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(57) Abstract

The invention of the present application concerns nucleotide probes for the diagnosis of trisomy 21. The probes are based on cosmid contigs. The cosmids contain polynucleotides mapping to 21q22.

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DETECTION OF TRISOMY 21; MATERIALS AND METHODS

This invention relates to the detection of trisomy 21.

Down's Syndrome is one of the most commonly occurring conditions resulting from a chromosomal abnormality. Most usually it is caused by the inheritance of three chromosome 21s instead of the usual two. In some cases, it is caused by the inheritance of two complete chromosome 21s plus the inheritance of a part of a further chromosome 21 which is connected to a part of another chromosome.

Prenatal diagnostic tests are available to detect trisomy 21, although there are a number of disadvantages with those available to date. Presently, a maternal blood sample is taken and investigated for levels of both the hormone human chorionic gonadotrophin (hCG) and alphafetoprotein (AFP). The relative levels of hCG and AFP in the blood sample at a given stage of pregnancy, and depending upon the mothers age and weight, enables the obstretician to determine the risk of the fetus having trisomy 21. Thus, the mother is told that she has a 1 in 2000 or a 1 in 500 etc. chance of carrying a trisomy 21 fetus. If the chances are sufficiently high, e.g. 1 in 300 or greater, the mother is then offered a fetal screen by amniocentesis. This is a technique in which a sample of the amniotic fluid surrounding the fetus is withdrawn for analysis. Obviously since the technique is invasive, there is some risk (albeit small)

that it could endanger the pregnancy.

The amniotic fluid contains small numbers of fetal cells. The test for trisomy 21 involves standard cytogenetic analysis on these cells. The cells have to be cultured, both to increase the cell sample size and so that they are actively dividing. This is because the tests depend on the analysis of metaphase chromosomes in hypotonic-treated, acetic-alcohol-fixed, air-dried preparations. The number of chromosome 21s within each cell are then simply counted. The problem with the fetal screen by amniocentesis is that it is time consuming and mothers have to wait for 2-3 weeks for results. The wait is understandably harrowing. Even more importantly, if trisomy 21 is detected, the pregnancy will be already somewhat advanced making elected abortion a more difficult and painful experience.

In view of these problems, attention has turned to the possibility of using labelled nucleotide probes to test the fetal cells for chromosome abnormalities. Since such a test would not necessarily require the culture of fetal cells, the results could be quickly available.

Although it has been shown that fluorescence in situ hybridization with chromosome-specific probes can detect the number of copies of a particular chromosome in interphase nuclei (e.g. Tkachuk D.C., et al 1991, Genet. Anal. Tech. Appl. 8, 67-74), unfortunately chromosome 21 analysis in interphase appears to be more difficult than for many other chromosomes because no reliable chromosome 21-specific repeat probe is available.

A number of nucleotide probes have already been used in an attempt to establish a quick, reliable diagnostic test for trisomy 21.

A successful diagnosis of trisomy 21 has been reported by using a biotinylated probe produced from pre-reassociated restriction-digested DNA from flow-sorted 21 chromosomes (Julien C., et al 1986, Lancet ii, 863-864). However, this procedure is cumbersome and unsuitable for routine diagnostic use.

Complex nucleotide probes comprising chromosome 21 libraries have been used for this purpose. However, they have not been entirely satisfactory. This appears to be due partly to the fact that chromosome 21 is the smallest chromosome and partly because it shares sequences preferentially with the other satellited chromosomes 13,14,15 and 22. Also, the association of satellited chromosomes within nuclei will increase the tendency of individual hybridization domains to merge with one another. This makes techniques which depend on chromosome painting with chromosome 21 specific libraries and chromosome 21-only interspecific somatic cell hybrids difficult to interpret. Also techniques which seem to work satisfactorily in lymphocytes sometimes fail in amniotic fluid cells. Trisomy 21 has been demonstrated by chromosome painting (Pinkel et al 1988 Proc. Natl. Acad. Sci., USA Vol 85, pp.9138-9142; Lichter, P., et al 1988 Hum. Genet 80: 224-234 and Proc. Natl. Acad. Sci. USA 85: 9664-9668; Kuo, W.L. et al 1991 Am.J. Hum. Genet. 49: 112-119) however, the frequencies of trisomic cells

showing three domains was low and wildly variable (10% to 50%). The main problems were that the hybridisation signals were weak in some samples and that domain definition was unclear in many cells. Therefore, the techniques described are clearly not yet robust enough to be reliable for routine screening in prenatal diagnosis.

Alternatively, unique probes comprising selected single copy sequences from chromosome 21 may be used. Plasmid based probes can only hold insert base sequences in the order of 5kb. The signals using such plasmid probes are quite small and difficult to detect in comparison to non-specific background signals. Cosmid based probes which can hold base sequence inserts in the order of 40kb are more useful. Nevertheless, they have been found to be only 50-70% reliable at best and this is still unsatisfactory for diagnostic purposes. Yac (yeast artificial chromosome) based probes have the advantage that they can hold base sequence inserts of large size e.g. several hundred kb. In theory then, such probes should be more specific and therefore reliable. In practice however, it has been found that a lot of yeast nucleotide sequences cross-react with human nucleotide sequences leading to non-specific background signals.

The present invention overcomes these problems by use of a nucleotide probe based on a cosmid contig. A cosmid contig comprises 2 or more cosmids each holding a base sequence insert. The individual base sequence inserts of each cosmid of the contig overlap with one another. In this way, the cosmid contig provides a probe

which spans a stretch of nucleotide sequence greater than each individual cosmid insert.

The cosmid contig probe is for chromosome 21. It maps to 21q22 of chromosome 21 and may comprise a nucleotide sequence which hybridises with the chromosome 21 nucleotide sequence provided by probe pGSB3.

Thus, the present invention provides a nucleotide probe for the diagnosis of trisomy 21 which comprises a cosmid contig having at least first and second cosmids which contain different, but overlapping respective first and second polynucleotides which map to 21q22 and wherein at least one of the first and second polynucleotides comprises a nucleotide sequence which hybridizes with part or all of the 6.4kb EcoRI fragment in D21S19 which is characteristic of 21q22.3.

Both of the first and second polynucleotides may comprise a nucleotide sequence which hybridizes with part or all of the 6.4kb EcoRI fragment.

The cosmid contig may comprise at least a further cosmid which contains a respective further polynucleotide which overlaps with at least one of said first and second polynucleotides. The further polynucleotide may overlap with both of said first and second polynucleotides. The further polynucleotide may comprise a nucleotide sequence which hybridizes with part or all of said 6.4kb EcoRI fragment.

The present invention also provides a cosmid contig which comprises a plurality of cosmids which each contain different polynucleotides, each of the polynucleotides

overlapping the polynucleotides of the other of said cosmids in the plurality, and mapping to 21q22 and wherein each of the polynucleotides comprises a nucleotide sequence which hybridizes with part or all of said 6.4kb EcoRI fragment.

The polynucleotides may comprise part or all of, or part or all of a substantially homologous variant of a chromosome 21 specific sequence as present in cosmids cCMP21.2, cCMP21.3, cCMP21.4, cCMP21.5 and cCMP21.6 which are each characterised by the restriction map as shown in figure 1.

The present invention also provides a nucleotide probe for the diagnosis of trisomy 21 which comprises a cosmid contig having at least first and second cosmids which contain different, but overlapping respective first and second polynucleotides which map to 21q22 wherein at least one of said polynucleotides comprises part or all of, or part or all of a substantially homologous variant of, a chromosome 21 specific sequence as present in cosmids cCMP21.2, cCMP21.3, cCMP21.4, cCMP21.5 and cCMP21.6, each being characterized by the restriction map as shown in figure 1.

The cosmid contig may comprise cosmids cCMP21.2 and cCMP21.6, the polynucleotides of which span a substantially 55kb portion of 21q22 of chromosome 21.

The cosmid contigs may be tagged with a detectable label.

Also provided are diagnostic preparations e.g. kits and reagents, having as a component a cosmid contig as

provided by the present invention. The preparations may contain other materials for detecting the specific binding of the cosmid contig to chromosome 21.

Also provided are methods which comprise using a cosmid contig as provided by the present invention in a diagnostic technique.

Also provided are ex vivo methods for the diagnosis of trisomy 21 which comprise the steps of (a) isolating a sample of cells of fetal origin; (b) applying a cosmid contig as provided by the present invention to the cell sample; and (c) detecting the number of hybridization signals indicating the specific binding of the probe to chromosome 21 in the nuclei of individual cells in the sample.

The method may also comprise the step of washing to cell sample to remove any cosmid contig not hybridized to chromosome 21.

Of course, the cosmid contig probe as herein provided, enables the identification of other sequences specific to chromosome 21 (and more specifically to 21q22 of chromosome 21). These sequences will locate to either side of the chromosome 21 sequence which hybridises with the cosmid contig herein provided. This can be achieved by chromosome walking using cosmid end clones (Wahl GM et al 1987 Proc. Natl. Acad. Sci 84: 2160-2164). Any such sequences identified by use of the cosmid contig and which are specific to chromosome 21 and more particularly specific to the region 21q22 will also be useful as diagnostic probes.

For example, the cosmid contig can be further extended by chromosome walking in which overlapping cosmids at the extremes of the contig are examined by restriction enzyme digestion and gel electrophoresis for the presence of fragments derived from the non-overlapping extremes of the two cosmids. These fragments are then excised from the gel, labelled and suitable fragments used to screen a cosmid library for new cosmids which will overlap with but extend out from the current contig. Alternatively, cosmids from the extremes of the contig can be partially sequenced and oligonucleotides synthesised to allow polymerase chain reaction (PCR) amplification of DNA sequences unique to the original cosmids. Further cosmids from a library can then be screened by PCR with such primers to identify new cosmids which will overlap with but extend out from the current contig.

Thus the present invention also provides these further probes for trisomy 21 identified by use of the cosmid contig herein provided.

In order that the present invention is more fully understood it will be described in more detail with reference to the following figures and examples provided by way of illustration only.

Figure 1 shows a restriction map for a 55kb cosmid contig based on restriction enzyme digestion with EcoRI, BamHI, ClaI, XhoI and EcoRV in single and double digest combinations. The figure also shows the relative location of the EcoRI 6.4kb D21S19 insert;

Figure 2 shows flow-sorted chromosome 21 library, pBS-21, and DOP-PCR 21 paints hybridized with normal cultured cells. (a,b) Hybridization with the chromosome 21 library showed signals on chromosome 21 and cross-hybridization signals on the other acrocentric chromosomes and diffuse domains in many interphase cells. (c,d) Hybridization with DOP-PCR 21 paints showed problems similar to those of the pBS-21 library;

Figure 3 shows distributions of signals in cultured normal interphase cells (lymphocytes) hybridized with different types of chromosome 21-specific probes;

Figure 4 shows hybridization of a YAC clone, HY70, with normal cultured cells. (a,b) Hybridization with HY70 using human placental DNA as a competitor showed signals on chromosome 21q22 and cross-hybridization on the short arms of other acrocentric chromosomes; these cross hybridization signals in the interphase cells are similar to the true chromosome 21 signals. (c,d) Hybridization with HY70 using total yeast DNA as a competitor showed that these cross-hybridizations can be suppressed;

Figure 5 shows a comparison of the hybridization efficiency of cosmids containing different sizes of insert. Hybridization with the cosmid cCMP21.2 (insert size = 46kb) showed two signals in 69% of normal interphase cells, whereas in hybridization with the cosmid cCMP21.5 (insert size = 35kb) only 56% of normal interphase cells showed two signals. The results suggest that the larger the insert, the more cells that showed

complete hybridization;

Figure 6 shows the cosmid contig hybridization. (a,b) Normal cultured cells showed signals on chromosome 21q22 and two compact signals in interphase nuclei. (c,d) Uncultured amniotic fluid cells showed three signals in trisomy 21 cells and two signals in normal cells.

Obtaining Cosmid Contigs

All procedures are standard and as described by Sambrook J. et al in "Molecular cloning a Laboratory Manual (2nd Ed) Cold Spring Harbor Laboratory Press.

A cosmid library was prepared from total human genomic DNA extracted from a lymphoblastoid cell line, although any tissue providing cells containing chromosome 21 would be acceptable for use.

Broadly, genomic DNA for library construction was partially digested with MboI, dephosphorylated and fractionated on a 25-45% sucrose velocity gradient. The appropriate fractions were ligated into the Bam HI site of the cosmid vector pWE15, packaged and then plated out at a density of 125000 colonies per 200mm x 200mm nitrocellulose filter. Replica filters were produced from the primary filters for screening purposes and the master filters were stored on a 25% glycerol-containing medium at -70°C. Library construction was essentially as described by Warner, J.P. et al 1992 in Molecular Genetic Analysis of Populations: a Practical Approach, Oxford IRL Press 189 223.

Approximately 10^6 primary recombinants (8 x 125000)

were screened using the 6.4kb EcoRI fragment from the nucleotide probe D21S19 (D21S19 is the Genome Database Accession number; the probe also has the designation pGSB3) specific to chromosome 21. Further information on the probe D21S19 is given in Human Gene Mapping II, London Conference, Cytogenet. + Cell Genet. Vol.58, Nos.3-4, 1991 Ed. Klinger H.P. D21S19 is on public deposit at European Collection of Animal Cell Cultures (ECACC), PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts SP4 OJG, United Kingdom under accession number P92022713 (deposit made on 27 February 1991). Details of the probe are given below:

Chromosome region	21q 22.3-qter
Name	pGSB3 or D21S19
Vector	pUC9
Site	EcoRI
Size	6.4
POLYMORPHISM	
Enzyme	Pst 1
Constant bands	1.2
Allele	A1, A2
Length	3.6, 2.2
Frequency	0.4, 0.96
N	58
PIC	0.07

Further details of the probe are also given by Stewart G.D. et al 1985. Nucl. Acids Res. 13, p4125-4132. The first screening with D21S19 enabled the

identification of 9 positive colonies. Cosmid DNA was prepared from 5 of the recombinants following secondary screening and clone purification. These five cosmids were mapped to 21q22 by fluorescence in situ hybridization. The presence of the D21S19 insert was shown by Southern analysis of EcoRI digested cosmid DNA. The five cosmids were restriction=mapped and shown to be a set of overlapping clones making up a 55kb contig containing the full 6.4kb EcoRI fragment from D21S19. Figure 1 shows the five cosmids, their inter-relationships and restriction enzyme maps. The complete 55kb contig is contained in the two overlapping cosmids cCMP21.2 and cCMP21.6. These two cosmids were used together as a probe as described hereafter. The cosmid DNA was prepared according to a standard alkaline lysis method.

As can be seen from figure 1, each individual cosmid contains a nucleotide sequence insert of approximately 40kb. Together however, they map a chromosome 21 nucleotide sequence of approximately 55kb. The nucleotide sequence inserts of cosmids cCMP21.3 and cCMP21.4 map within the nucleotide sequence insert of cosmid cCMP21.2. Likewise, the nucleotide sequence insert of cosmid cCMP21.5 maps within the nucleotide sequence insert of cosmid cCMP21.6. Therefore a contig based on cosmids cCMP21.2 and cCMP21.6 alone, will be sufficient to give the maximum available base coverage of chromosome 21.

The restriction enzyme maps of the cosmids as shown

in figure 1 provides a finger print which characterises the cosmids. Restriction enzymes were supplied by Boehringer Mannheim and used according to the manufacturers instructions.

Use of the Cosmid Contig and comparitive studies

Aneuploidy analysis of chromosome 21 by in situ hybridisation with fluorescent labelled probes based on chromosome 21 specific libraries, DOP-PCR paints, YAC clones and a cosmid contig as herein provided, was carried out as a comparative study.

1. Probes

(i) The cosmid contig used was based upon cosmids cCMP21.2 and cCMP21.6 as provided above.

(ii) The chromosome 21 specific library (pBS-21) was constructed by subcloning Hind III digested inserts from a recombinant DNA phage library containing large inserts from chromosome 21, into Bluescribe plasmids. The plasmid DNA was prepared according to standard methods.

(iii) Chromosome 21 specific YACs HY70, HY94, HY7 and HY8 which map to 21q22 were supplied as a gift. The insert sizes of both HY70 and HY94 are approximately 300kb. The insert sizes of HY7 and HY8 are approximately 90kb. Yeast cells growing in 100ml of YEPD (1% yeast extract, 2% trytone, 2% glucose) overnight, were centrifuged down at 5000 rpm for 5 min. Cells were resuspended in 3.0ml of 0.9 M sorbitol, 0.1 M EDTA pH 7.5. 15 ul of 10mg/ml zymolyase 100T was then added and the mixture incubated at 30°C for 60 min. The cells were then centrifuged at 4000 rpm for 5 min. The cell pellet

was resuspended in 5.0ml of 50 mM Tris pH 7.4, 20 mM EDTA, 1% SDS and incubated at 65°C for 30 min and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a clean centrifuge tube and 2 x volumes of ethanol were added and the DNA pellet resuspended in 3.0ml of 10 mM Tris pH 7.4, 1 mM EDTA, 50 ug/ml RNase and incubated at 37°C for 30 min. Phenol/chloroform extraction was carried out, followed by precipitation with isopropanol. The DNA was centrifuged down and the pellet air dried and resuspended in TE (50 mM Tris pH8.0, 1 mM EDTA).

The probe DNA was chemically modified by nick translation with biotin-11-dUTP (Sigma Chemical Co., Poole, Dorset, UK) and precipitated by ethanol. Labelled probe DNA was resuspended in TE to a final concentration of 100 ng/ul.

(iv) Chromosome 21 DOP-PCR paints. The paints were generated by DOP-PCR (Degenerate Oligonucleotide Primed Polymerase Chain Reaction) direct amplification of a small number of flow-sorted chromosome 21s. Biotinylation was achieved by subjecting 300 ng of the primary PCR product in reaction supplemented with dUTP-11-biotin to further amplification.

2. In Situ Hybridisation and Detection

Cells

Uncultured amniotic fluid samples were obtained from East Anglian Regional Cytogenetics Laboratory. Approximately 1.0-2.0 ml of each amniotic fluid sample was used for in situ hybridisation. Samples were

centrifuged down at 400g for 8 min. The cell pellet was resuspended in 2 ml of saline. 2 ml of methanol: acetic acid (3:1) was then added. Cells were re-centrifuged down, resuspended in 0.5 ml of methanol: acetic acid (3:1) and stored at -20°C for use. All the samples had been stored at least 6 months. The longest storage was 10 months. Before in situ hybridisation, cells were pelleted by gentle centrifugation for 30 seconds in a microcentifuge. 40-100 ul of the supernantant was retained for making the cell suspension, which was used for making air-dried drop preparations. Usually 2-3 slides were made from each sample.

Lymphoblastoid cell lines were used for chromosome preparation according to standard methods. Briefly, amethopterin to a final concentration 10^{-7} molar was added to cultures which were ready for harvesting. Cultures were then incubated for a further 16-18 hours, BrdU (5-bromo,2-deoxyuridine) was added to the cultures to a final concentration of 10 ug/ml, followed by further incubation for 5 hours. Finally, Colcemid was added to a final concentration of 10µg/ml followed by incubation for a further 30 min. before harvesting. Cells were centrifuged at 200g for 8 min., resuspended in 75 mM potassium chloride solution and incubated at 37°C for 8 min. These cells were fixed in 2 changes of methanol: acetic acid (3:1) and stored at -20°C for use. Before in situ hybridisation, the cell suspension was dropped onto ethanol cleaned slides.

Hybridisation

Hybridisation of the cell and chromosome preparations was carried out using a modification of the procedure described by Pinkel et al 1988 Proc. Natl. Acad. Sci Vol 85, P.9138-9142. The slides were treated in methanol: acetic acid (3:1) for 60 min at room temperature (RT), and then air dried. The target DNA was denatured by immersing the slides in 70% formamide/2xSSC (1xSSC = 0.15 M NaCl and 0.15 M sodium citrate, pH 7.0) for 2 min at 65°C and then immediately quenched in cold 70% ethanol. The slides were dehydrated in ethanol series (70%, 70%, 90%, 90%). The hybridisation mixture (15 µl total volume consisting of 50-100 ng biotinylated probe DNA, 5-10 ug of human placental DNA, 50% formamide, 2xSSC, 10% dextran sulphate, 1% SDS and 1x Denhardt's) was denatured at 70°C for 10 min and incubated at 37°C for 30-60 min. The hybridisation reaction was sealed under a coverslip and incubated at 42°C for 14-16 hours. After hybridisation, the coverslips were removed by rinsing in 2xSSC and the slides were washed twice on 50% formamide/1xSSC at 42°C for 5 min, washed twice in 2xSSC at 42°C for 5 min and blocked in 4XTNFM (4xSSC, 0.05% Tween-20, 5% non-fat milk, spun to remove solid before use) at 37°C for 20-30 min. The slides were then treated with alternating layers of fluoresceinated avidin and biotinylated goat anti-avidin, both at 5 ug/ml concentration in 4XTNFM buffer, for 30 min at 37°C until two layers of avidin were applied. After each incubation in avidin or anti-avidin, the slides were washed three times at 42°C in 4XTNFM for 5 min. After the last washes

in 4XTNFM, the slides were rinsed in 4XSCC, 0.05% Tween-20 for 5 min, dehydrated by passage through an ethanol series and then mounted in 0.6 ug/ml propidium iodide (PI) and 3 ug/ml DAPI in Citifluor (Citifluor Ltd) for chromosome preparations and 0.6 ug/ml PI in Citifluor for preparations of uncultured cells.

For the cosmid contig, equal amounts of cosmids cCMP21.2 and cCMP21.6 were used to provide the total DNA of 50-100ng per slide.

Microscopic Analysis

Fluorescence microscopy with appropriate filters (480nm/546nm) was used to analyse the slides. Confocal laser scanning microscopy (MRC-600, Biorad Microscience Ltd) was used to collect and store the images. Approximately 200 cells were scored to give a distribution of signals in cultured interphase cells hybridised with the different types of chromosome 21 specific probes. The study of uncultured amniotic fluid cells by in situ hybridisation with the chromosome 21 cosmid contig was carried out in a blind study. Samples were coded and scored without knowledge of the karyotype. Approximately 50 cells per slide were counted, although approximately 100 cells were counted if the signal distribution suggested an abnormal result. Degenerate nuclei without signals, and nuclei that overlapped or which were partly covered by cytoplasm were not scored and are therefore not included in the data presented. In addition, closely paired signals from G2 nuclei were scored as indicating one chromosome.

Results

(A) Cultured lymphoblastoid cells were used to assess the usefulness of different types of chromosome 21 probes for interphase analysis.

Hybridisations with the pBS-21 library revealed hybridisation signals on chromosome 21 except 21p and cross hybridisation signals on the short arms of chromosome 13,14,15 and 22 (Fig.2a) in metaphase chromosomes and diffuse signals in many nuclei (Fig.2b). The signal distribution in interphase nuclei showed one signal in 35% of cells, two signals in 53% of cells, and three signals in 6% cells (Fig.3). The 53% of cells which could not be analysed because of the diffuse nature of the signals was often as high as 25%.

DOP-PCR paints showed more intense signals on chromosome 21, but suffered from the same problem of cross hybridisation signals as the pBS-21 library (Figs. 2c and 2d). The results for signal distribution in the nuclei were similar to those obtained using the pBS-21 library (Fig 3).

Hybridisation with YACs HY70, HY94, HY7 and HY8 on metaphase spreads showed hybridisation signals on chromosome 21q22. Hybridisation signals were also shown on the short arms of other acrocentric chromosomes when human placental DNA was used as a competitor DNA (Fig.4a). The hybridization signals from YACs HY70 and HY94 were brighter on chromosome 21q22 than those from YACs HY7 and HY8. The signals in the interphase nuclei could not be interpreted, as cross hybridisation signals

on other acrocentric chromosomes were easily mistaken for real chromosome 21 signals (Fig.4b). Hybridisation with YAC HY70 using total yeast DNA as competitor, only showed signals on chromosome 21q22 (Fig.4c) and two clear signals in approximately 65% normal interphase cells (Fig.4d and Fig.3).

Hybridization with each of the five cosmids, which have different sizes on insert, showed that the larger the insert, the more cells that showed two signals in normal interphase cells (fig.5). Hybridization with the cosmid cCMP21.5 (insert size 35kb) showed two signals in 56% of normal cultured interphase cells and hybridization with the cosmid cCMP21.2 (insert size 46kb) showed two signals in 69% of normal cultured interphase cells. However, hybridization with the cosmid contig showed two signals in 85% of normal cultured interphase cells. The hybridization signals produced by the contig were intense and compact on chromosome 21q22 (fig.6a) and in interphase nuclei (fig.6b).

Fifty samples of uncultured amniotic fluid cells were studied by fluorescence in situ hybridization with the cosmid contig. The samples were randomly selected and the slides were coded and scored without knowledge of the karyotypes. One sample could not be analysed as it contained only a few degenerate and squamous cells, which are unsuitable for in situ hybridization. In the remaining 49 samples, four trisomy 21 cases were detected and confirmed later by reference to the previous cytogenetic analysis. The distribution of signals in the

nuclei of trisomic cells showed one signal in 8-11% of cells, two signals in 31-39% of cells, three signals in 34-51% of cells (fig.6c) and four signals in 7-10% of cells. This compared with one signal in 27% of cells, two signals in 61% of cells (fig.6d), three signals in 6% of cells and four signals in 4% of cells in normal samples (table 1). No false-positive or false-negative diagnoses were made.

A useful prenatal test should be reliable, robust, and reproducible. Fluorescence in situ hybridization with chromosome-specific repeat probes can detect the number of copies of particular chromosomes in interphase nuclei rapidly and without cell culture (e.g. Guyot B. et al., 1988, Prenat. Diagn. 8 435-493). This technique has practical application in prenatal diagnosis. However, the reliability of the analysis by fluorescence in situ hybridization depends largely on the specificity of the probe and the hybridization efficiency. Generally speaking, chromosome-specific repeat probes are almost ideal for interphase analysis, as the hybridization signals produced by these probes are intense and well localized (e.g. Guyot et al., 1988 supra). Although a repeat probe which hybridizes to both chromosome 13 and 21 is available (Devilee P. et al., 1986 Cytogenet. Cell Genet. 41, 193-210). It is difficult to use for interphase analysis as it does not distinguish between the chromosomes and twice the number of signals are usually produced. Besides, the copy number of the repeat sequence targeted by this probe on chromosomes 13 and 21

varies in size among normal individuals and may be very weak or totally absent (Weier and Gray, 1992 Annal. Cell Pathol. 4, 81-86). As a result, interphase analysis of chromosome 21 with this probe is unreliable. The successful detection of chromosome 21 abnormalities in metaphase and cultured interphase cells using a chromosome 21-specific library has been reported (Pinkel et al., 1988, Proc. Natl. Acad. Sci., 85, 9138-9142). However, the results have been found to be inadequate for uncultured amniotic fluid cells. In this application related to a cosmid contig nucleotide probe, a comparison is made for different types of chromosome 21-specific probes for aneuploidy analysis of chromosome 21 in interphase cells. The chromosome 21-specific library, pBS-21, showed hybridization signals on chromosome 21 and cross-hybridization signals on other acrocentric chromosomes. It gave diffuse signals in interphase nuclei partly because chromosome 21 shares repeat sequences with other acrocentric chromosomes, and partly because of the phenomenon of satellite association which increases the tendency of diffuse domains to coalesce. These problems make chromosome 21 libraries unsuitable for interphase analysis. The use of 21 DOP-PCR paints showed similar problems to those of the pBS-21 library, even though the DOP-PCR paints gave more intense signals on chromosome 21. The techniques described above are clearly not reliable enough for the analysis of uncultured amniotic fluid cells.

Cosmids and YAC clones have large inserts and could

be used as alternatives to repeat probes. The YAC clone (HY70) showed intense hybridization signals on chromosome 21q22, but also signals on the short arms of other acrocentric chromosomes when human placental DNA was used as a competitor. These cross hybridization signals in interphase cells can be easily mistaken for the true chromosome 21 signals and make it impossible to determine the copy number of chromosome 21. They were shown to arise from yeast ribosomal repeat sequences, as total yeast DNA used as a competitor was found to suppress them (figs.4c and 4d). The difficulty in obtaining sufficient total yeast DNA for in situ suppression and the instability of YAC clones renders YACs less suitable for general clinical use.

Hybridization with the 21q cosmid contig was clearly the most suitable of the alternatives tested, as it is highly chromosome 21-specific and it produced two clear and intense signals in more than 85% of normal interphase cells, compared with single cosmids in which only 56-69% normal interphase cells showed two signals (fig.5). The hybridization efficiency was highly reproducible and the signals produced by the cosmid contig were brighter than those produced by single cosmids. As this cosmid contig hybridizes to the subregion of chromosome 21q22, both signals could be resolved in the common centric translocations involving duplication of chromosome 21q. This makes the probe particularly useful, as it is able to detect trisomy 21 caused both by the abnormal inheritance of an entire chromosome 21 and the

inheritance of only an extra part of chromosome 21. In Robertsonian translocations, it is generally the long arm of chromosome 21 which is abnormally present. Inheritance of partial trisomies where part of chromosome 21 including 21q22 is translocated to another chromosome may also lead to Down's syndrome. Probes not mapping to this area, would not detect abnormalities caused by such translocations.

So far, the most common type of cells used for prenatal diagnosis of chromosomal abnormalities are amniotic fluid cells obtained by amniocentesis. Cells isolated from uncultured amniotic fluid are of variable quality. Most of the cells (>80%) are degenerate sqaumous epithelial cells which are unsuitable for in situ hybridisation; others are covered by cytoplasm, which causes unacceptable background signals. This means that to be diagnostically useful and to avoid the culture requirement, the nucleotide probe must be both efficient and highly specific for chromosome 21. The present applicants studied 49 samples of uncultured amniotic fluid cells by fluorescence in situ hybridization with the 21q cosmid contig. The signal distributions in uncultured amniotic fluid cells were more variable between individual samples compared with the signal distributions in cultured cells. Trisomy 21 cases can be detected easily, as 34-51% of trisomic cells showed three signals and 31-39% of cells showed two signals compared with <10% of normal cells which showed three signals and >60% of normal cells which showed two signals (table 1).

Only 50 cells need to be counted to give a diagnostic signal distribution. In two samples, only 20-30 cells were available for analysis but the signal distributions clearly showed them to be normal. The successful diagnosis of the four trisomy 21 cases in the 49 uncultured amniotic fluid samples with this contig suggests that it is a suitable probe for diagnostic use. As analysis of uncultured cells has the potential to provide a result within 48 h or amniocentesis, it should improve the patient acceptability of second-trimester prenatal diagnosis.

The above discussion only refers to the use of the contig probe on fetal cells derived from amniotic fluid. Of course the probe may be effectively used on any cells of fetal origin. For example, the contig probe could be used on fetal cells isolated from maternal blood thus providing a diagnostic procedure which does not endanger the pregnancy. Several methods exist for isolating fetal nucleated erythrocytes from maternal blood for use in prenatal diagnostic procedures. The probe can be used on any source of fetal cells.

As can be seen from the above, the use of the cosmid contig as herein described provides a reliable and robust probe for the diagnosis of trisomy 21. It offers the opportunity for rapid prenatal diagnosis of trisomy 21 as there is no requirement for cell culture.

The cosmid contig probe provided more reliable results than those obtained with the other types of probes.

Distribution of Signals in Uncultured Amniotic Fluid Cells

Hybridized with the 21q Cosmid Contig

TABLE 1

signals / cases Trisomy 21	1 no.	%	2 no.	%	3 no.	%	4 no.	%	5 no	%	6 no	%
F91/0728	8	(8.9)	32	(35.6)	38	(42.2)	10	(11.1)	2	(2.2)	0	
F91/0804	12	(11.3)	41	(38.7)	36	(34.0)	12	(13.2)	3	(2.8)	1	(0.9)
F91/0942	8	(8.3)	31	(31.6)	50	(51.0)	7	(7.1)	1	(1.0)	1	(1.0)
F91/1300	9	(7.6)	46	(38.7)	50	(42.0)	11	(9.2)	1	(0.8)	1	(0.8)
Normal	756	(27.4)	1695	(61.5)	169	(6.1)	116	(4.2)	17	(0.6)	2	(0.1)

CLAIMS:

1. A nucleotide probe for the diagnosis of trisomy 21 which comprises

a cosmid contig having at least first and second cosmids which contain different, but overlapping respective first and second polynucleotides which map to 21q22 and wherein

at least one of said polynucleotides comprises a nucleotide sequence which hybridizes with part or all of the 6.4kb EcoRI fragment in D21S19 which is characteristic of 21q22.3.

2. A nucleotide probe according to claim 1 wherein both of said the first and second polynucleotides comprise a nucleotide sequence which hybridizes with part or all of said 6.4kb EcoRI fragment.

3. A nucleotide probe according to claim 1 or claim 2 which comprises at least one further cosmid which contains a respective one further polynucleotide wherein said one further polynucleotide overlaps with at least one of said first and second polynucleotides.

4. A nucleotide probe according to claim 3 wherein said further polynucleotide overlaps with both of said first and second polynucleotides.

5. A nucleotide probe according to claim 3 or

claim 4 wherein said further polynucleotide comprises a nucleotide sequence which hybridizes with part or all of said 6.4kb EcoRI fragment.

6. A nucleotide probe according to any one of claims 1 to 5 which comprises

a plurality of cosmids each containing different polynucleotides, each of the polynucleotides overlapping with the polynucleotides of the other of said cosmids and mapping to 21q22 and wherein

each of said polynucleotides comprises a nucleotide sequence which hybridizes with part or all of said 6.4kb EcoRI fragment.

7. A nucleotide probe according to any one of the preceding claims wherein said polynucleotides comprise part or all of, or part or all of a substantially homologous variant of a chromosome 21 specific sequence as present in cosmids cCMP21.2, cCMP21.3, cCMP21.4, cCMP21.5 and cCMP21.6 each being characterised by the restriction map as shown in figure 1.

8. A nucleotide probe for the diagnosis of trisomy 21 which comprises

a cosmid contig having at least first and second cosmids which contain different, but overlapping respective first and second polynucleotides which map to 21q22 and wherein

at least one of said polynucleotides comprises part

or all of, or part or all of a substantially homologous variant of, a chromosome 21 specific sequence as present in cosmids cCMP21.2, cCMP21.3, cCMP21.4, cCMP21.5 and cCMP21.6, each being characterised by the restriction map as shown in figure 1.

9. A nucleotide probe according to claim 7 or claim 8 which comprises cosmids cCMP21.2 and cCMP21.6, the polynucleotides of which together span a substantially 55kb portion of 21q22 of chromosome 21.

10. A nucleotide probe according to any one of claims 1 to 9 or a nucleotide probe obtained by the process of claim 14 which is tagged with a detectable label.

11. A diagnostic preparation having as a component a nucleotide probe according to any one of claims 1 to 10 or a nucleotide probe obtained by the process of claim 14.

12. A method which comprises using a nucleotide probe according to any one of claims 1 to 10 or a nucleotide probe obtained by the process of claim 14 in a diagnostic technique.

13. An ex vivo method for the diagnosis of trisomy 21 which comprises the steps of

(a) isolating a sample of cells of fetal origin,

(b) applying a nucleotide probe according to any one of claims 1 to 10 or a nucleotide probe obtained by the process of claim 14 to the cell sample,

(c) detecting the number of hybridization signals indicating the specific binding of the probe to chromosome 21 in the nuclei of individual cells in the sample.

14. A process for obtaining a nucleotide probe for the diagnosis of trisomy 21 which comprises

using at least part or all of, or part or all of a substantially homologous variant of, a chromosome 21 specific sequence as present in cosmids cCMP21.2, cCMP21.3, cCMP21.4, cCMP21.5 and cCMP21.6, each being characterised by the restriction map as shown in figure 1 to obtain one or more polynucleotide sequences characteristic of chromosome 21.

Fig. 1.

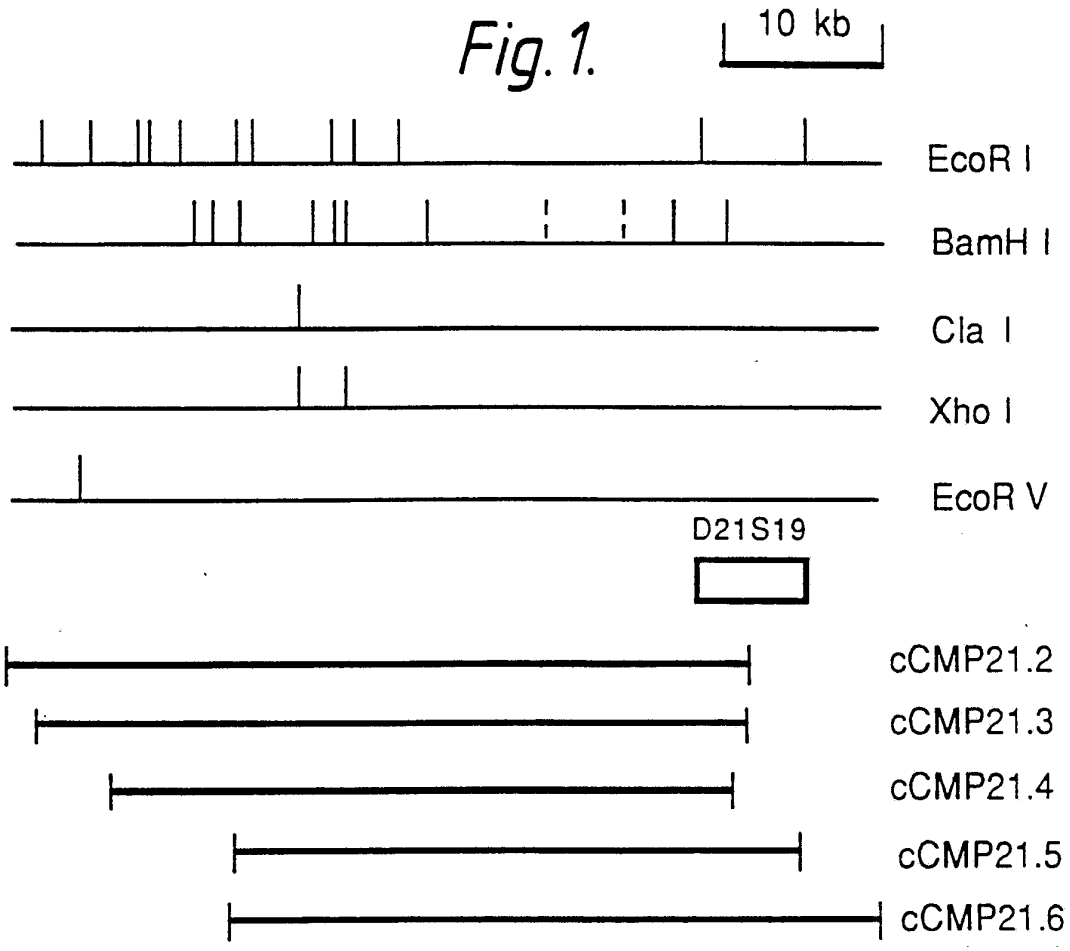

10 kb
EcoR I
BamH I
Cla I
Xho I
EcoR V
D21S19
cCMP21.2
cCMP21.3
cCMP21.4
cCMP21.5
cCMP21.6

Fig. 5.

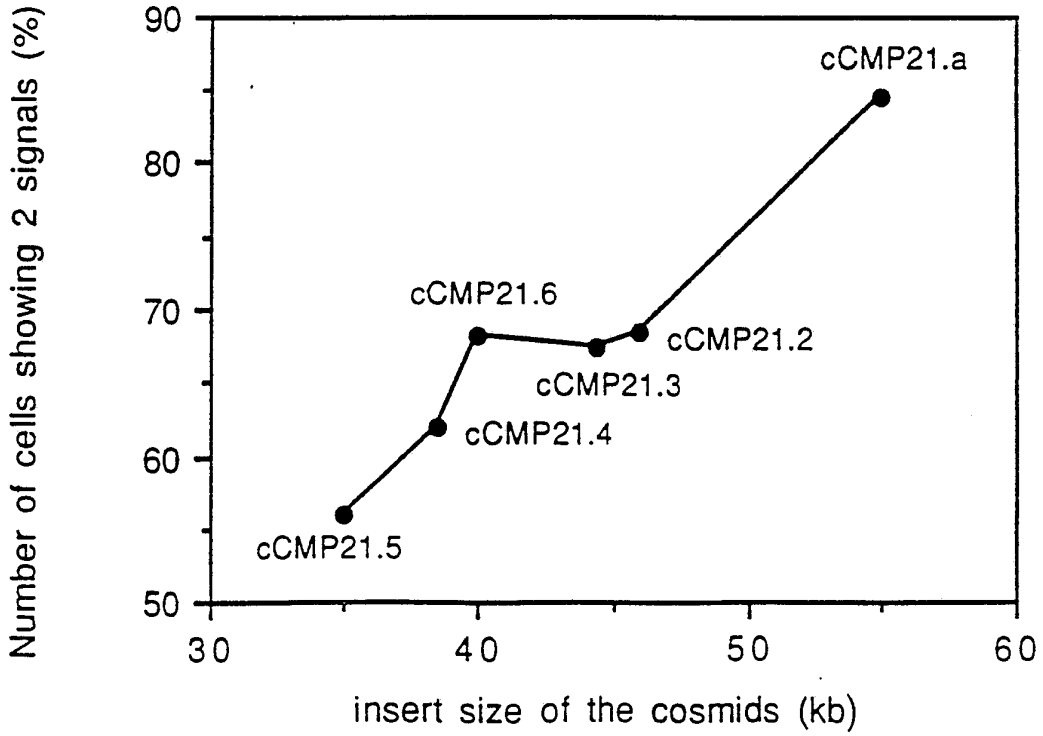

Number of cells showing 2 signals (%)
90
80
70
60
50
30
40
50
60
insert size of the cosmids (kb)
cCMP21.a
cCMP21.6
cCMP21.2
cCMP21.3
cCMP21.4
cCMP21.5

Fig. 2.

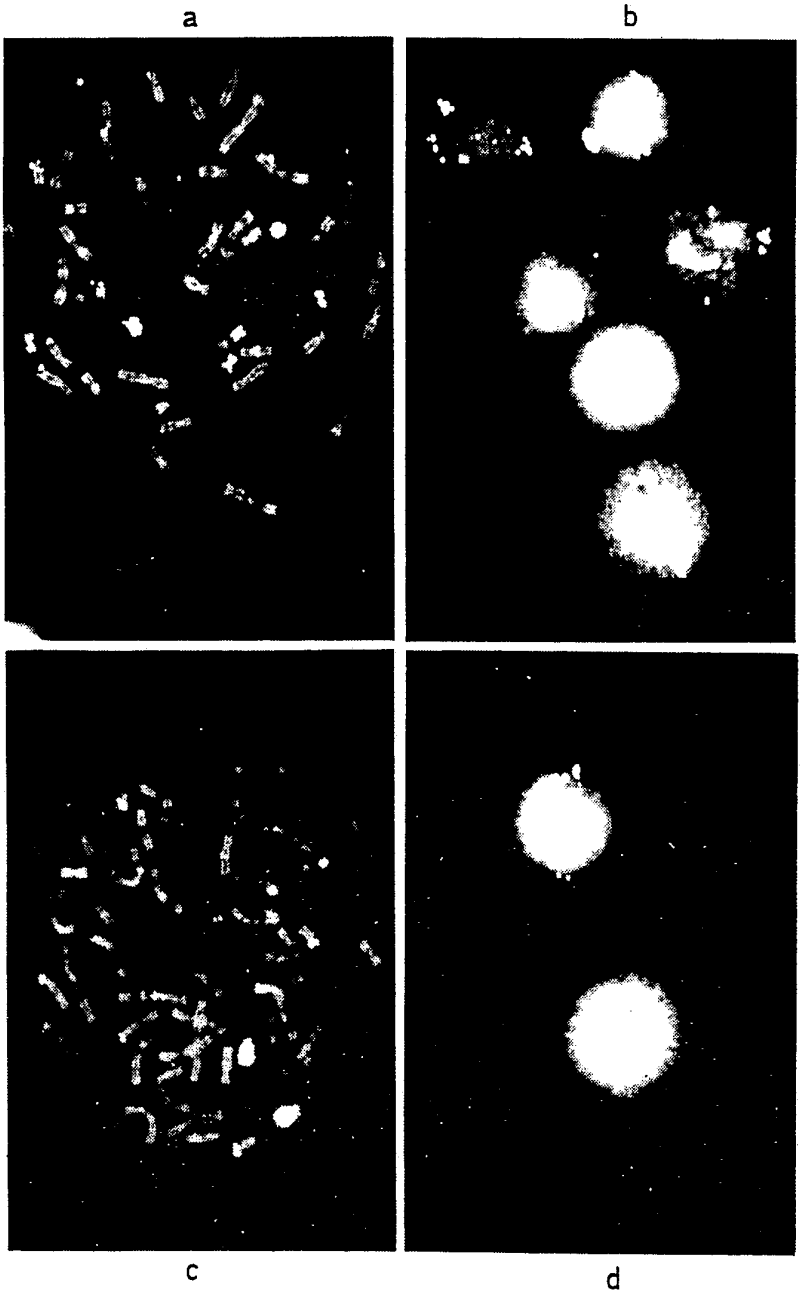


Fig.3.

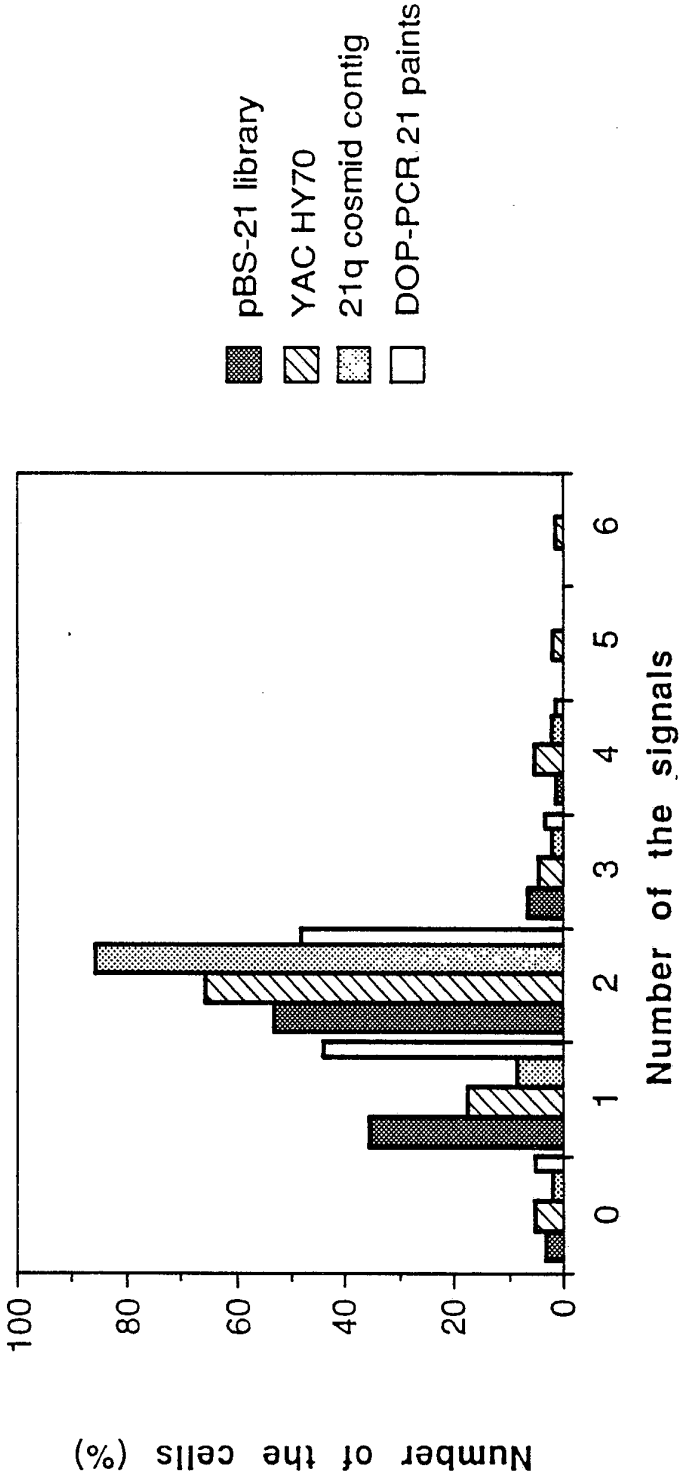

pBS-21 library
YAC HY70
21q cosmid contig
DOP-PCR 21 paints
Number of the signals
0
1
2
3
4
5
6
Number of the cells (%)
0
20
40
60
80
100

Fig. 4.

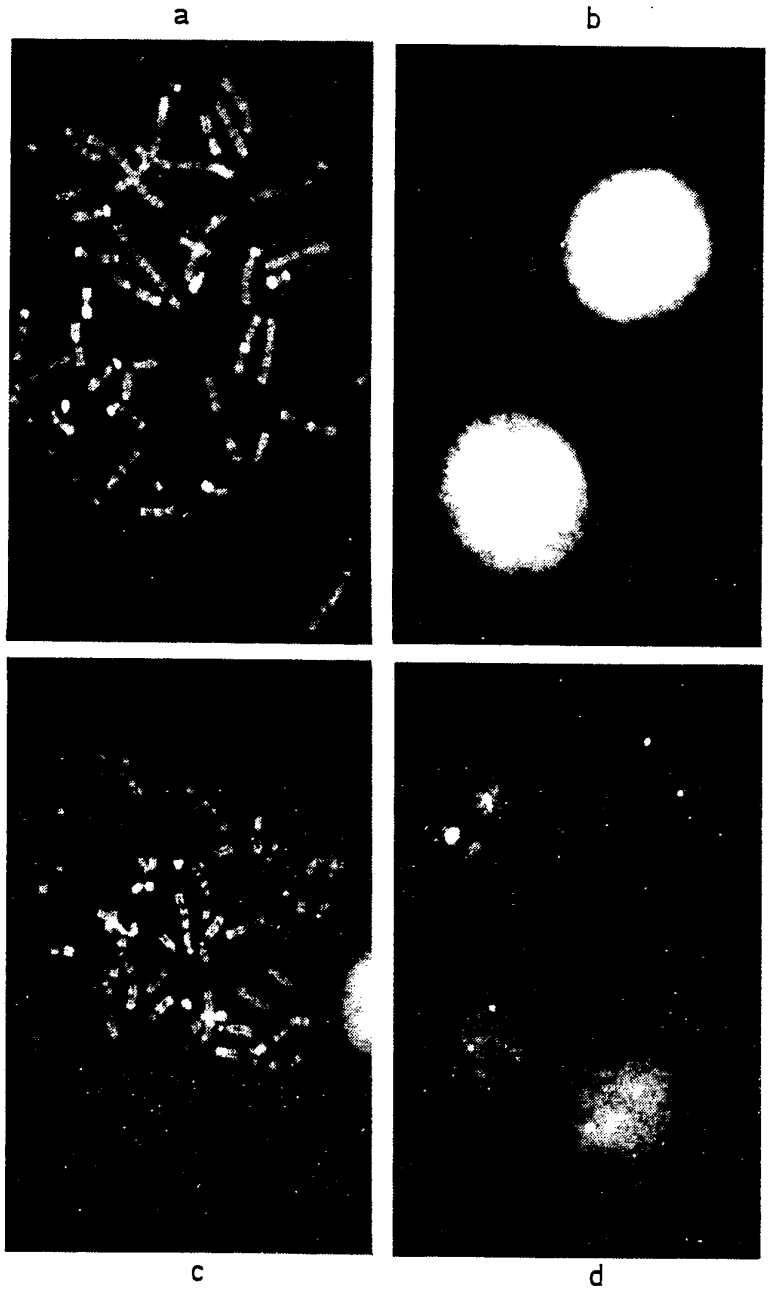
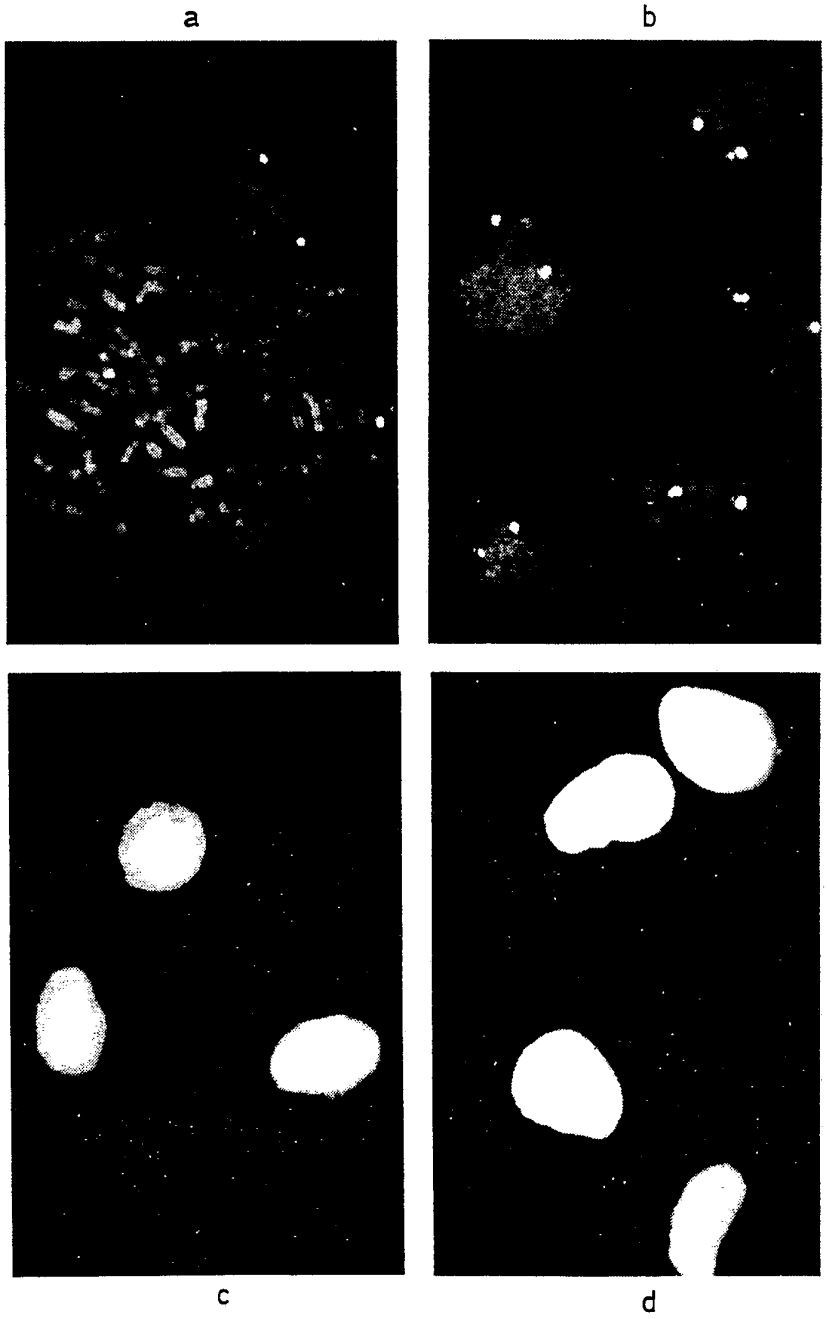


Fig. 6.



INTERNATIONAL SEARCH REPORT

PCT/GB 93/00437

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68; C12N15/11

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 85, December 1988, WASHINGTON US pages 9664 - 9668 P.LICHTER ET AL. cited in the application see the whole document ---	1
A	EP,A,0 430 402 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 5 June 1991 see page 5, line 2 - page 8, line 31 see page 13, line 31 - page 14, line 41 see page 28, line 5 - page 31, line 14; claims --- -/--	1

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26 APRIL 1993	18. 05. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	LUZZATTO E.R.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	NUCLEIC ACIDS RESEARCH. vol. 13, no. 11, 1985, ARLINGTON, VIRGINIA US pages 4125 - 4132 G.D.STEWART ET AL. cited in the application see the whole document	1
P,X	PRENATAL DIAGNOSIS vol. 12, November 1992, CHICHESTER,UK pages 931 - 943 Y.L. ZHENG ET AL. see the whole document	1
P,X	AMERICAN JOURNAL OF HUMAN GENETICS vol. 51, no. 1, July 1992, CHICAGO, USA pages 55 - 65 K. KLINGER ET AL. see the whole document, especially the abstract	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9300437
SA 70719

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CA-A- 2021489	20-01-91
		JP-A- 3224499	03-10-91