 0.7 \mu\text{m}$): $10 \mu\text{g/ml}$ EtBr for 2 h.

Table 1. Percentages of chromosomes with various length/metaphase plate, classified in categories: short, medium, long and very long

Treatment	Very long (%)	Long (%)	Medium (%)	Short (%)	Comments
Untreated PreB cells	0	8	38	54	
BrdU 1 h	0	7	43	50	Not long enough
BrdU 2 h	0	24	46	30	Not long enough
BrdU 16 h	12	30	36	22	Acceptable
EtBr $2.5 \mu\text{g/ml}$ 1 h	6	30	46	18	Not long enough
EtBr $5 \mu\text{g/ml}$ 1 h	10	50	27	13	Acceptable
EtBr $10 \mu\text{g/ml}$ 1 h	18	47	25	10	Overlaps
EtBr $2.5 \mu\text{g/ml}$ 2 h	16	40	40	4	Acceptable
EtBr $5 \mu\text{g/ml}$ 2 h	21	44	21	14	Overlaps
EtBr $10 \mu\text{g/ml}$ 2 h	34	26	24	16	Overlaps
BrdU 1 h + colcemid 1 h	nd*	70	nd*	nd*	Chosen method

* nd: not determined

calating agents, ethidium bromide and BrdU. Ethidium bromide produced efficient results upon shorter incubation time, which suggests it to be more suitable. For experiments that require staining with red fluorochromes, BrdU is more suitable than ethidium bromide (excitation 302 nm and emission 595 nm) that interferes with hybridizations using red fluorochrome(s). However, for

FISH using fluorochromes outside this range, ethidium bromide will be suitable. Increasing the concentration of BrdU did not improve the length of the chromosomes, which might be due to the limited capacity of the cells to uptake the thymidine analogue and incorporate it during replication (Sawyer et al. 1989). On the other hand with ethidium bromide, the resolution increased as a

Figure 3. FISH using co-hybridization with chromosome 11 paint (red) and subteleric probe (green) on mouse Pre-B lymphocytes. The size bar equals 10 μm . Both pictures were taken at the identical magnification ($63 \times$ oil) and acquisition time. No image enhancements were performed. (a) Representative image showing FISH on non-elongated mouse Pre-B chromosomes. The subteleric probe (green) localizes to the end of chromosome 11 (red) in a metaphase plate that harbors trisomy 11. Note that the subteleric probe is also found on extrachromosomal elements (EEs). (a'): Enlarged chromosome 11 from (a) showing two subteleric signals (green), see arrow. (b) Representative FISH image showing FISH on elongated mouse Pre-B chromosomes. The elongation involved BrdU and colcemid as outlined in the text. (b') Enlarged chromosome 11 from (b). The subteleric probe (green) is seen as a doublet on an elongated chromosome 11 (4 signals) (see arrows). An additional copy of the subteleric probe (1 signal) is inserted into one sister chromatid. This duplication pattern suggests an inversion of the telomeric end of chromosome 11. (b'') Single chromosome 11 from a different metaphase showing the duplication of the subteleric probe on chromosome 11 (see arrows).

function of concentration, most likely due to the electrostatic attractions between the DNA and ethidium bromide (Paoletti 1979; Tomiyasu and Testa 1988; Lemke et al. 2001).

Finally, a combination of short-term incubation of cells with both BrdU and colcemid for the preparation of elongated chromosomes was the method of choice in our study. Using this technique to prepare chromosomes from mouse PreB cells for FISH experiments allowed us to identify a duplicated region on mouse chromosome 11, that was not identified using conventional methods for chromosome preparation (Figure 3a, b). This method for the preparation of elongated mouse chromosomes is applicable for any other molecu-

lar cytogenetic study that requires the analysis of small duplications, deletions, insertions and/or translocations.

Acknowledgments

The authors thank Dr Francis Wiener for discussions and critical reading of this manuscript. This study was performed with financial support from NSERC (Canada) and Cancer Research Institute/Concern Foundation (Sweden). SL is funded through a fellowship funded by the CIHR Strategic Training Program entitled "Innovative Technologies in Multidisciplinary Health Research Training".

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