Identification and Cytogenetic Analysis of an Abnormal Pig Chromosome for Flow Cytometry and Sorting

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Z. Naturforsch. 48c, 645–653 (1993); received February 26/June 4, 1993

Pig Chromosomes, Translocation Chromosomes, Flow Karyotyping, Slit-Scan Flow Cytometry, Banding of Flow Sorted Chromosomes

For cytogenetics of pig (Sus scrofa domestica) and the influence of chromosome aberrations on pig production, high interest exists in flow sorted chromosomes for gene mapping, to establish DNA-libraries, or to produce DNA-probes. Flow karyotyping and sorting as well as slit scan flow analysis of metaphase chromosomes of an abnormal cell type carrying a translocation marker chromosome 6/15 are described. Flow sorting of the largest chromosomes of these cells was performed. After sorting the chromosomes still had a well preserved morphology and were identified microscopically by G-banding. The quality of the band pattern of the sorted chromosomes was compatible to that of isolated chromosomes not subjected to flow cytometry. The sorted fraction showed an enrichment of chromosome 6/15 and chromosome 1 which have quantitatively about the same integrated fluorescence intensity. Slit scan flow analysis was performed to discriminate these two chromosomes. Metacentric and submetacentric chromosomes were analyzed according to their bimodal slit scan profiles. Profiles of the largest chromosomes were distinguished by their different centromeric indices. Two groups were interpreted as the normal chromosome 1 and the translocation chromosome 6/15.

Introduction

Gene mapping in pig is not very developed [1]. Since one of the first genes, the SLA (swine leukocyte alloantigen), was localized on chromosome 7 [2, 3] more than 100 other loci were assigned on pig chromosomes [1, 4]. Among them the gene responsible for halothane sensitivity (HAL) [5] and some other genes coding for different enzymes, e.g. glucose phosphate isomerase (GPI) [6], or phosphogluconate dehydrogenase (PGD) [7], were found on chromosome 6.

To further analyze chromosome 6 for gene mapping or specific DNA probes, it is advantageous to use sorted chromosomes. For this purpose fluorescence activated flow analysis and sorting (“flow cytometry”) offers an important tool. Frequency histograms of the fluorescence intensity of isolated chromosomes, stained in suspension with DNA specific fluorochromes, can be registered (“flow karyotyping”). These flow karyotypes show a high individual reproducibility and a species characteristic peak pattern [8–13]. Flow sorting has meanwhile been performed with considerable success to establish chromosome specific libraries in human molecular genetics [14–16].

Univariate flow cytometry and chromosome sorting of normal and abnormal pig karyotypes has also been described [17–19]. Also two parameter flow karyotyping showing a resolution of 19–20 peaks has been published [20, 21]. The one parameter flow karyotypes suggest that there may exist principle limitations to sort chromosome 6 with higher purity. The normal pig flow karyotype shows 12 (female) or 13 (male) peaks, respectively. A rough estimate of the peak position as a function of chromosome length shows the presumptive chromosome 6 peak closely correlated to other chromosome peaks (e.g. chromosome 2, 3, 4, 7, 8, 9, 15, X) [17]. Therefore a cell line with a translocated chromosome consisting of the total chromosome 6 and 2/3 of chromosome 15 was used [22]. This translocated chromosome became one of the largest chromosomes and was easier to distinguish from the other ones, in particular the middle sized chromosomes.

For flow sorting a detailed examination to identify this marker chromosome will be described.
Since there are only a few specific DNA-probes localized on chromosome 6 and 15, classical G-banding technique was preferred to be applied instead of fluorescence in situ hybridization [23, 24]. So far banding is still not common for fluorescence stained chromosomes after flow sorting; protocols have been described for Chinese hamster and human chromosomes [25–27].

Compared to flow karyotyping that only registers the integrated fluorescence intensity of a chromosome, slit scan flow cytometry [29–31] produces detailed morphological information. For each chromosome of a suspension, an intensity profile (fluorescence intensity vs. time of flight) is measured to determine morphological features of chromosomes such as the number and relative positions of centromeres (centromeric index) [28, 32–35], or a fluorescence banding pattern [36]. Principally, slit scan flow cytometry may offer a tool to increase sorting purity of chromosomes, if two chromosomes of about the same DNA content differ significantly in their centromeric index. The high flow velocity (up to 10 m/sec) and event rate (up to hundreds of chromosomes per sec) of slit scanning, however, requires high computational effort in online data evaluation for slit scan sorting [35, 37–40]. Thus, slit scan sorting is still in the beginning of the development [28]. Before slit scan sorting procedures can be implemented, e.g. for pig chromosomes, it has to be shown that the slit scan profiles of the objects to be sorted can be discriminated from the profiles registered from other particles of a chromosome suspension.

The results presented here show how the marker chromosome 6/15 can be sorted, identified after sorting by G-banding, and distinguished from chromosome 1 by slit scan analysis.

Materials and Methods

Chromosome preparation for cytogenetic analysis

Fibroblast cell cultures obtained from the abnormal pig cell line and from a normal pig cell line [22] were incubated for 7 h with BrdUrd solution (final concentration 10 μg/ml). After 30 min colchicine treatment and harvesting, the cells were treated according to a routine protocol and stained with acridine orange to obtain RBA-bands [41] in order to identify the chromosomes involved in the translocation.

Chromosome preparation for flow karyotyping

For flow cytometry analysis and sorting, logarithmically growing fibroblast cell cultures were used. Demecolchmine (Colcemid Gibco, final concentration 10 μg/ml) was added to the cultures. After 5 h incubation the mitotic cells were collected and resuspended in a hypotonic solution (7.5 mM KCl) for 10 min to isolate the metaphase chromosomes. According to a slightly modified hexandiol method [42] the cell pellet was drained carefully and resuspended in 1 ml of TAcCaM buffer (25 mM Tris/HAc, 5 mM CaCl₂, 5 mM MgCl₂, pH 3.2) [43]. The chromosome suspension in TAcCaM buffer can be stored for several months at 4 °C. Staining of the chromosomes was performed with ethium bromide (10 μl/ml at a final concentration of 100 μg/ml) for flow karyotyping and sorting and with propidium iodide (about 200–250 μM) for slit scanning. Before flow sorting the samples were filtered through one layer 10 μm mesh sieve in order to eliminate large particles of cell debris and agglomerates.

Chromosome banding after flow sorting

The flow sorted chromosomes were deposited on glass slides and additionally stained with Giemsa. After localization of the chromosomes on the slide by microscopy they were destained by washing with absolute ethanol (p.a.); the slides were then air-dried. To identify the chromosomes of the sorting fractions unequivocally, G-banding was applied. For this purpose, the slides were treated with 0.25 percent trypsin solution in PBS for 30 sec and restained with Giemsa for 10 min.

Flow karyotyping and sorting

Flow karyotyping and chromosome sorting were performed on an EPICS V flow cytomter (Coulter, Hieleah, Fl., U.S.A.) equipped with one argon ion laser (Spectra Physics, Mountain View, Ca., U.S.A.). Ethidium bromide fluorescence was excited by the 488 nm line of the laser operating with a power of 500 mW. A dichroic 560 nm filter was used to collect the red fluorescence of the ethidium bromide stained material on the corresponding photomultiplier. The passing fluorescence was registered with a 590 nm long pass filter. Monoparametric distributions of forward scatter (FSC), peak height of red fluorescence
(HRFL), and logarithm of the integral of red fluorescence (LIRFL) were measured using list mode and stored in 256 channel arrays. To eliminate background, gating on channel 30-255 (HRFL, LIRFL) were set. About 2000–3000 particles were measured per flow karyotype. The histogram informations were analyzed and plotted using the Coulter MDADS and EASY 88 system.

The chromosomes were sorted with PBS (137 mM NaCl, 2.68 mM KCl, 8.09 mM Na2HPO4 \((12 \text{ H}_2\text{O})\), 1.47 mM KH2PO4, 11 H2O, pH 7.2) sheath buffer using a 76 \(\mu\)m nozzle and three droplet sorting. Particle coincidence was eliminated electronically. Sorting was performed on microscope slides as well as in tubes containing TAcCaM buffer to stabilize the chromosome morphology.

Slit scan flow cytometry

Slit scan flow cytometry was performed on the Heidelberg slit scan flow cytometer that is based on an EPICS V flow sorter equipped with a 5 W argon ion laser (Spectra Physics 2020). A detailed description of the present state of the instrument is published elsewhere [35]. Chromosomes stained with propidium iodide were measured “jet in air” with a flow speed of about 10 m/sec. For slit scanning a “direct through” optics was used. For the used wavelength of 488 nm a theoretical beam width of 28 \(\mu\)m \(\times\) 2 \(\mu\)m (full width at the 1/e\(^2\) points of the laser beam intensity profile) at the intersection point with the flow jet was calculated. The experimentally achieved resolution was sufficient to obtain bimodal profiles for doublets of fluorescence beads of 3 \(\mu\)m diameter each. The laser excitation power was about 700 mW. Chromosome fluorescence was detected by a photomultiplier that was read out by a 100 MHz analog-digital-converter. Thus, data were acquired with a time resolution of 10 nsec [44]. During the measurement the profiles were stored on the harddisk of a PC (80386 SX). Data, evaluation was performed offline [40]. The profiles were classified according to profile length, integrated fluorescence intensity, and centromeric index.

Results

A) Cytogenetics

Fig. 1 shows a RBA-banded karyotype of the abnormal pig cell line. A segment of two thirds of the acrocentric chromosome 15 is translocated to the short arm of chromosome 6 resulting in a metacentric marker chromosome. From 5 normal and 5 abnormal karyotypes the average relative length was evaluated using a MIMAL analyzer software Biocom. These data were included in the karyogram (relative length vs. chromosome number) recently published [19]. Fig. 2 shows the karyogram of the normal pig chromosomes and the abnormal ones 6/15 and 15-. These results indicate that the size of the translocated chromosome 6/15 is close to that of the normal chromosome 1. The centromeric indices (length of the long chromosome arm / total chromosome length) of chromosome 1 and the 6/15 were calculated from the microscope images to be 0.65 and 0.52, respectively.

B) Flow karyotyping and sorting

Fig. 3 shows a one parameter flow karyotype of a chromosome suspension of the abnormal pig cell line. Several different peaks corresponding to one or several chromosome types were distinguished. The flow karyotype is compatible to other flow karyotypes of normal cells previously published [17, 18]. Assuming that the peak position in one parameter flow karyotype is correlated with the DNA content and thus with the chromosome length, this result is supported by the karyogram of Fig. 2.

From the flow karyotype, a sorting window was determined, which was around the largest chromosomes. The sorted fraction was collected on slides and inspected microscopically. According to their size and their centromeric indices, the sorted chromosomes shown in Fig. 4 appeared to be chromosome 6/15 and chromosome 1.

G-banding of the sorted chromosomes provided an accurate identification. As shown in Fig. 5 the banding quality of the chromosomes after flow sorting was compatible to banded chromosomes not subjected to flow cytometry. Many sorted chromosomes were identified to be chromosome 1 and 6/15.

C) Slit scan flow cytometry

There existed several limitations to distinguish chromosome 1 and 6/15 by one parameter flow karyotyping and to sort them with high purity. From karyotyping it was known that there is a sig-
Fig. 1. RBA-banded karyotype of an abnormal pig cell line carrying a 6/15 translocation (arrow) and the corresponding 15-.

Fig. 2. Karyogram of pig chromosomes supplemented by the abnormal chromosomes 6/15 and 15-.
Fig. 3. One parameter flow karyotype of the abnormal cell line carrying the translocation chromosome 6/15. Ordinate: Particle frequency; abscissa: Relative fluorescence intensity (ethidium bromide staining). The sorting window for the largest chromosomes is indicated.

Fig. 4. Flow sorted chromosomes of the sorting window show (a) submetacentric (presumptive chromosome 1) and (b) metacentric chromosomes (presumptive chromosome 6/15).

significant difference in the centromere indices of these chromosomes. Here, 1800 slit scan profiles were registered from a chromosome suspension from the abnormal cell line. The fluorescence intensity was triggered at a certain minimum so that profiles with an integrated intensity compatible with the flow karyotype were measured. Fig. 6 shows several profiles classified to be chromosome 1 and chromosome 6/15 according to their fluorescence intensities and different centromeric indices.

From the registered profiles only those bimodal profiles showing a significant (automatically detectable) centromeric dip were selected as fully intact chromosomes. This means that together with
non-chromosomal fragments as well as acrocentric chromosomes were excluded from consideration, too. The criterion of bimodality of a profile also excluded profiles of chromosomes which were not sufficiently well aligned in the flow system.

This reduction of data to 186 profiles was not a limitation for our examination because chromosome 1 as well as chromosome 6/15 were long, metacentric or submetacentric chromosomes. From the remaining profiles a plot centromeric index vs. fluorescence intensity was calculated (data not shown). All profiles with a high fluorescence intensity (> half maximum intensity) were evaluated according to their centromeric indices and inspected manually. Being aware that the number of evaluated profiles has not yet a statistical significance, three CI-values were found with an accumulated number of profiles. For the presumptive chromosome 1 the CI values 0.69 and 0.62 were found resulting in an average of 0.65. For the presumptive chromosome 6/15 an average CI of 0.52 was found. These results were compatible to the microscopically obtained centromeric indices for chromosome 1 and chromosome 6/15.

Discussion

To produce chromosome specific libraries and for gene mapping, in humans a well established method is to use flow sorted chromosomes [14–16, 45–47]. In this case the library is represented by many chromosomes and the quality of the library
is directly correlated to the sorting purity of a chromosome type, i.e. the discrimination of the chromosome peaks in the flow karyotype. To increase the quality of a library aberrant chromosomes may be sorted (e.g. [48]) and the library might be established from microdissected parts of these chromosomes. This requires only a few chromosomes but the degree of representation of the library is not known. For a detailed molecular analysis, polymerase chain reaction (PCR) and cloning might be insufficient. Thus it might be necessary to perform the molecular analysis directly from the chromosome (see e.g. human chromosome 19 in the Human Genome Project).

In pig the gene map is not well developed. A recently initiated European collaborative project (PiGMaP) should improve our knowledge on gene mapping [49]. It is envisaged to establish a low resolution genetic and physical map and in a final stage to map genes with major economic interest.

Fig. 6. Slit scan profiles of isolated pig chromosomes (ordinate: relative fluorescence intensity; abscissa: time of flight of the chromosome passing the detection volume in units of 20 ns corresponding to chromosome length). 6 bimodal profiles of a data set are shown. The profiles a-c (interpreted to be chromosomes 1) and d-f (interpreted to be marker chromosomes 6/15) were selected by the gating criteria (fluorescence intensity, centromeric index). The horizontal dotted lines in the profiles determine the level of the fluorescence background; the vertical dashed lines show the calculated beginning, intensity maximum, and end of a profile. The big arrow indicates the automatically calculated position of the centromere and the small arrows label the positions of the neighbouring maxima in a bimodal profile.
In the future these localized genes should be used in marker-assisted selection. In this context, the chromosome 6 in pig is important because it carries a gene responsible for a genetic defect known as malignant hyperthermia (MH) [4]. This syndrome found in human and several mammalian species is triggered by halothane anesthesia (HAL) [50]. It is a member of a linkage group including several biochemical markers as blood groups and erythrocyte enzymes.

The sorted chromosome 6/15 is an example of an aberration which might be used for chromosome sorting to produce DNA libraries of pig chromosome 6. One parameter flow sorting has still some limitations in sorting this chromosome with high purity because its fluorescence intensity is similar to chromosome 1 and close to chromosome 2, 6, and 13. Bivariate flow cytometry may give a better discrimination of the chromosomes [20, 21] but requires a two laser flow cytometer. Here it was shown that slit-scan flow cytometry also provides an additional parameter in a one laser system. The centromeric index can be used to distinguish those chromosomes. Because the Heidelberg slit scan flow cytometer (sorter) is still in development [35] only slit scan analysis of a limited number of profiles was performed. The results were calculated automatically and controlled by manual inspection. The results suggest that there is a well detectable difference in the centromeric indices of chromosome 1 and 6/15. The different accumulative values 0.62 and 0.69 for chromosome 1 may be due to chromosome polymorphism. To establish this assumption would require a higher number of slit scan profiles to achieve a better statistic. However, from the results of the slit scan analysis shown here, it seems to become feasible that slit scan sorting will lead to sorting of chromosome 6/15 with higher purity than by univariate flow sorting alone.

Acknowledgements

There is a close correlation to the National Program of Sow Herd Management that examines hypoprolifer boars in France. This collaboration of INRA, Jouy-en-Josas, and the Institute of Applied Physics, Heidelberg, was supported by the PROCOPE program. We thank Burkhard Wickert for his assistance in slit scan measurements. The support of the Deutsche Forschungsgemeinschaft for the Heidelberg slit scan flow cytometer is gratefully acknowledged.
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