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(54) Title: SPECIFIC DNA MOLECULAR PROBES FOR BOS-TYPE MALE GENOME

(54) Titre: SONDES MOLECULAIRES D'ADN SPECIFIQUE DU GENOME MALE DU GENRE BOS

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PATENT OFFICE

5'

ATCAGTGCAGGGACCGAGATGTGCTCCAAGGAGTGTTTATCGGCTGCTT

(A)

(57) Abstract

Synthetic oligonucleotides consist of fragments of a molecular probe comprising a nucleoside sequence of 49 pairs of bases of formula (A). Application: as a molecular probe for sexing the embryos or foetuses of ruminant mammals or as a molecular probe for detecting Y chromosomes in a spermatozoid population.

(57) Abrégé

Oligonucléotides synthétiques constitués par des fragments d'une sonde moléculaire comprenant une séquence en nucléotides de 49 paires de bases, de formule (A). Application: en tant que sonde moléculaire pour la détermination des sexes d'embryons ou de fœtus de mammifères ruminants ou en tant que sonde moléculaire pour le contrôle de la présence de chromosomes Y dans une population de spermatozoïdes.

The present invention relates to specific DNA molecular probes useful for the determination of the sex - or sexing - of ruminant embryos or foeti, particularly of the sub-family of bovines, and in particular of the bos genus, having DNA sequences specific of the male sex, as well as to flanking or primer sequences for the genetic amplification of these probes ; the present invention also encompasses the applications of said probes preferably amplified by means of the above-said flanking sequences, and particularly their applications to the determination of the sex of embryos and of foeti of the above-said ruminants, and for checking the presence of the Y chromosome in a spermatozoa population as well as to the separation of said population into two groups comprising respectively to the Y chromosome and the X chromosome, for artificial insemination.

The international application PCT 86/07095 in the name of THE SALK INSTITUTE BIOTECHNOLOGY INDUSTRIAL ASSOCIATES, filed 30 May 1986 claiming a priority US Serial Number 739 817 of 31 May 1985 and published 4 December 1986, describes nucleic acid probes for the pre-natal sexing of males of the Bos genus, more particularly of the Hereford and Holstein breeds. These probes which have the characteristic of being formed of nucleic acids which hybridize significantly with the total DNA of males of one of the aforesaid breeds of the Bos genus, comprise :

a) Either a segment which has substantially the same sequence as :

- . the smallest PstI fragment of the plasmid pES5 (2),
- . or the smallest fragment PstI of the plasmid PES8,
- . or the 6.2 kbp EcoRI fragment of the genome of lambda-ES6.0 ,

- b) or any segment greater than length of 20 bp of anyone of the segments enumerated under a) ;
- c) or either of the strands of one of the segments enumerated under a) and b) ;
- d) or the single or double stranded RNA, having the same sequence as one of the segments enumerated under a), b) and c) above.



However, although the RNA is mentioned in this PCT International Application (see d) above), it seems that the preferred nucleic acids are the DNA and are constituted by the EcoRI fragment of 6.2 kbp, 4.0 kbp or 2.2. kbp of the genome of the lambda-ES6.0.

In accordance with this International Application, the method of sexing embryos or foeti of the Bos genus consists of :

- placing in contact the DNA of cells of the embryo or of the foetus, in hybridization conditions, with one or several hybridization probes as defined above, each labelled detectably ;
- verifying a significant hybridization is produced between the DNA of the cells of the embryo or of the foetus and the one or more hybridization probes.

According to the PCT International Application concerned, the hybridization probes are obtained by nick-translation and the embryo or foetus DNA would only come from at least 4, but preferably from at least 10, embryo cells.

The PCT International Application 86/07095 describes in addition a method for isolating a DNA clone coming from a genome bank of the males of the species concerned, which consists of :

- screening the genomic library by hybridization of nucleic acids with a male-specific probe comprising the total male DNA and the total female DNA of the species, to identify the clones of the library which comprise the DNA which hybridizes with a fragment containing a male specific DNA segment said screening being performed on a solid support pre-hybridized with the total female DNA of the species, fragmented randomly to have an average length of about 20 or 1000 nucleotides ;
- then identifying the clones of the library identified in the course of the screening, which shelter the DNA which hybridizes considerably more with the total male DNA of the species than with the total female DNA of said species.

In practice, to permit detectable hybridization with the small amounts of embryonic DNA available in the small number of cells which can be taken out from the embryos or foeti to be sexed, it is preferred for the nucleic acids used as probes to be labelled



with radioactive atoms and it seems also preferable to use conjointly two of the above defined probes.

Three male-specific probes were obtained, of which the first corresponds to the RsaI fragment of 5-6 kbp of male bovine DNA, the second to the RsaI-EcoRI fragment of 4 kbp of male bovine DNA and the third to the EcoRI fragment of 2.2 kbp of male bovine DNA, and their sequence in nucleotides has been at least in part identified.

However, due to their length itself, it is difficult to envisage synthesizing such probes, of which nothing indicates, after all, in said PCT International Application, what are the segments of these sequences which are "specifically"

male-specific and are in fact useful for the sexing of embryos, and which have manifestly not been identified.

Applicants have, on their side, isolated a specific DNA molecular probe of DNA specific male genome of mammals, particularly of the Bos genus - which the Inventors have besides succeeded in synthesizing -, of which the hybridization profile (determined by hybridization of said probe with the male genomic DNA digested with EcoRI) shows the presence of a band of the order of 7 kb specific for the male genome of the Bos genus (and absent from the female genomic DNA), which probe - which has received the name bc.1.2. - comprises 49 pairs of bases whose sequence in nucleotides (A) is as follows :

5' 3'
ATCAGTGCAGGGACCGAGATGTGCTCCAAGGAGTGTTTATCGGCTGCTT (A)

or a fragment of said sequence comprising at least 11 nucleotides.

This probe is described and claimed in a Patent Application filed in France in the name of the Applicants on the date of 28 February 1986, under no. 86 02811, and its applications are described and claimed, on the one hand, in said application, and, on the other hand, in a Certificate of Addition to this application, which was filed in France on 9 September 1986, under no. 86 12616.



Within the compass of pursuing their research with the object of stating whether the specificity of the recognition of the male genome could be identified in a segment of the sequence of 49 nucleotides which constitutes the probe according to French Patent Application no. 86 02811, the Inventors have established that within this sequence, segments or fragments of several consecutive nucleotides show the hybridization profile defined in their Patent Application of February 1986 and are specific to the male genome of the Bos genus.

The possibility of having available a probe comprising a sequence of nucleotides of short-length has considerable interest, since it permits the synthesis at will, at industrially acceptable cost prices, of such a probe which, through this fact, is completely pure and does not cause background noises.

In addition, the Inventors have succeeded in identifying the flanking segments of said probes, which has permitted them to amplify the latter and has the consequence of increasing their sensitivity, of ensuring a reliability in the determination of the sex of embryos of the order of 100 %, and of reducing the time necessary for such a determination.

Moreover, the Inventors have been able to establish that the segments or fragments of DNA of short-length which are male-specific, that they have identified and synthesized can play the role both of probes and of flanking segments, or primers.

It is an object of the present invention to provide synthetic oligo-nucleotides which are characterized in that they are constituted by fragments of a molecular probe comprising a sequence of nucleotides of 49 pairs of bases, of the Formula A, below :

$$\begin{array}{ccc} 5' & & 3' \\ \text{ATCAGTGCACGGACCGAGATGTGCTCCAAGGAGTGTTTATCGGCTGCTT} & & \end{array} \quad (A)$$



known in itself, and constituting a DNA segment specific of the male genome of ruminant mammals, particularly of the Bos genus, which fragments are themselves characterized in that they comprise at least 10 bases of said sequence (A), among which are at least 5 consecutive bases of the latter, as well as their complementary fragments or any fragment having at least 60 % homology with said oligo-nucleotides.

According to an advantageous embodiment of the invention, the synthetic oligo-nucleotides according to the invention are characterized in that they are constituted by fragments of the molecular probe (A) which comprise 17 bases, among which are at least 5 consecutive bases of the latter and in that they correspond to formulae (1) to (8) below :

- 15
- (1) $\begin{array}{cc} 5' & 3' \\ \text{GATCAGTGCAGGGACCG} \end{array}$
- (2) $\begin{array}{cc} 5' & 3' \\ \text{CAGGGACCGAGATGTGC} \end{array}$
- 20
- (3) $\begin{array}{cc} 5' & 3' \\ \text{GGACCGAGATGTGCTCC} \end{array}$
- (4) $\begin{array}{cc} 5' & 3' \\ \text{AGATGTGCTCCAAGGAG} \end{array}$
- 25
- (5) $\begin{array}{cc} 5' & 3' \\ \text{CTCCAAGGAGTGTATTAT} \end{array}$
- (6) $\begin{array}{cc} 5' & 3' \\ \text{AGGAGTGTATTATCGGCT} \end{array}$
- 30
- (7) $\begin{array}{cc} 5' & 3' \\ \text{TTATCGGCTGCTTGATC} \end{array}$
- (8) $\begin{array}{cc} 5' & 3' \\ \text{TGGTTGAGCTCAGGGAC} \end{array}$



as well as their complementary fragments or any fragment having at least 60 % homology with said oligo-nucleotides. In another advantageous embodiment of the invention, the synthetic oligo-nucleotides according to the invention are characterized in that they are constituted by fragments of the molecular probe (A) which comprise 20 bases each and have the sequences of nucleotides shown by formulae (9) to (11), below :

(9) $\begin{array}{ccc} 5' & & 3' \\ \text{ATCAGTGCAGGGACCGAGAT} \end{array}$

(10) $\begin{array}{ccc} 5' & & 3' \\ \text{GTGCTCCAAGGAGTGTATTAT} \end{array}$

(11) $\begin{array}{ccc} 5' & & 3' \\ \text{GGACCGAGATGTGCTCCAAG} \end{array}$

as well as their complementary fragments or a fragment of said sequences comprising at least 5 consecutive nucleotides or any fragment having at least 60 % homology with said sequences.

It is emphasized that the sequence (9) corresponds to a fragment of the probe of 49 bp of formula (A) which extends from base 1 to base 20, that sequence (10) corresponds to a fragment of the probe of 49 bp formula (A) which extends from base 21 to base 40 and that the sequence (11) corresponds to a fragment of said probe which extends from the base 11 to base 30.

The International PCT Application no. 86/07095 in the name of THE SALK INSTITUTE BIOTECHNOLOGY INDUSTRIAL ASSOCIATES INC. (abbreviated "SIBIA") filed on 30 May 1986 claiming the priority of an initial application of 31 May 1985 in the United States, described, as indicated above, the partial sequence of total (chromosomic) DNA extracted from bovine cells and particularly describes sequences constituting respectively the *RsaI* fragment of 5-6 kbp and the *EcoRI* fragment of 4 kbp .. the bovine male DNA and this application has given the partial sequence comprising these two fragments.



15

(12)

(13)

(14)

(15)



5' 3'
ACACTGCTGAGCCCGCATCTTGATCCCCGCGCTAAACCTGGCCGGCTCCTACCCCCAGG

(16)

5' 3'
5 ACCGGTCTAGGTCTGGCCCTTGTTGATTCACTCAGCACAGACTCCTGAGCCCCCATCTC

(17)

5' 3'
AGCTGTGATCCCGCAGCTCANTCCCGGCACTGAAGCCACCTCTGGGGCTTGGCCTGCTTC

(18)

10 5' 3'
AGAAGCAGTCTAGGGCCTGCCTTGTTGGAATTCGGATGGATGCTGCCCTTGGGCAACAC

(19)

5' 3'
TTCGGAATTCGGATGGATGCTGCCCTTGGGCAACACCCCCACCGCCCCCAAACTCACACA

15 (20)

5' 3'
AGCTCAACCACGGTGGTTTCTGCCTCTGGCCAAGGCCCTCGACAATCAATCCTGAGCCCA

(21)

5' 3'
20 ATCTCACGCGCTGCACTAAACCACGCAGAGTTCGGCCCTTCCTGAAGTGCCCGTCTAAAG

(22)

5' 3'
ACCAGTCGAGGTCCTGCCCTTTGTGCGGGCCCCCTAGCACAGACTCCCGACCCAAACCTCT

(23)

25 5' 3'
ACACTGCTGAGCCCGCATCTTGATCCCCGCGCTAAACCTGGCCGGGCTCCTACCCCCAGG

(24)

5' 3'
ACCGGTCTAGGTCTGGCCCTTGTTGATTCACTCAGCACAGACTCCTGAGCCCCCATCTC

30 (25)

5' 3'
CCCCCGCCCCGGTGTCTGCACTGATCCAGGCTGGCTCCTGCCCTCGGTCAAGACCCTGG

(26)

5' 3'
35 AGCAGGCTGGCTCCTGCCTTCGTTCAAGCACCTGGCAAAGTAACCGCCGGGAGACTCCAG

(27)



5' 3'
 AGCTGTGATCCCGCAGCTCACTCCCGGACTGAAGCCACCTCTGGGGCTTGGCCTGCTTC
 (28)

5' 3'
 5 AGAAGCAGTCTAGGGCCTGCCTTCTTCGATTCCCGCGACACACACTCCTGAGCCACAGCT
 (29)

It is also an object to the present invention to provide a method of a genic amplification of a molecular probe of DNA specific of the male genome of mammals, particularly of ruminants, more particularly of the sub-family of bovines and specifically of the genus Bos, which probe has one the sequences of nucleotides of formulae (A) and (1) to (29) above, which method of amplification consists in fixing at each of of the ends, respectively, of said molecular probe, a flanking sequence judiciously selected from among the DNA fragments of formulae (1) to (29) such as defined above, by hybridization, then in applying a procedure of enzymatic extension by means of DNA polymerase, followed by a denaturation process, and in repeating the cycle hybridization-extension-denaturation, known under the name of PCR cycle, a sufficient number of times to increase the amount of the sequence constituting the starting molecular probe in an exponential proportion with respect to the number of cycles employed.

The bovine DNA fragments useful as molecular probes of DNA specific of the male genome of the mammals mentioned above, or as flanking segments of these probes, identified above, can be, as the case may require, advantageously amplified according to the present invention, and duly labelled with a radio-isotope or with a suitable non-radioactive substance, are used as described in French Patent Application no. 86 02811 and/or in its Certificate of Addition no. 86 12616, for the sexing of embryos or of foeti or for checking the presence of the Y chromosome in a spermatozoa population, or again for the separation of spermatozoa into two populations of spermatozoa



bearing respectively the Y chromosome and the X chromosome for their use in artificial insemination.

It is also an object to the present invention to provide a method for the preparation of monoclonal or polyclonal antibodies by immunisation of rodent mammals with the spermatozoa fragment bearing the Y or X chromosome checked by hybridization with a bovine DNA fragment of formula (1) to (29), or a probe of formula (A), amplified by one or several primers - or flanking segments - of formulae (1) to (29).

The present invention has in addition, the object of providing specific immunological reagents recognizing antigenic determinants checked by the X or Y chromosomes which have served for obtaining said reagents and expressed at the surface of the spermatozoas, characterized in that they are constituted by antibodies obtained by employing the aforesaid method, if necessary purified.

Besides the foregoing features, the invention comprises yet other features which will emerge from the description which follows.

The invention will be better understood by means of the additional description which follows, which refers to examples of practicing the present invention.

It must however be understood, that these examples are given purely by illustration of the invention, of which they do not constitute in any way a limitation.

EXAMPLE 1

SYNTHESIS AND PURIFICATION OF SYNTHETIC OLIGONUCLEOTIDES OF FORMULAS (1) TO (8) ACCORDING TO THE INVENTION

These eight oligonucleotides of 17 pairs of bases each, comprising at least 5 consecutive bases of the molecular probe (A), which correspond respectively to the formulae (1) to (8) given below :



- (1) 5' GATCAGTGCAGGGACCG 3'
- (2) 5' CAGGGACCGAGATGTGC 3'
- 5 (3) 5' GGACCGAGATGTGCTCC 3'
- (4) 5' AGATGTGCTCCAAGGAG 3'
- 10 (5) 5' CTCCAAGGAGTGTATTAT 3'
- (6) 5' AGGAGTGTATTATCGGCT 3'
- (7) 5' TTATCGGCTGCTTGATC 3'
- 15 (8) 5' TGGTTGAGCTCAGGGAC 3'

are fragments of the segments of 49 bp which has the nucleotide composition (A) indicated above.

- 20 Their chemical synthesis was performed by means of an automatic synthesizer "APPLIED BIOSYSTEMS" by the solid phase phosphoramidite technique.

- The reaction being 95-97 % effective at each step, it was necessary to purify the final product (sub-fragments (1) to (8)) of the intermediate oligonucleotides. About 7 mg of the oligonucleotides mixture in 60 μ l H₂O were separated by electrophoresis in a 20 % polyacrylamide gel + 8 M urea at 700 V, 38mA for 3 hours. The bands corresponding to the 17 mer are marked by means of an aluminum foil coated with silica gel, fluorescent at 254 nm (Merck).
- 30

This portion of the gel was cut off, then the DNA was eluted in 1.5 ml of 0.5 M CH₃COONH₄ solution + 5 mM EDTA with stirring at 37° C for 16 hours.

The supernatant is concentrated to about 300 μ l and the urea



is removed by chromatography on a column 10 cm X 1 cm of Sephadex G-50 (Pharmacia) equilibrated with the 10 mM Tris-HCl pH 7.5 + 1 mM EDTA buffer. The DNA thus isolated is freeze-dried and stored at -20° C.

5 RADIOACTIVE LABELLING (³²p) OF THE SYNTHETIC FRAGMENTS (1) TO (8) (Monostrand)

10 to 20 pM of fragments (1) to (8) in a volume of 3 µl are added to 23 µl of the reaction mixture composed of :
100 mM dithiothreitol (2.5 µl), 10 mM spermidine (2.5 µl),
10 500 mM Tris-HCl incubation buffer pH 7.5 + 100 mM MgCl₂ (2.5 µl), 100 µCi (γ-³²P) adenosine triphosphate-ATP 3000Ci/mmol (Amersham) and 20 U T4 polynucleotide kinase (Boehringer).
After incubation for 30 mn at 37° C, the reaction is stopped with 2µl 400 mM EDTA and the labelled oligonucleotide is separated from the ATP by chromatography on a column with Sephadex G-25.

15 PREHYBRIDIZATION AND HYBRIDIZATION

The filters or blots are prehybridized from 42 to 65° C for 1 to 4 hours with a mixture 5xSSPE containing 0.9 M - NaCl ;
20 50 mM NaHPO₄ pH 7.4 ; 5 mM EDTA pH 7.4 ; 5 X Denhart ; 0.3 mg/ml of sonicated and hydrolyzed salmon sperm DNA ; 0.5 % sodium dodecyl sulfate. The hybridization is carried out in the same mixture. Each of the probes radiolabelled with ³²P, denaturated at 100° C for two minutes, is added to the hybridization solution at 1 to 6x10⁶ pm/ml.

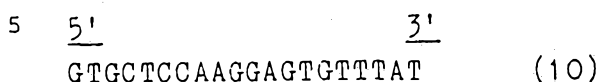
25 The hybridization is performed at 40 to 65° C for at least 1 hour. After hybridization, the filters are washed in a 2 X SSC buffer plus 0.5 % SDS, one or twice at 45° C for 30 minutes. The filters are then placed in a cassette equipped with "CRONEX" (DUPONT) intensifiers, placed in contact with
30 a Kodak XAR-5 film for autoradiography for 1 to 16 hours at -70° C.

EXAMPLE 2

SYNTHESIS AND PURIFICATION OF SYNTHETIC OLIGONUCLEOTIDES OF FORMULAE (9) TO (11) ACCORDING TO THE INVENTION



These three oligonucleotides of 20 pairs of bases each which correspond respectively to the formulae (9) to (11) below :



are fragments of the sequence of 49 pairs of bases of formula (A) of which sequence (9) comprises the bases 1 to 20 of the sequence (A), whilst the sequence (10) comprises the bases 21 to 40 of said sequence (A) and the sequence (11) comprises the bases 11 to 30 of said sequence (A).

The synthesis of the fragments (9) to (11) was carried out by taking the sequence (A) as a model.

This chemical synthesis was performed by means of an "Applied Biosystems" automatic synthesizer by the solid phase phosphoramidite technique.

The reaction was 95-97 % effective for each probe, it was necessary to purify the final product (sub-fragments (9) to (11) - 20 mer) from the intermediate oligonucleotides. About 7 mg of oligonucleotide mixture in 60 μ l H_2O are separated by electrophoresis in the gel of 20 % polyacrylamide + 8 M urea at 700 V, 38 mA for 3 hours. The bands corresponding to the 20 mer are marked by means of an aluminum foil coated with silica gel, fluorescent at 254 nm (Merck). This portion of the gel is cut off, then the DNA eluted in 1.5 ml of the solution 0.5 M $\text{CH}_3\text{COONH}_4$ + 5mM EDTA with stirring at 37° C for 16 hours.

The supernatant is concentrated to about 300 μ l and the urea is removed by chromatography on a 10 cm x 1 cm Sephadex G-50 (Pharmacia) column equilibrated with the 10 mM Tris-HCl pH 7.5 + 1 mM EDTA buffer. The DNA thus isolated is freeze-dried and stored at -20° C.

RADIOACTIVE LABELLING (^{32}P) OF THE (9) to (11) SYNTHETIC
(Single strand FRAGMENTS)



10 to 20 pM of the (9) to (11) fragments in a volume of 3 μ l are added to 23 μ l of reactional mixture composed of : 100 mM dithiothréitol (2.5 μ l), 10 mM spermidine (2.5 μ l), incubation buffer 500 mM Tris-HCl pH 7.5 + 100 mM $MgCl_2$ (2.5 μ l 100 μ Ci ($-^{32}P$) adenosine triphosphate-ATP 3000Ci/mole (Amersham) and 20 U T4 polynucleotide kinase (Boehringer). After incubation for 30 mn at 37° C, the reaction is stopped with 2 μ l 400 mM EDTA and the labelled oligonucleotide is separated from the ATP by chromatography on a column with Sephadex G-50.

PREHYBRIDIZATION AND HYBRIDIZATION

The filters or blots are prehybridized at 55° C for 4 hours with a 5xSSPE mixture containing 0.9 M NaCl ; 50 mM $NaHPO_4$ pH 7.4 ; 5 mM EDTA PH 7.4 ; 5 X Denhart ; 0.3 mg/ml of sonicated and hybridized salmon sperm DNA ; 0.5 % sodium dodecyl sulfate. The hybridization is performed in the same mixture. Each of the fragments radiolabelled with ^{32}P denaturated at 100° C for two minutes, is added to the hybridization solution at 1 to 2 X 10^6 cpm/ml.

The hybridization is performed at 55° C for at least 1 hour. After hybridization, the filters are washed in the 6 x SSC plus 0.1 % SDS buffer, once or twice at 65° C for 20 minutes. The filters are then placed in a cassette equipped with "CRONEX" (DUPONT) intensifiers, placed in contact with a Kodak XAR-5 film for autoradiography for 7 to 36 hours at -70° C.

EXAMPLE 3

SYNTHESIS AND PURIFICATION OF THE SYNTHETIC OLIGONUCLEOTIDES OF FORMULAE (12) TO (29)

Procedure is as described in Example 1, to prepare DNA fragments of formulae (12) to (29).

EXAMPLE 4

DETERMINATION OF THE SEX OF BOVINE EMBRYOS BY HYBRIDIZATION IN SITU WITH THE BIOTINYLATED DNA FRAGMENTS (NON-RADIOACTIVE) OF FORMULAE (1) TO (29)



A. Preparation of the cells

The cells used come from bovine embryos aged 7 to 8 days gestation. After having been dissected, the cells are deposited on a slide and incubated for 10 to 15 minutes in a fixing solution such as the mixture alcohol-acetic acid (3 : 1 v/v) then dried in air. Slides are used immediately or, in the case of postponed sexing, stored at 4° C protected from dust.

B. Biotinylation of the DNA fragments of formulae (1) to (29).

The biotinylation of said fragments is performed by the addition at the 3' end of a biotinylated dUTP or any other biotinylated deoxynucleotide by means of terminal-transferase enzyme (Boehringer).

10 to 20 pM respectively of the synthesized fragments are added to 100 µl of reactional mixture composed of : 140 mM of potassium cacodylate ; 30 mM Tris-HCl pH 7.6 ; 0.1 mM dithiothreitol ; 1 mM CoCl_2 + 0.02 mM of biotinylated dUTP and 1 to 2 units of terminal-transferase enzyme.

After incubation for 1 hour at 37° C, the labelled oligonucleotide is separated by chromatography on a column with superfine Sephadex 25.

C. Technique of Hybridization on slides.

Slides are incubated with 90 to 100 µl of hybridization liquid and coated with a plastic film (to avoid evaporation).

After denaturation for 10 mn in an oven at 100° C, the slides are placed in a moist chamber and incubated at 55° C for 16 hours.

Final composition of the hybridization liquid is :

- . 0.9 M NaCl
- . 5 mM EDTA
- . 50 mM NaHPO_4 pH 7.4
- . 5 x Denhart
- . 0.5 % SDS



(5 x Denhart = 0.1 % BSA, or 0.1 % powdered skimmed milk ;
0.1 % polyvinylpyrrolidone
0.1 % Ficoll) ;

After having removed the plastic film, the slides are washed
5 successively in a 6 X SSC solution (2 X 30 minutes at 55° C),
then in PBS pH 7.4 supplemented with 0.1 % v/v of Triton
X - 100 and 0.5 % (p/v) of powdered skimmed milk (Régilait).

D. Development

The still wet slides after removing the excess fluid are then
10 incubated for 1 hour at 37° C with a goat antibody antibioline or
with a rabbit antibody anti-biotine. This antibody is sold purified
by affinity chromatography (Vector Laboratories ref. SP-3000)
and is used in our tests, diluted to 1:350 in PBS pH 7.4
+ 0.1 % (v/v) Triton X - 100 + 0.5 % (p/v) powdered skimmed
15 milk.

After washing 15 minutes at: ~~the~~ room temperature in PBS pH 7.4 + 0.1 %
(v/v) Triton X - 100 + 0.5 % (p/v) powdered skimmed milk,

the slides are then incubated 1 hour at 37° C, with a conven-
tional rabbit anti-goat antibody or anti-rabbit goat coupled

20 with peroxidase (Biosys ref. BI 2403) diluted 1:40 in PBS pH 7.4
+ Triton 0.1 % + 0.5 % powdered skimmed milk.

The slides are then washed twice for 15 minutes in PBS pH 7.4
containing this time 0.1 % (v/v) of Tween - 20 and finally in
PBS pH 7.4 for 5 minutes.

25 Incubation for 5 to 8 minutes at room temperature follows
with a diaminobenzidine solution (DAB SIGMA ref. D 8001) at
0.5 mg/ml in PBS pH 7.4 containing 0.01 % of H₂O₂.

The precipitate formed by the action of peroxidase on the DAB
is amplified by successive precipitation of gold and silver
30 salts by the method described by BURNS et al. (Journal Clinical
Pathology 1985, 35, 1085-1092) :

In a first stage, 50 to 100 µl per slide of a 2.5 mM solution of
mM solution of Na (NaAuCl BDH ref. 30125 2R)

Na (NaAuCl BDH ref. 30125 2R) are deposited and they are incu-
35 bated 5 mn at room temperature. After washing for 5 mn in



distilled water, the slides are incubated 5 mn at room temperature with 50 to 100 μ l of a 0.1 M solution of Na Sulfide (Na_2S , SIGMA ref. S 2006), rinsed 5 mn with distilled water and incubated 4 to 8 mn with 100 to 300 μ l of silver reagent.

5 The composition of the silver reagent is as follows :

A Na Carbonate	0.24 M	(SIGMA ref. S 4132)
B1 Ammonium Nitrate	13 mM	(SIGMA ref. A 9642)
B2 Silver Nitrate	6 mM	(BDH ref. 303873N)
B3 Dodecatungsto-		

10 silicic acid 1.5 mM (BDH ref. 305453R)

B4 Formaldehyde 37 % 0.6 μ l/ml (SIGMA ref. F 1635)

The silver reagent is prepared by mixing successively and with stirring the solutions B1 - B2 - B3 - B4. The mixture obtained is added 1:1 to the solution A and the reagent thus prepared is used immediately.

15 After the treatment with the silver reagent, the slides are washed with distilled H_2O for 15 minutes, then in acetic acid 1 % (v/v) twice for 15 minutes and finally dyed for 1 minute in Pyronin Y (SIGMA ref. P 7017) 1 % in H_2O , rinsed
20 for some seconds with distilled water, dried in hot air and mounted on DPX.

EXAMPLE 5

CONTROL OF THE QUALITY OF THE SORTING OF A BOVINE SPERMATOZOA
POPULATION WITH THE DNA PROBES SPECIFIC OF THE MALE SEX
25 ACCORDING TO THE INVENTION

Preparation of the cells

Cells used are bullspermatozoa frozen and stored in liquid nitrogen. A small flake is thawed, being about 13,000,000 living spermatozoa. The flakes are reheated for 30 seconds
30 at 37° C and then the sperm diluted in 1 ml of sterile PBS. A series of centrifugations for the purpose of maximum removal of the the freezing medium follows. The suspension of spermatozoa in PBS is first centrifuged for 10 mn at 500 g, the supernatant is sucked out and the spermatozoa taken up
35 again in 1 ml of 0.11 M sodium citrate. They are centrifuged



10 mn at 500 g. The supernatant is sucked out, and the spermatozoa are again in 1 ml of 0.11 M sodium citrate + 5 % DMSO, centrifuged 10 mn at 500 g. The supernatant is sucked out, the spermatozoa are resuspended in 1 ml of 0.11 M sodium citrate + 15 % DMSO. They are centrifuged 10 mn at 500 g. The supernatant is sucked out, the spermatozoa resuspended in 1 ml of 0.11 M sodium citrate + 50 % DMSO. They are centrifuged 10 mn at 500 g. The supernatant is sucked out ; the spermatozoa are resuspended in 1 ml of 0.11 M sodium citrate in Tris-HCl 0.1 M pH 7.4 and centrifuged 10 mn at 500 g. The supernatant is sucked out and the spermatozoa resuspended in 250 µl of the same buffer.

At this stage, the cells are either deposited on a slide (30 - 40 µl of spermatozoa suspension per slide) and treated by hybridization in situ with one of the biotinylated probes of formulae (1) to (29), or deposited on a filter and hybridization by the dot-blot technique with one of the probes of formulae (1) to (29) labelled by means of radio-active nucleotides.

EXAMPLE 6

GENIC AMPLIFICATION IN VITRO OF THE MOLECULAR PROBES OF DNA SPECIFIC OF THE MALE GENOME OF RUMINANT MAMMALS, PARTICULARLY OF THE BOS GENUS.

The genic in vitro amplification process is based on successive cycles - of hybridization of probes with the oligonucleotidic "primers" (the "primers" are flanking segments and, specifically, in the present case, anyone of the fragments of formulae (1) to (29), - extension from the "primers", by means of DNA-polymerase enzyme and - denaturation, for example by a procedure called PCR ("Polymerase chain reaction") described by the CETUS CORPORATION COMPANY (cf. particularly MULLIS et al. , COLD SPRING HARBOR SYMPOSIA ON QUANTITATIVE BIOLOGY, Vol. I-I, 1986, pages 263-272). The cells of a biopsy are deposited in a microtube under a volume of 10 µl in PBS - 35 µl of H₂O are added, then the tube is placed at 95° C for 10 minutes to lyse the cells



and denature the DNA. In the lysate is added 55 μ l of the PCR buffer (50 mM KCl, 10 mM Tris pH 8.3, 8 mM $MgCl_2$, 1 μ M of primer A to be fixed to one of the ends of the probe, 1 μ M of primer B to be fixed to the other end of the probe, 1.5 mM of ATP, 1.5 mM of dCTP, 1.5 mM dTTP, 1.5 mM dGTP. The specimens are then transferred at a suitable temperature for 2 minutes so that the primers are fixed. The extension of the strands is primed by the addition of 4 units of "Taq Polymerase" (CETUS CORPORATION) and incubation at 69° C for 2 minutes. This cycle is repeated 30 to 40 times. The samples are then denaturated in a solution 0.4 N NaOH, 25 mM EDTA and deposited on a nylon membrane manually or by means of a dot-blot apparatus. The repetition of this cycle 30 to 40 times procures an increase of 1×10^{12} times the amount of the starting sequence which constitutes the probe proper. The membranes are then neutralized and dried at 80° C, prehybridized and hybridized at 40 or at 65° C according to the primer employed, in a buffer 5 X SSPE, 5 X Denhart, 0.5 % SDS containing the probes radio-labelled at 5' - end with γ - ^{32}P /ATP by means of the polynucleotide-kinase enzyme, Probes (49 mer or 17 mer or 20 mer) may be either radio-active, or labelled with biotine or with another cold compound. The hybridization is carried out from 40 to 60 ° C for 1 hour. The membranes are then washed twice in 2 X SSPE, 0.1 % SDS at room temperature, followed by the washing at 45° C in the same buffer for 10 minutes. For the radio-active probes, the autoradiography is performed for 30 minutes to 10 hours at -80° C with an intensifying screen. For the cold probes, the demonstration of the hybridization is done by a sequence of immunocytochemical reactions described in Example 3 above. In the case of a specific example in which the primers A and B are respectively the fragment of formula (7) and the fragment of formula (1) :

Amorce A : $\begin{array}{c} 5' \\ \text{TTATCGGCTGCTTGATC} \\ 3' \end{array}$ (7)

Amorce B : $\begin{array}{c} 5' \\ \text{GATCAGTGCAGGGACCG} \\ 3' \end{array}$ (1)



the PCR cycle progresses in the following manner :

The biopsy cells are deposited in a microtube in a volume of 10 μ l in PBS with the addition of 35 μ l of H_2O , as described above ; however, the tube is placed at 95° C for only 2 minutes and the operations are continued as described above, except that the samples are transferred for 2 minutes at 55° C and the incubation with the "Taq Polymerase" takes place at 69° C for 2 minutes and the cycle is repeated 40 times.

With other primers it will be recommended to perform the incubation with the "Taq Polymerase" at a lower temperature, of the order of 37° C for example, or to perform the incubation in certain cycles at this temperature, and at 55° C in other cycles, the durations being opportunely slightly prolonged or reduced according to the primers employed.

The probe employed may be a probe selected from among the probes or fragments of formulae A and 1 to 29. It will be possible, for example, with the primers of formulae (7) and (1) to use a probe of formula (4) :

5' 3'
AGATGTGCTCCAAGGAG (4)

In such a case, the prehybridization is carried out as well as the hybridization, at 65° C.

With this system, a very intense male signal is obtained, distinct from that of females which is very weak, starting from 125 picograms of DNA, namely the equivalent of 25 cells and directly from cells, with 50 male cells, after 30 minutes autoradiography.

In the same manner, by using, for example, as a probe a fragment of formula (2) or formula (3), with the primers respectively of formulae (8) and (5), a specifically male signal is obtained, from 125 picograms of DNA.

Although the foregoing description has essentially taken account of the part played by the probes of formulae (1) to



(29), for the determination of the sex of bovine embryos, the checking of the presence of the Y chromosome in a spermatozoa population and the separation of the latter into two groups comprising respectively the Y chromosome and the X chromosome for their use in artificial insemination,

5 easily understood that this role may extend to other animals to the extent that the latter show DNA sequences specific of the male sex similar to those of said bovine embryos ; in the same way, the role of probes of formulae (1) to (29) as described in the foregoing, is given purely by way of non-limiting example and may be extended to the research of other specific sequences of the male sex.

10 Thus as emerges already from the foregoing, the invention is in no way limited to those of these types of performing embodiments and uses which have just been described more explicitly ; it encompasses on the contrary all modifications which may come to the spirit of a technician in the art, without departing from the scope, nor of the range of the present invention.



THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A synthetic oligonucleotide specific for the male genome of ruminants, particularly of the Bos genus comprising 17 bases and having a nucleotide sequence selected from the group consisting of the formulae 1 to 8 below:

- | | | |
|-----|-------------------|-----------|
| | <u>5'</u> | <u>3'</u> |
| (1) | GATCAGTGCAGGGACCG | |
| | <u>5'</u> | <u>3'</u> |
| (2) | CAGGGACCGAGATGTGC | |
| | <u>5'</u> | <u>3'</u> |
| (3) | GGACCGAGATGTGCTCC | |
| | <u>5'</u> | <u>3'</u> |
| (4) | AGATGTGCTCCAAGGAG | |
| | <u>5'</u> | <u>3'</u> |
| (5) | CTCCAAGGAGTGTTTAT | |
| | <u>5'</u> | <u>3'</u> |
| (6) | AGGAGTGTTTATCGGCT | |
| | <u>5'</u> | <u>3'</u> |
| (7) | TTATCGGCTGCTTGATC | |
| | <u>5'</u> | <u>3'</u> |
| (8) | TGGTTGAGCTCAGGGAC | |

and their complementary sequences, or any fragment having at least 60% homology with said oligonucleotides.

2. A synthetic oligonucleotide comprising 20 bases and having a nucleotide sequence selected from the group consisting of the formulae (9) to (11) below:

- | | | |
|------|----------------------|-----------|
| | <u>5'</u> | <u>3'</u> |
| (9) | ATCAGTGCAGGGACCGAGAT | |
| | <u>5'</u> | <u>3'</u> |
| (10) | GTGCTCCAAGGAGTGTTTAT | |
| | <u>5'</u> | <u>3'</u> |
| (11) | GGACCGAGATGTGCTCCAAG | |

and their complementary sequences, or any fragment having at least 60% homology with said oligonucleotides.

3. A synthetic oligonucleotide constituted by a DNA fragment specific for the male genome of a ruminant mammal, particularly of the Bos genus, adapted to be associated with the ends of the nucleotides according to Claim 1 or Claim 2



for the purpose of amplification, which fragment has a nucleotide sequence selected from the group consisting of the formulae (12) to (29) below:

5' 3'
ACCGGTCTAGGTCTAGCCCTTGTTTCGGGACGCACATCACAGGCTCCTGAGCCCCCATCTC
(12)

5' 3'
GGTAGAGCCCGCATCTCGGTCCCCGCGGTGAACCTGGCCCGGTTCTACCCAGTC
(13)

5' 3'
CTGAGGCCTCCTCTCGCACTCTGCCCTGAAGCCGTGCGCGTCCTGCCCTCGATCAGACCT
(14)

5' 3'
GACAGGGTGGGCCGTGCCCTCGTTCAGTCACCCGGGAAACACTGCTGAGCCCGCATCTTG
(15)

5' 3'
ACACTGCTGAGCCCGCATCTTGATCCCCGCGCTAAACCTGGCCGGCTCCTACCCCCAGG
(16)

5' 3'
ACCGGTCTAGGTCTGGCCCTTGTTTCGATTCACTCAGCACAGACTCCTGAGCCCCCATCTC
(17)

5' 3'
AGCTGTGATCCCGCAGCTCANTCCCGGCACTGAAGCCACCTCTGGGGCTTGGCCTGCTTC
(18)

5' 3'
AGAAGCAGTCTAGGGCCTGCCTTGTTTCGGAATTCGGATGGATGCTGCCCTTGGGCAACAC
(19)

5' 3'
TTCGGAATTCGGATGGATGCTGCCCTTGGGCAACACCCCCACCGCCCCAACTCACACA
(20)

5' 3'
AGCTCAACCACGGTGGTTTCTGCCTCTGGCCAAGGCCCTCGACAATCAATCCTGAGCCCA
(21)

5' 3'
ATCTCACGCGCTGCACTAAACCACGCAGAGTTCCGCCCTTCCTGAAGTGCCCGTCTAAAG
(22)

5' 3'
ACCAGTCGAGGTCCTGCCCTTTGTGCGGGCCCCTAGCACAGACTCCCGACCCAAACCTCT
(23)



5' 3'
ACACTGCTGAGCCCGCATCTTGATCCCCGCGCTAAACCTGGCCGGGCTCCTACCCCCAGG
(24)

5' 3'
ACCGGTCTAGGTCTGGCCCTTGTTGATTCACTCAGCACAGACTCCTGAGCCCCCATCTC
(25)

5' 3'
CCCCCGCCCGGTGTCTGCACTGATCCAGGCTGGCTCCTGCCCTCGGTCAAGACCCTGG
(26)

5' 3'
AGCAGGCTGGCTCCTGCCTTCGTTCAAGCACCTGGCAAAGTAACCGCCGGGAGACTCCAG
(27)

5' 3'
AGCTGTGATCCCGCAGCTCACTCCCGGACTGAAGCCACCTCTGGGGCTTGGCCTGCTTC
(28)

5' 3'
AGAAGCAGTCTAGGGCCTGCCTTCTTCGATTCCCGCGACACACACTCCTGAGCCACAGCT
(29)

and their complementary sequences, or any fragment having at least 60% homology with said oligonucleotides.

4. A method for the gene amplification of a DNA target sequence specific to the male genome of mammals, particularly ruminants, comprising the steps of fixing to each of the ends, respectively, of the sequence to be amplified, a flanking sequence selected from the DNA fragments of formulae (1) to (29) according to any one of Claims 1 to 3, by hybridization, then applying an enzymatic extension procedure by means of DNA-polymerase, followed by a denaturation procedure, and repeating the hybridization-extension-denaturation cycle a sufficient number of times to increase the amount of the target sequence in an exponential proportion with respect to the number of cycles employed.

5. A method according to Claim 4 in which the target sequence is specific for the sub-family of bovines.

6. A method according to Claim 4 or claim 5 in which the target sequence is specific to the genus *Bos*.

7. Use of a fragment of DNA of any one of the formulae (1) to (29) according to any one of Claims 1 to 3, labelled with a detectable marker, as a molecular probe for



the determination of the sex of embryos or of fetuses of a ruminant mammal.

8. Use of a DNA fragment of any one of the formulae (1) to (29) according to any one of Claims 1 to 3, labelled with a detectable marker, as a molecule probe for checking the presence of the Y chromosome in a spermatozoa population.

9. Use of a DNA fragment of any one of the formulae (1) to (29) according to any one of Claims 1 to 3, labelled with a detectable marker, as a molecular probe for checking the sorting of spermatozoa in two populations of spermatozoa bearing respectively the Y chromosome and the X chromosome for their use for artificial insemination.

10. Use of a DNA fragment of any one of the formulae (1) to (29) according to any one of Claims 1 to 3, advantageously suitably labelled and amplified by means of one or several of the DNA fragments of formulae (1) to (29) according to any one of Claims 1 to 3, for the determination of the sex of embryos or of fetuses of ruminant mammal.

11. Use of a DNA fragment of any one of formulae (1) to (29) according to any one of Claims 1 to 3, advantageously suitably labelled and amplified by means of one or several of the DNA fragments of formulae (1) to (29) according to any one of Claims 1 to 3, for checking the presence of the Y chromosome in a spermatozoa population.

12. Use of a DNA fragment of any one of the formulae (1) to (29) according to any one of Claims 1 to 3, advantageously suitably labelled and amplified by means of one or several of the fragments of formulae (1) to (29) according to any one of Claims 1 to 3, for checking the sorting of spermatozoa into two spermatozoa populations bearing respectively the Y chromosome and the X chromosome for their use for artificial insemination.

13. Use according to any one of Claims 7 to 12 in which the mammal is of the sub-family bovines.

14. Use according to Claim 12 in which the mammal is of the genus Bos.

15. A process for preparation of monoclonal or



polyclonal antibodies comprising the step of immunization of a rodent with a fraction of spermatozoa bearing chromosome Y or X as checked by hybridization with a DNA fragment of any one of formulae (1) to (29) according to any one of Claims 1 to 3.

16. A process for the preparation of monoclonal or polyclonal antibodies comprising the step of immunization of a rodent with a spermatozoa fraction bearing the Y or X chromosome as checked by hybridization with a DNA fragment of any one of the formulae (1) to (29), according to any one of Claims 1 to 3, or a probe of formula (A) amplified by one or several DNA fragments of formulae (1) to (29) according to any one of Claims 1 to 3.

17. A monoclonal or polyclonal antibody produced by the method of Claim 15 or Claim 16.

18. An immunological reagent comprising an antibody according to Claim 17, which recognizes an antigen expressed at the surface of spermatozoa.

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