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Original article

A repetitive probe for FISH analysis of bovine interphase nuclei

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Abstract – The purpose of this study was to generate repetitive DNA sequence probes for the analysis of interphase nuclei by fluorescent in situ hybridisation (FISH). Such probes are useful for the diagnosis of chromosomal abnormalities in bovine preimplanted embryos. Of the seven probes (E1A, E4A, Ba, H1A, W18, W22, W5) that were generated and partially sequenced, five corresponded to previously described *Bos taurus* repetitive DNA (E1A, E4A, Ba, W18, W5), one probe (W22) shared no homology with other DNA sequences and one (H1A) displayed a significant homology with *Rattus norvegicus* mRNA for secretin receptor transmembrane domain 3. Fluorescent in situ hybridisation was performed on metaphase bovine fibroblast cells and showed that five of the seven probes hybridised most centromeres (E1A, E4A, Ba, W18, W22), one labelled the arms of all chromosomes (W5) and the H1A probe was specific to three chromosomes (ch14, ch20, and ch25). Moreover, FISH with H1A resulted in interpretable signals on interphase nuclei in 88% of the cases, while the other probes yielded only dispersed overlapping signals.

satellite DNA / FISH / bovine / centromere

Résumé – Génération d'une sonde bovine à séquences répétées pour l'analyse en FISH des noyaux bovins en interphase. L'objectif de cette étude est d'isoler des sondes nucléiques bovines spécifiques d'un faible nombre de chromosomes permettant une analyse par hybridation in situ fluorescente (FISH) des noyaux en interphase. De telles sondes présentent un outil précieux pour l'étude d'anomalies chromosomiques d'embryons chez les bovins. Sept sondes ont été générées (E1A, E4A, Ba, H1A, W18, W22, W5) et partiellement séquencées : cinq d'entre elles correspondent à des séquences répétées d'ADN génomique bovin déjà décrites (E1A, E4A, Ba, W18, W5),

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la sonde W22 ne présente à ce jour aucune homologie avec les séquences connues dans "Genbank" et la dernière, H1A (3,5 kb isolée après digestion par l'enzyme *Hin*dIII) présente une homologie significative sur 158 paires de base avec l'ARNm codant pour le 3^{e} domaine transmembranaire du récepteur de la secrétine de rat (*Rattus norvegicus*). L'hybridation in situ fluorescente sur des fibroblastes bovins en métaphase a montré que cinq sondes (E1A, E4A, Ba, W18, W22) hybrident la plupart des centromères, que la sonde W5 marque les bras de tous les chromosomes, et que la sonde H1A est spécifique de trois chromosomes bovins (ch14, ch20 et ch25). De plus, sur noyaux interphasiques, l'utilisation de H1A a permis d'obtenir des signaux interprétables dans 88 % des cas, contrairement aux autres sondes qui donnent des signaux superposés difficiles à interpréter.

ADN satellite / FISH / bovin / centromère

1. INTRODUCTION

Highly repetitive DNA represents a large fraction of most eukaryotic genomes. In mammals, these DNA components are either dispersed throughout the genome or arranged in tandem in large blocks known as satellite DNA [4, 31], which often localise to pericentromeric areas. Probes containing such sequences are considered as powerful tools for detecting numerical chromosome abnormalities in eukaryotic cells [14]. After fluorescent in situ hybridisation (FISH), they display distinct bright signals in metaphase as well as interphase cells [11, 25]. These probes are now routinely used in human clinical cytogenetics for various applications such as studying cellular disorders associated with tumoral cells [1, 24], or genetic aberrations in tumours by analysing a single cell suspension isolated from solid cancer. Thus, this method avoids cell culture that may lead to selective growth of cells with the highest mitotic index [26].

FISH using repetitive probes has also been successfully applied for prenatal diagnosis by analysing uncultured amniotic fluid samples as well as for human preimplantation embryo diagnosis [15, 16, 23]. Recently, the use of repetitive probes enabled preconception diagnosis by FISH analysis of both human oocytes [23, 35] and spermatozoa [25, 32].

In the bovine species, very few repetitive DNA probes are available [33]. This is partly due to the fact that, in animal research programmes, FISH is mainly used for physical genome mapping which involves unique sequence probes to localise genes or genetic markers on metaphase plates [5, 30]. However, in interphase nuclei, FISH using such probes does not allow accurate screening, as signals are usually very weak and may be confused with the background.

With the recent advances in biotechnology associated with embryo transfer in cattle, the study of genetic disorders in preimplantation embryos is becoming highly relevant. Until now, chromosomal abnormalities in bovine embryos have mainly been studied by karyotyping. Whole embryos incubated overnight in colchicine [18, 20], have yielded very variable estimates of the aberrations. For example, the incidence of chromosomal abnormalities in 2-cell bovine embryos has been estimated at 12% by Iwasaki and Nakahara [17] and at 36% by Iwasaki et al. [18]. In the same way, results on karyotypes at the blastocyst stage differ according to studies. The main category observed is mixoploidy, which varies between 44% [18] and 99% [9]. This variability is mainly due to the fact that only a minor proportion of embryonic cells can be analysed. Our study aims

to generate repetitive bovine DNA probes for the screening of chromosomal abnormalities. In this work, we isolated and cloned seven repetitive probes, one of which hybridised to a limited number of chromosomes. This probe represents a promising tool for characterising the genetic status of interphase nuclei, particularly for diagnosing embryos deriving from transgenesis or cloning biotechnologies.

2. MATERIALS AND METHODS

2.1. Probes generation

Bovine genomic DNA was prepared according to Jeanpierre [19], and was digested in six independent reactions by one of six different restriction endonucleases (*Bam*H1, *Sac*I, *Stu*I, *Eco*RI, *Eco*RV and *Hin*dIII). After digestion, restricted genomic DNA was run on 1% agarose gel in 1XTBE buffer overnight at 60 V and stained with ethidium bromide. Prominent bands corresponding to repetitive DNA elements were cut out from the gel, extracted, and purified by the deep freeze / phenol technique [34]. Purified bands were ligated into pGEM4Z (Promega) (pre-disgested with restriction enzymes yielding compatible ends and dephosphorylated) in 20 µL total volume at 16 °C overnight. One µL of ligation was electroporated into 20 µL home-made DH10B electro competent cells grown in 3 mL of LB.

White colonies which likely corresponded to positive transformants containing the ligated band were selected after plating the transformation product on LB plates with Ampicilline (100 $\mu g \cdot mL^{-1}$). DNA was extracted from the positive colonies, digested with the same enzyme used for plasmid digestion, and electrophoresed on a 1% agarose gel to check that the insert was cloned. This was done by ascertaining that the molecular weight of the band was the same as that of the DNA fragment in the ligation product.

Roughly 500 bp of each probe were sequenced from one strand using a universal primer vector. The DNA sequences obtained were compared with Genbank/EMBL using the Blast program.

2.2. Probes labelling

Probes were labelled with biotin 16-dUTP (Boehringer Mannheim, Germany) using a nick translation kit (GIBCO - BRL). Usually, 200 ng of DNA were labelled in a 50 µL mixture containing (1) 0.2 mM each of dATP, dCTP, dGTP, (2) 0.35 mM biotin 16-dUTP, (3) 2.5 U DNA polymerase I, (4) 2 mU DNAse I.

Labelled DNA samples were ethanol precipitated in the presence of sonicated salmon sperm DNA (100 µg). Precipitates were dissolved in 20 µL of hybridisation mixture {60% formamide (Sigma) in SSP (Saline Sodium Phosphate) and 10% dextran sulfate (Pharmacia)} at 37 °C, approximately 30 min prior to denaturation (6 min at boiling temperature) and chilled rapidly on ice.

2.3. In situ hybridisation (ISH)

In situ hybridisation was performed on bovine fibroblasts from a cell line available in the laboratory (59 XX, t4.10). Subsequently, a normal 60XX cell

line was used to validate the results. After BrdU incorporation during the late S phase, air-dried chromosomal preparations were obtained using standard procedures [7]. Protocols used for FISH were as previously described in Bahri-Darwich et al. [2]. Chromosomal DNA was counterstained and R-banded by the direct fluorescent technique described by Lemieux et al. [21]. The slides were screened with a Leica fluorescence microscope and photomicrographs were taken with a Fujichrome 400 Asa colour slide film.

Chromosome identification was performed according to the Texas nomenclature [27]. FISH experiments were carried out with the seven probes and the specificity of each probe was determined on both metaphase and interphase bovine fibroblasts. Each probe was used in five independent experiments, each of which involved scoring 50 interphase and 10 metaphase cells from the 59 XX, t4.10 cell line. In addition, each probe was subsequently hybridised on the 60XX cell line.

3. RESULTS

After digestion of genomic DNA samples by six different enzymes, 11 prominent bands ranging from 0.8 to 3.5 kb were clearly identified. Seven out of the 11 bands were cloned. DNA sequence analysis revealed 90 - 95% identity with Bos taurus repetitive sequences (satellite or SINE) for five of the seven probes. One probe (AF124263) revealed no homology with previously described DNA sequences, and another one (AF118556) derived from the 3.5 kb *Hin*dIII fragment, displayed 90% identity with a *Rattus norvegicus* secretin receptor transmembrane domain 3 from which 158 bp out of 500 were sequenced (Tab. I).

After FISH, all these probes gave clear fluorescent signals in fibroblast metaphases derived from the 59 XX, t4.10 as well as the 60XX cell lines. Each one consisted of a set of small clustered spots producing a bright distinct signal. The W5 probe, which corresponded to the SINE sequence, hybridised to all chromosomes from each metaphase, and painted the entire arms of chromosomes except for the centromeres (Fig. 1a); five other probes hybridised to a large number of centromeres (Fig. 1b), and appeared to be specific for about 25 of the 30 pairs of chromosomes. The last probe (H1A) hybridised to subcentromeric regions of only three chromosomes and provided six strong signals which where clearly identified on all the metaphases analysed (Fig. 1c and 1d).

FISH in interphase fibroblast nuclei showed that all probes except H1A yielded overlapping signals which were widely dispersed through the optical section of the nuclei, making an accurate count impossible (Fig. 2a). In contrast, six fluorescent signals (Fig. 2b) could be clearly counted in 70% (175/250) of the nuclei when hybridised with the H1A clone. Carefull screening of labelled nuclei made it possible to identify 10% (25/250) nuclei with three spots (haploid) and 8% (20/250) nuclei with 12 signals (hyperploid). Some cells (12%; 30/250) could not be scored because of intermingling signals.

Table I. Probe sequence analysis.

Probes	Homologous to*	Localisation
E1A (EcoRI)	Bovine 1.715 satellite (embV00124)*	Most centromeres
E4A (EcoRI)	*Bos taurus* microsatellite DVEPCO46 (gb/U95979)	Most centromeres
Ba (*Bam*H1)	Bovine genomic fragment for 1.709 satellite DNA (emb/X00979)	Most centromeres
H1A (*Hin*dIII)	*Rattus norvegicus* mRNA for secretin receptor (emb/X59132)	Subcentromeric regions of chromosomes 14, 20, 25
W18 (*Sac*I)	Bovine satellite DNA fragment(emb/VOO122)	Most centromeres
W22 (*Eco*RV)	No homology	Most centromeres
W5 (*Stu*1)	*Bos taurus* DNA for SINE sequence Bov-1D (emb/X64126)	All chromatids

* Figures in brackets indicate accession numbers with EMBL/GenBank data base.

4. DISCUSSION

In genomic cytogenetic mapping studies, the objective is to locate a given genomic sequence in a specific chromosomal region; therefore, the most suitable probes are those derived from a unique sequence [30]. Conversely, it has been demonstrated that probes containing centromeric repetitive sequences are the most adequate for detecting numerical aberrations in interphase nuclei [8, 10, 28, 37]. In humans, repetitive sequence probes specific for each chromosome are now commercially available [14], whereas bovine chromosome specific probes come mainly from cosmid and YAC/BAC libraries and are only recently available in a few laboratories [5, 12, 13, 22, 30].

The approach used in this study made it possible to isolate a probe (H1A) suitable for analysing interphase nuclei. Using this probe, abnormal ploïdies were detected in interphase nuclei where 8% and 10% of cells yielded more or less than six signals, respectively. Since no abnormal numbers were detected in metaphases, these discrepancies could stem from the fact that we scored only complete metaphases, incomplete ones being discarded to avoid confusion with a hypotonic treatment artefact. The abnormal numbers observed on interphase nuclei could correspond to hyper- and hypoploid nuclei respectively, and be attributed to the cell line used [6]. However, a hybridisation artefact which may be due to superimposition of signals when two labelled chromosomes lie immediately adjacent or on top of each other, should not be disregarded [11].

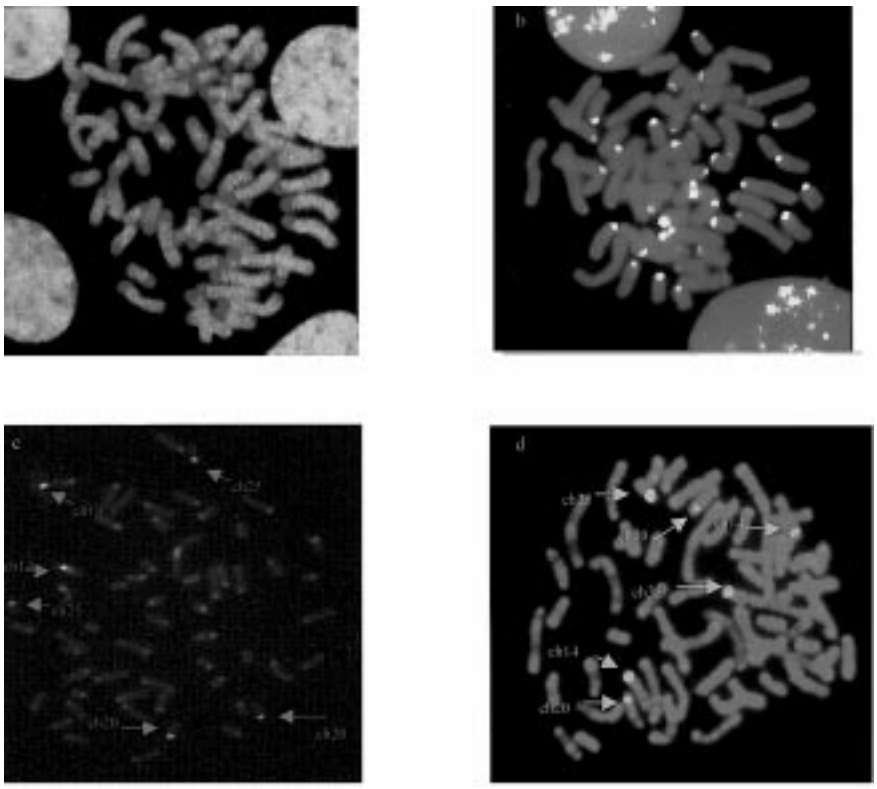

Figure 1. Signals obtained following hybridisation on metaphase cells.
a. A 59XXt4.10 cell line hybridised with the W5 probe.
b. A 59XXt4.10 cell line hybridised with the E1A probe.
c. A 59XXt4.10 cell line hybridised with the H1A probe.
d. A normal 60XX cell line hybridised with the H1A probe.

Apart from these limitations, the H1A probe offers the advantage of hybridising to three chromosome pairs simultaneously. It thus provides an accurate assessment of aneuploidies in interphase nuclei, since it discriminates between monosomy for a single chromosome and complete haploidy.

In conclusion, our work has enabled us to isolate a probe specific to a limited number of chromosomes. Increasing the number of restriction enzymes for a more complete digestion of genomic DNA would probably generate a panel of new probes which could be used for routine detection of numerical chromosome abnormalities in cattle cells. Alternatively, the isolation of sequences specific to chromosome fragments or whole chromosome painting probes by microdissection might be the method of choice to generate a complete set of chromosome specific probes. The feasibility of such an approach is widely reported in humans [3, 29, 36]. These probes would constitute promising tools for analysing the chromosomes of embryonic cells which are now widely used in biotechnology.

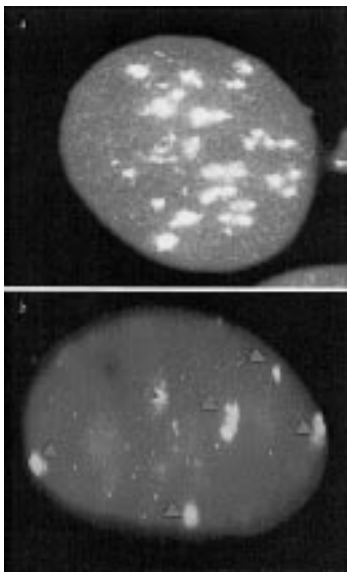

Figure 2. Signals obtained following hybridisation on interphase nuclei.
a. A 59XXt4.10 cell line hybridised with the Ba probe.
b. A 59XXt4.10 cell line hybridised with the H1A probe.

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