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(54) Titre : LIGNEE DE CELLULES RENFERMANT UN EXCES DE CENTROMERES MAMMALIENS

(54) Title: CELL LINE CARRYING AN EXCESS OF MAMMALIAN CENTROMERES

(57) Abrégé/Abstract:

DNA fragments and methods for obtaining them are disclosed which when put into mammalian cells together with a dominant marker gene are able to form functional centromeres. The sequences can be used to generate probes for these centromeres. Cell lines containing the functional centromeres are also provided. Methods are taught for isolating mammalian centromeric DNA as well as for producing cell lines carrying an excess of mammalian centromeres linked to a dominant selectable marker gene.

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ABSTRACT OF THE DISCLOSURE

DNA fragments and methods for obtaining them are disclosed which when put into mammalian cells together with a dominant marker gene are able to form functional centromeres. The sequences can be used to generate probes for these centromeres. Cell lines containing the functional centromeres are also provided. Methods are taught for isolating mammalian centromeric DNA as well as for producing cell lines carrying an excess of mammalian centromeres linked to a dominant selectable marker gene.

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CELL LINE CARRYING AN EXCESS  
OF MAMMALIAN CENTROMERES

**BACKGROUND OF THE INVENTION**

The centromere is a specialized region of the eukaryotic chromosome. It is the site of kinetochore formation, a structure which allows the precise segregation of chromosomes during cell division. In addition to this, a possible structural role in the higher-order organization of eukaryotic chromosomes has also been suggested (Hadlaczky (1985), Internat'l. Rev., 94:57-76).

The isolation and cloning of centromeres is crucial, not only to understand their molecular structure and function, but also for the construction of stable artificial chromosomes. Taking advantage of the existence of centromere-linked genes, functional centromeres of lower eukaryotes (yeast) have been successfully isolated (Blackburn, et al. (1984) Ann. Rev. Biochem., 53:163-194; Clarke, et al. (1985), Ann. Rev. Genet., 19:29-56). The combination of a functional centromere with telomeres, which stabilize the chromosome ends, permitted the construction of yeast artificial chromosomes (Murray, et al. (1983) Nature, 305:189-193; Burke, et al. (1987), Science, 236:806-812). This initiated a new era in the study of chromosome function and in genetic manipulation.

Higher eukaryotes (e.g., mammals), in contrast to yeast, contain repetitive DNA sequences which form a boundary at both sides of the centromere. This highly repetitive DNA interacting with certain proteins, especially in animal chromosomes, creates a genetically inactive zone (heterochromatin) around the centromere. This pericentric heterochromatin keeps any selectable marker gene at a considerable distance, and thus repetitive DNA prevents the isolation of centromeric sequences by chromosome "walking."

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Thus there is a need in the art for methods of isolating higher eukaryotic centromeric DNA. Isolation of such DNA is necessary for construction of artificial mammalian chromosomes. Use of such chromosomes could overcome problems inherent in present techniques for introduction of genes to mammalian cells, including the concomitant creation of insertional mutations, size limitations on introduced DNA, and imperfect segregation of plasmid vectors.

**SUMMARY OF THE INVENTION**

It is an object of the present invention to provide a method for isolating centromeric DNA from a mammal.

It is another object of the invention to provide a DNA element which will insure faithful segregation of inserted DNA in meiosis and mitosis.

It is yet another object of the invention to provide a DNA element for formation of vectors to insert large amounts of DNA into mammalian cells.

It is still another object of the invention to provide a DNA element which binds mammalian centromere proteins.

These and other objects are provided by one or more of the embodiments described below.

In one embodiment a non-human cell line is provided that contains an excess of centromeres.

In another embodiment a nucleic acid probe is provided which hybridizes to a DNA molecule having the sequence shown in Figure 1.

In yet another embodiment a method of isolating centromeric DNA from a mammal is provided comprising:

isolating metaphase chromosomes of a mammalian cell line;  
fragmenting the chromosomes to form a suspension containing chromosome fragments;

incubating the suspension with human serum containing anti-centromere antibodies to bind chromosome fragments to the antibodies;

separating antibody-bound chromosome fragments from the suspension; and

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deproteinizing said bound fragments to provide a preparation of centromeric DNA.

In still another embodiment a method is provided of producing a cell carrying an excess of mammalian centromeres, comprising:

cotransfected cells with: (1) DNA carrying mammalian centromeric DNA; and (2) DNA carrying a dominant selectable marker;

selecting cells which express the dominant selectable marker;

detecting cells which carry an excess of mammalian centromeres.

These and other embodiments will be described in more detail below. The present invention thus provides the art with methods to access and isolate the important centromeric DNA of mammalian cells. In particular, a human DNA fragment CM8 is provided which can be used to create artificial chromosomes for gene therapy.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides the sequence of a 13,863 bp fragment of DNA identified in a  $\lambda$  Charon 4A human genomic library.

Figure 2 shows the results of agarose gel electrophoresis of DNA fragments obtained by immunoprecipitation.

Lanes A and B: DNA isolated from chromosome fragments remaining unbound to anti-centromere Sepharose.\*

Lanes C and D: DNA isolated from chromosome fragments bound to anti-centromere Sepharose\*. Note the presence of a population of high molecular weight DNA fragments. Samples of lanes B and D were treated with 100  $\mu$ g/ml RNase-A prior to electrophoresis.

Lane M:  $\lambda$  HindIII marker.

Figure 3 shows a restriction map of the human genomic DNA insert of CM8  $\lambda$  Charon 4A clone. The arrow shows the position of a 300 bp Alu repeat deficient in the flanking direct repeat sequences.

Figure 4 shows the results of *in situ* hybridization with  $^{3}H$ -thymidine labelled CM8 DNA to human metaphase chromosomes.

Panel A: Preferential localization of silver grains at the centromeres of human chromosomes (arrowheads).

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Panel B: Diagram showing the distribution of silver grains (•) on 131 metacentric chromosomes. Numbers indicate the frequency of silver grain localization to certain regions of the chromosomes.

Figure 5 shows the detection of dicentric and minichromosome of the EC3/7 cells by indirect immunofluorescence (panels A and B) with anti-centromere antibodies, and by *in situ* hybridization with biotin labelled CM8 probes (panel C) and with a 1 kb SmaI/BglII fragment of APH-II gene (panel D).

Panels E and F: DNA staining with Hoechst 33258;

Panels G and H: DNA staining with propidium iodide. Panels E-H correspond to A-D, respectively. Arrowheads point to dicentric and minichromosomes.

Figure 6 shows the duplication of the extra centromere in the EC3/7 cell line.

Panels A-C: *In situ* hybridization with biotin labelled CM8 probe.

Panels D-F: Corresponding DNA staining of A-C, respectively.

Figure 7 demonstrates the colocalization of the integrated DNA sequences with the centromere region detected by immunostaining with anti-centromere serum (Panels A and D) and subsequent *in situ* hybridization with biotin labelled CM8 (panel B) and APH-II probe (panel E) on the same metaphases of the EC3/7 cells.

Panels C and F: DNA staining.

#### DETAILED DESCRIPTION

It is the discovery of the present invention that a segment of human DNA can be isolated and introduced into mouse cells and result in a functional centromere. The functional centromeres containing DNA of the present invention are preferably linked to a dominant selectable marker. This can be a resistance marker, such as the aminoglycoside-3' phosphotransferase-II which provides resistance to G418 (Sigma). Other such markers known in the art may be used.

The method of isolating centromeric DNA of the present invention can be applied to any higher eukaryote, especially mammals. Preferably a human cell line will be employed. Metaphase

chromosomes are isolated according to techniques known in the art. The chromosomes are then fragmented. Endonuclease digestion and mechanical shearing can be used to fragment the chromosomes. Desirably the majority of the fragments will be in the size range of less than 1  $\mu$ m and some chromosomes will remain unbroken. Unbroken chromosomes can be readily removed from the preparation by centrifugation at about 1,500 g for about 10 minutes.

A human serum containing anti-centromere autoantibodies can be employed in the method of the invention. This is available from patients with CREST syndrome. Alternative sources of antibody may be used, such as monoclonal or animal derived polyclonal sera containing anti-centromere antibodies. The antibodies are incubated with the preparation of chromosome fragments under conditions where antibody-antigen complexes form and are stable. It is convenient if the antibodies are bound to a solid support. Preferably a support such as Protein-A Sepharose CL4B (Pharmacia) is used to facilitate separation of bound from unbound chromosomal fragments. However other methods to accomplish this goal can be used, as are known in the art, without employing an antibody bound to a solid support.

The DNA fragments comprising centromere DNA are liberated from the antibodies and centromeric proteins by a deproteinization treatment. Ultimately the DNA is purified from all proteins, by degrading the proteins and extracting them from the chromosome fragment preparation. Any such treatment known in the art may be used including but not limited to proteases and organic solvents such as proteinase K and phenol.

The centromeric DNA fragments can be used for any purpose or application known in the art. For example, they can be labelled and used as probes; they can be ligated to vectors to clone or all part of the sequences; and they can be used to purify centromeric proteins by attachment to a solid support.

In one particular embodiment of the invention the centromeric DNA fragments are used to probe a library of genomic DNA from humans or other mammals for clones which hybridize. Hybridizing

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clones can be analyzed for their ability to perform functions which centromeric DNA possesses. One such function is to bind to centromeric proteins. Another such function is to form a structure in cells which can be cytologically detected using appropriate immunostaining with anti-centromere antibodies which particularly stain centromeres.

According to another method of the present invention a cell carrying an excess of mammalian centromeres is formed. The cell may be human or other mammalian. The centromere may comprise DNA isolated from the same or a different mammalian species as the cell. The method involves cotransfection of a cell with two DNA molecules: one is a DNA carrying centromeric DNA; the other is a DNA carrying a dominant selectable marker. Preferably these two DNA molecules contain sequences which allow concatamer formation, for example phage DNAs such as  $\lambda$  phage. The first DNA molecule may be isolated from a library of genomic DNA using, for example, as a probe the centromeric fragments taught above. Alternatively the first DNA molecule may result from cloning the centromeric fragments taught above into a phage, for example  $\lambda$ , after manipulations to create fragments of the appropriate sizes and termini. The second DNA molecule is readily within the reach of those of skill in the art, for example a  $\lambda$  phage carrying a drug resistance marker.

It is believed to be desirable to employ  $\lambda$  phage DNA because it concatemerizes, however the absolute necessity of this has not been determined. Further, even if this property is necessary, other viral DNAs or DNA constructs may be able to supply this function. Such other means of achieving concatemerization are also contemplated within this method.

After cotransfection, cells are selected which express the dominant selectable marker, for example by growth in amounts of G418 which are cytotoxic for the cells without the marker. This selected population of cells is further screened to detect cells with an excess of mammalian centromeres. This screening can be done by standard cytogenetic techniques, as well as by immunostaining with

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anti-centromere antibodies. Desirably the lambda, marker, and centromeric DNA (from the  $\lambda$  clone) will all be localized at the site of the extra centromere. This can be determined by in situ hybridization studies, which are well known in the art.

One cell line made by the methods described above is EC3/7 which has been deposited at the European Collection of Animal Cell Cultures, Porton Down, U.K. under accession no. 90051001 (deposit date October 5, 1990) under the  
10 conditions of the Budapest Treaty.

The sequence of the DNA insert in the lambda phage which was used to make the EC3/7 cell line, (referred to as CM8) was determined by standard techniques and is shown in Figure 1. The sequence does not correspond to any in DNA sequence banks.

The present invention also contemplates nucleic acid probes, preferably of at least 10 nucleotides, which  
20 hybridize to a DNA molecule having the sequence shown in Figure 1. One such molecule is CM8, the lambda phage clone from which the sequence was derived. Probes can be radiolabeled, biotin labeled or even unlabeled, for example, depending on the use for which they are desired.

The following examples do not limit the invention

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to the particular embodiments described, but are presented to particularly describe certain ways in which the invention may be practiced.

**Example 1**

This example demonstrates the isolation of human DNA from centromeres.

Human colon carcinoma cell line (Colo 320) was grown as a suspension in RPMI medium supplemented with 10% foetal calf serum (FCS). Metaphase chromosomes of Colo 320 10 cells were isolated by our standard method (Hadlaczky, et al. (1982), Chromosomes, 86:643-659). Isolated metaphase chromosomes were resuspended in 1 ml of buffer (105 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 5 mM

2-mercaptoethanol) at a concentration of 1 mg/ml DNA and digested with 500 u EcoRI restriction endonuclease for 1 h. The suspension was diluted with 4 ml of IPP buffer (500 mM NaCl, 10 mM Tris-HCl, 0.5% NP-40\*, pH 8.0) and sonicated for 5x50 s with an MSE 5-70 sonicator. This treatment resulted in a suspension containing chromosome fragments and a few (< 1%) unbroken small chromosomes. The suspension was centrifuged at 1500 g for 10 min to remove unbroken chromosome fragments. The supernatant contained only small (< 1  $\mu$ m) chromosome fragments as judged by light microscopy.

Two hundred fifty mg of Protein-A Sepharose<sup>\*</sup> CL4B (Pharmacia) was swollen in IPP buffer and incubated with 500  $\mu$ l human anti-centromere serum LU851 (Hadlaczky, et al. (1989), Chromosoma, 97:282-288) diluted 20-fold with IPP buffer. Suspension of sonicated chromosome fragments (5 ml) was mixed with anti-centromere Sepharose (1 ml) and incubated at room temperature for 2 h with gentle rolling. After 3 subsequent washes with 25 ml IPP buffer the Sepharose was centrifuged at 200g for 10 min.

Isolation of DNA from the immunoprecipitate was carried out by Proteinase-K treatment (Merck, 100  $\mu$ g/ml) in 10 mM Tris-HCl, 2.5 mM EDTA, pH 8.0 containing 1% SDS, at 50°C overnight, followed by repeated phenol extractions and precipitation with isopropanol. All general DNA manipulations were done according to (Maniatis, et al. (1982) Molecular Cloning—A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Results of electrophoresis of immunoprecipitated and supernatant DNA are shown in Figure 2. The bulk of DNA from chromosome fragments which did not bind to the anti-centromere Sepharose<sup>\*</sup> (supernatant) ranged from several hundred base pairs to 5 kb (Fig. 2, lanes A and B), while DNA from chromosome fragments which bound to the anti-centromere Sepharose<sup>\*</sup> contained an additional population of high molecular weight (9-20 kb) fragments (Fig. 2, lanes C and D). This distribution of fragments sizes is consistent with the notion that the centromeric DNA is in the structurally most stable region of mammalian chromosomes (Hadlaczky, et al. (1981), Chromosoma,

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81:557-567), thus rendering this DNA relatively resistant to enzymatic digestion and mechanical shearing.

Example 2

This example demonstrates the use of the high molecular weight immunoprecipitated DNA as a hybridization probe to screen a genomic DNA library.

The high molecular weight DNA was isolated from the agarose gel described in Example 1, by electroelution, labelled with  $^{32}\text{P}$ -dATP by random oligonucleotide priming (Feinberg, et al. (1983), Anal. Biochem., 132:6-13) and used as a probe for screening a  $\lambda$  Charon 4A human genomic library (Maniatis, et al. (1978), Cell, 15: 687-701). A hybridizing clone (CM8) was obtained which contains a 14 kb human DNA insert. The restriction map of this insert for some restriction endonucleases is shown in Figure 3. Southern hybridization of parts of the 14 kb insert to human lymphocytic genomic DNA indicates that the 14 kb insert represents a continuous piece of DNA in the genome and is not the ligation product of a number of fragments.

Example 3

This example demonstrates that the copy number of the 14 kb insert of clone CM8 is consistent with it being present on each chromosome in the human genome.

Southern blotting experiments were performed in which a single copy DNA probe (XV2C) (Estivill, et al. (1987), Nature, 326:840-845) and the central Xhol-EcoRI fragment of the CM8 insert (Fig. 2) simultaneously hybridized with serial dilutions of human peripheral lymphocyte DNA. The probes were labelled by random oligonucleotide priming (Feinberg, et al. (1983), Anal. Biochem., 132:6-13). By comparing the signal of the CM8 probe to the known single copy probe, the copy number of CM8 was estimated to be 16-32 per haploid genome.

Example 4

This example shows the use of the CM8 DNA as a probe to human metaphase chromosomes.

Radioactive in situ hybridization with  $^3\text{H}$ -thymidine labelled CM8 DNA to human (Colo 320) metaphase chromosomes was

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performed according to the method of Pinkel, et al. (1986), Proc. Natl. Acad. Sci. USA, 83:2934-2938. A preferential centromeric localization of silver grains was observed (Fig. 4).

In non-radioactive *in situ* hybridization according to the method of (Graham, et al. (1973), Virology, 52:456-467), using biotin-labelled subfragments or the whole CM8 insert it was not possible to detect a positive hybridization signal by our standard method. Furthermore, using a hybridization method which is suitable for single copy gene detection with a biotin-labelled probe (Lawrence, et al. (1988), Cell, 52:51-61), apart from the typical R-band like Alu hybridization pattern (Korenberg, et al. (1988), Cell, 53:391-400), no specific hybridization signal was detected on any of the chromosomes with the whole 14 kb CM8 insert. Possible explanations for this negative result are that these sequences are virtually inaccessible to the hybridization probe, due to their compact packing in the midst of the centromere structure, and that the biotin system is less sensitive than the radioactive one.

#### Example 5

This example discloses the sequence of the human CM8 clone.

The sequence of the human genomic insert of  $\lambda$  CM8 was determined using the dideoxy method (Sanger, et al. 1977), J. Mol. Biol., 143:161-178; Biggin, et al. (1983), Proc. Natl. Acad. Sci. USA, 80:3963-3965). See Figure 1.

The sequence of the 13,863 bp human CM8 clone was compared with a complete nucleic acid data bank (MicroGenie<sup>\*</sup>, Beckman) and showed no homology to any known sequence. However, a 300 bp Alu repeat deficient in the flanking direct repeat sequences was found in the 2.5 kb EcoRI-XhoI fragment (Fig. 3), which explains the Alu type *in situ* hybridization pattern.

#### Example 6

This example demonstrates the use of the CM8 DNA to form centromeres in mammalian cells.

In order to detect any *in vivo* centromere function of the CM8 DNA, it was introduced with the selectable APH-II gene into mouse

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LMTK<sup>-</sup> fibroblast cells. The mouse fibroblast cells were maintained as a monolayer in F12 medium supplemented with 10% FCS. The calcium phosphate method (Harper, et al. (1981), Chromosoma, 83:431-439) was used to transfect the cells with 20 µg λ CM8 and 20 µg λ gtWESneo DNA per Petri dish (80 mm). A 2 minute glycerol shock was used. The λgt WESneo was made by cloning the pAG60 plasmid (Colbere-Garapin, et al. (1981), J. Mol. Biol., 150:1-14) into a λ gtWES (Leder, et al. (1977), Science, 196:175-177) bacteriophage vector.

The whole λ CM8 and λ gt WESneo constructions were used for transfections for two reasons. First, to separate the marker gene from the CM8 sequences, in order to avoid inactivating the APH-II gene, a process which may occur during centromere formation. Second, λ DNA is capable of forming long tandem arrays of DNA molecules by concatamerization. Concatamerization was postulated as being necessary to form centromeres since, in S. pombe 4 to 15 copies of conserved sequence motifs form centromeres (Chikashige, et al. (1989), Cell, 57:739-751). Considering these two facts a multiplication of the putative centromeric DNA by concatamerization might increase the chance of centromere formation.

Transformed cells were selected on growth medium containing 400 µg/ml G418 (Genticin, Sigma). Individual G418 resistant clones were analyzed. The presence of human sequences in the transformed clones was monitored using Southern blots probed with subfragments of the CM8 insert. Screening for excess centromeres was achieved by indirect immunofluorescence using human anti-centromere serum LU851 (Hadlaczky, et al. (1989), Chromosoma, 97:282-288). The chromosomal localization of "foreign" DNA sequences was determined by in situ hybridization with biotin labelled probes.

Eight transformed clones have been analyzed. All of the clones contained human DNA sequences integrated into mouse chromosomes. However, only two clones (EC5/6 and EC3/7) showed the regular presence of dicentric chromosomes. Individual cells of clone EC5/6 carrying di-, tri-, and multicentromeric chromosomes exhibited extreme instability. In more than 60% of the cells of this cell

line the chromosomal localization of the integrated DNA sequences varied from cell to cell. Due to this instability, clone EC5/6 was deemed to be unsuitable. However, cells of clone EC3/7 were stable, carrying either a dicentric (85%) or a minichromosome (10%). Centromeres of dicentric chromosomes and minichromosomes were indistinguishable from the normal mouse centromeres by immunostaining with anti-centromere antibodies (Fig. 5A and B).

#### Example 7

This example shows that the newly introduced DNA in the EC3/7 cell line contributes to centromere formation.

In situ hybridization with biotin labelled CM8, APH-II gene, and  $\lambda$  phage DNA were carried out. Chromosomes were counterstained with propidium iodide (Pinkel, et al. (1986), Proc. Natl. Acad. Sci. USA, 83:2934-2938) for in situ hybridization experiments while in indirect immunofluorescence with DNA binding dye, Hoechst 33258 used. All observations and microphotography were made by using an Olympus<sup>\*</sup> AHBS Vanox microscope. Forte 400 Professional black and white, and Fujicolor<sup>\*</sup> 400 Super HG<sup>\*</sup> colour film were used for photographs.

Without exception these three probes hybridized onto the same spots: either on the distal centromere of the dicentric chromosome (Fig. 5C) or on the centromere of the minichromosome (Fig. 5D). In less than 5% of the EC3/7 cells an alternative localization of the hybridization signal was found. These included cells with more than one integration site, cells without a detectable signal, or cells where the hybridization was found on chromosomes other than that identified as the dicentric chromosome.

In less than 0.5% of the cells a tandem array of the hybridization signal was observed on the dicentric chromosomes (Fig. 6A-C), suggesting that the additional centromere was capable of autonomous "duplication." At least some of these duplicated centromeres appeared to be functional. This was indicated by the existence of a minichromosome with double centromeres. Both centromeres of this minichromosome showed positive immunostaining with anti-centromere antibodies (Fig. 7A). Minichromosomes carrying

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double centromeres might be breakage products of multicentromeric chromosomes.

Indirect immunofluorescence of mouse metaphase cells was performed as described by Hadlaczky, et al. (1989), Chromosoma, 97:282-288. When indirect immunofluorescence and in situ hybridization were performed on the same metaphases, mitotic cells were resuspended in a glycine-hexylene glycol buffer (Hadlaczky, et al. (1989), Chromosoma, 97:282-288), swollen at 37°C for 10 min followed by cytocentrifugation and fixation with cold (-20°C) methanol. After the standard immunostaining (Hadlaczky, et al. (1989), Chromosoma, 97:282-288) metaphases were photographed, then coverslips were washed off with phosphate buffered saline and slides were fixed in ice-cold methanol-acetic acid, air-dried and used for in situ hybridization.

To demonstrate the integration of the human CM8 clone and the APH-II gene in the centromere region, immunostaining of centromeres with anti-centromere antibodies followed by in situ hybridization with CM8 and APH-II probes was carried out on the same metaphase plates of EC3/7 cells. The in situ hybridization signals with both biotin-labelled CM8 and APH-II probes showed a colocalization with the immunostained centromeric region of the chromosomes carrying additional centromeres (Fig. 7).

#### Example 8

This example describes the stability of the EC3/7 cell line.

Forty-six independent subclones derived from a single cell were isolated and analyzed. Each of the subclones carried the dicentric chromosome. The percentage of minichromosome-containing cells varied between 2% and 30% in different subclones. We were unable to isolate a subclone which carried the additional centromere exclusively in a minichromosome. This result suggested that the minichromosomes were unstable and they can be regarded as the products of regular breakages of the dicentric chromosomes.

A preliminary analysis by immunostaining of EC3/7 cells (103 metaphases) cultured for 46 days in non-selective medium showed that 80.6% of the cells contained either a dicentric (60.2%) or a

minichromosome (20.4%). Subsequent in situ hybridization with biotin labelled probes proved the presence of the "foreign" DNA in the additional centromere. These results indicate that no serious loss or inactivation of the additional centromeres had occurred during this period of culture under non-selective conditions.

Example 9

This example shows that the CM8 insert concatamerized to form the functioning centromere of cell line EC3/7.

DNA of the EC3/7 cell line and human lymphocyte DNA were digested with restriction endonucleases and probed with subfragments of the CM8 insert in a Southern hybridization experiment. Comparing the intensity of the hybridization signal with EC3/7 DNA to that with the human DNA, the minimum number of integrated human sequences in the additional centromere was estimated to be  $\geq 30$ . The copy number of CM8 in human lymphocytic DNA was determined as described above in Example 3.

**The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:**

1. A non-human mammalian cell line, comprising cells that contain an excess of centromeres, wherein said cells comprise human DNA having the restriction fragment map as set forth in Figure 3 or a fragment thereof.
- 5 2. A rodent cell line, comprising cells that contain an excess of centromeres, wherein:
  - a) said cells comprise human DNA having the restriction fragment map as set forth in Figure 3 or a fragment thereof; and
  - b) human autoantibodies isolated from CREST syndrome patients bind to one or more chromosomes in cells in the cell line.
- 10 3. A cell that is selected from cells that have all of the identifying characteristics of the cells deposited at the European Collection of Animal Cell Cultures (ECACC) under accession no. 90051001.
- 15 4. A cell line having all of the identifying characteristics of the cells deposited at the European Collection of Animal Cell Cultures (ECACC) under accession no. 90051001.
- 20 5. A rodent cell line produced by a method comprising the steps of:
  - a) cotransfected cells with a DNA fragment comprising human DNA and a DNA fragment encoding a dominant selectable marker, wherein said human DNA has the restriction fragment map as set forth in figure 3 or a fragment thereof;
  - b) growing the cells and selecting cells that express the dominant selectable marker;
  - c) detecting among the cells that express the dominant selectable marker those cells with an excess of mammalian centromeres.
- 25 6. A rodent cell line produced by a method comprising the steps of:

- 5           a) cotransfected cells with a DNA fragment comprising human DNA and a DNA fragment encoding a dominant selectable marker, wherein said human DNA has the restriction fragment map as set forth in figure 3 or a fragment thereof;
- b) growing the cells under selective conditions and selecting cells that express the dominant selectable marker;
- c) detecting among the cells that express the dominant selectable marker those cells with an excess of mammalian centromeres that include a chromosome with two centromeres.

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7.       A rodent cell line produced by a method comprising the steps of:
- a) cotransfected cells with a DNA fragment comprising human DNA and a DNA fragment encoding a dominant selectable marker, wherein said human DNA has the restriction fragment map as set forth in figure 3 or a fragment thereof;
- b) growing the cells under selective conditions and selecting cells that express the dominant selectable marker;
- c) detecting among the cells that express the dominant selectable marker those cells with an excess of mammalian centromeres that include a minichromosome, wherein the minichromosome is smallest chromosome in the cell.

20           8. A cell or cell line according to any one of claims 1 to 7, wherein said human DNA comprises the nucleotide sequence set forth in Figure 1.

25

9.       A cell or cell line according to claim 8, wherein the cells are mouse cells.

30

10.      A method of producing a mammalian cell containing an excess of functional centromeres, comprising:

              a) cotransfected cells with a DNA fragment comprising human DNA and a DNA fragment encoding a dominant selectable marker, wherein said human DNA comprises CM8;

- b) growing the cells and selecting cells that express the dominant selectable marker;
- c) detecting among the cells that express the dominant selectable marker those cells with an excess of mammalian centromeres.

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11. The method of claim 10, wherein said human DNA comprises the nucleotide sequence set forth in Figure 1.

10 12. The method of claim 11, wherein the cells that express the dominant selectable marker and have an excess of mammalian centromeres are cells that have all of the identifying characteristics of the cells deposited at the European Collection of Animal Cell Cultures (ECACC) under accession no. 90051001.

15 13. The method of claim 12, wherein the human DNA is contained in the clone λ CM8 and the selectable marker is encoded by λ gt WESneo.

14. A method of producing mammalian cells containing a dicentric chromosome, comprising:  
20 a) cotransfected cells with a DNA fragment comprising human DNA and a DNA fragment encoding a dominant selectable marker, wherein said human DNA has the restriction fragment map as set forth in Figure 3 or a fragment thereof;

25 b) growing the cells under selective conditions and selecting cells that express the dominant selectable marker;  
c) detecting among the cells that express the dominant selectable marker those cells with an excess of mammalian centromeres that include a chromosome with two centromeres.

30 15. A method of producing mammalian cells containing a minichromosome that contains heterologous DNA, comprising:

- a) cotransfected cells with a DNA and a DNA fragment comprising human DNA and a DNA fragment encoding a dominant selectable marker,

wherein said human DNA has the restriction fragment map as set forth in Figure 3 or a fragment thereof;

b) growing the cells under selective conditions and selecting cells that express the dominant selectable marker;

5 c) detecting among the cells that express the dominant selectable marker those cells with an excess of mammalian centromeres that include a minichromosome, wherein the minichromosome is smallest chromosome in the cell.

10 16. A method of producing a mammalian cell containing an excess of centromeres, comprising:

a) cotransfected cells with a DNA and a DNA fragment comprising human DNA and a DNA fragment encoding a dominant selectable marker, wherein said human DNA has the restriction fragment map as set forth in Figure 15 3 or a fragment thereof;

b) growing the cells and selecting cells that express the dominant selectable marker;

c) detecting among the cells that express the dominant selectable marker those cells with an excess of mammalian centromeres.

20 17. The method of any of claims 14 to 16, wherein said human DNA fragment comprises the human DNA in the clone λ CM8.

25 18. The method of any of claims 14 to 16, wherein said human DNA comprises the nucleotide sequence set forth in Figure 1.

19. The method of any of claims 11-18, wherein the selectable marker encodes aminoglycoside-3' phosphotransferase-II.

20. A DNA molecule having the restriction map as set forth in Figure 3 or a fragment thereof.

5

21. A DNA molecule having the nucleotide sequence as set forth in Figure 1 or a fragment thereof.

22. A minichromosome derived from the cell line of claim 4.

10

23. A minichromosome isolated from the cells produced by the method of claim 15.

24. A dicentric chromosome derived from the cell line of claim 4.

15

25. A minichromosome isolated from the cells produced by the method of claim 14.

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**FIG. I (A)**

10	20	30	40	50	60	70	80	90	100
GAATTCAATGC CAACTGCAAG TCTGGGGTC ACCTTGACTG GACACCCCTCC TCCCCGCCACG TTCTTGAAC TTCCCTCCAT CGCGCTCCAAG TCTCTCCAA									
110	120	130	140	150	160	170	180	190	200
TGCCTCAG AGCCCTGCAG CAGCCCTCAC TCCCAGATGCC TTCCCACCTC CTCACCACTC TGCCCCCACC TGGCCAGGCC ATCACCTCCA GGGCCCAACT									
210	220	230	240	250	260	270	280	290	300
TGGAGCCCCC AGGACCTCCC CGTCCCCCTGC CTGATGTCCC GCCGTCCCC ACAGAGCCTC ACTTGGTCAC CACCCAGTCC TGGCCCTTGC TTACTGTGGC									
310	320	330	340	350	360	370	380	390	400
TGCACCCCCA GGTGTCTTAG GGTCTAGGAG GTGGCTGCC AGACATGGAG GTAGAGGAAG GAGTGGTGG GGATGGCTT GTCCGTGCCA GGCGTCCCTG									
410	420	430	440	450	460	470	480	490	500
CCYGTCTGCC TGGCCACAGC CTTGGCTTGC CCAGGAGAAC CCATGGGCCA CACATCCCAC TGCCAATCCC ACACGTCTT TCTCGGGAAC ACCGTGGGA									
510	520	530	540	550	560	570	580	590	600
AACCTGTGGC ACCAGCTCCT TCCTTTGCA ACTCTGATGA ATCTCACCCA GGGATTTCAA GGCCCCCTGGT CACACCAGGA TCATAGGCC CCCCCATCCC									
610	620	630	640	650	660	670	680	690	700
CTGGACACAC AGAGACACAC CTGGATTCAAG GTCAAGGCCTC GCCCACTCTC GGCTATATTCT CTCCTCAAGC CGTGTGTCTT CAGCTGTAGA ATCAGGACCA									
710	720	730	740	750	760	770	780	790	800
TAAGGAAGTT CCCTCATAGG GTTCTTGTCA GGACGGCAG ATTACCTAG GGGATGCTCA CACCGTGCCT GGCAAGCTGGG AGCGACTCCA CCCGGGGCAG									
810	820	830	840	850	860	870	880	890	900
CCCGGTCCCCA TGGCTTCTCA GTGACTTTTC CAGCCACACT GCACCTCTTA GACAGGAACA CTCCATACGA TGTCCCTCTC CTCCACTGGAA TGGCCCCAAA									
910	920	930	940	950	960	970	980	990	1000
ATCTGAAATA AGAGGAGGAG TGCCTGTGAA GCTCCCAGTG GACCGTTGG CACCTGTCCA GCATGTCCCC AAGGGCAAGT CACGGCTCTG AGATTCACTG									
1010	1020	1030	1040	1050	1060	1070	1080	1090	1100
TCTCCTCTG CAAGTAGGGC CAATAGTGGT TCCCTCCCTCC CAGGGCTGAA GTGAGGATGA GATGGATAA TCCACCCCCCG TCCCCACACC CTGCAGGTCA									
1110	1120	1130	1140	1150	1160	1170	1180	1190	1200
TCATCATTCGC TAGCAGTTGT GTGGTGGAGC AGGTGCTCTT GAGGGAGCGA CACCTCCAGG TGCTCCCTG CCCTGCTGGC CCCTCTGCCAG GAGGTGACAC									
1210	1220	1230	1240	1250	1260	1270	1280	1290	1300
CCAGGGCCCT TTCCCCCTGGG GCAGCCAGCT CAGCCCTCTC CTCTCCCACA GGTGCCGCTG CAGTTCTTT GGCAGTAAGT AACAGCGCCT GGGGAGGGTG									
1310	1320	1330	1340	1350	1360	1370	1380	1390	1400
CCCACGGCCC CCACCTGCACG CGCCCTCTTG CATGTCTGG AAAAACAGG AGAGAAAAAA GGGGCTTCAG TGTCCCTCT GGGACTTGGG CCATCACTC									
1410	1420	1430	1440	1450	1460	1470	1480	1490	1500
CCTCCTCTAA TTACACCCCT ACTGCTTCTC CACCTCTCCC CCCTCCACCT CCCCCCTCTC ACCCATCCCC ACTTCACATC ATATGCCGTA TAGCCATGTG									
1510	1520	1530	1540	1550	1560	1570	1580	1590	1600
TCATTTGCT GTGCCCTGTG CCCAGGAATC TCTAGGCTCT CCCAGGAGCT CCATCACTGC TGCTTTGGAA AACGGGACAG GACTTTTGC AGGTCTCTG									
1610	1620	1630	1640	1650	1660	1670	1680	1690	1700
GCCCTGGGT GGCCTCCCTG CTCCCTCTGC CACCCACGCC ACTCTCTCA CCTGGGATCT GGAGAGCAGT CTCTCTGCC AGTCAAGAGT GGGGTGACCT									
1710	1720	1730	1740	1750	1760	1770	1780	1790	1800
TCCCCCACCA GGCAGAAATCC ACCCCCTAGC CTAACCATGG CGGCAGGCCCTC CCTCTGGCAG CCTCTGCAGC CAGCTGTCC CAGGGCTCTG CTCGTCCAGG									
1810	1820	1830	1840	1850	1860	1870	1880	1890	1900
TCAGCTCAGG TCCCAGGGGA GTCGGACCAAG GGACGGCAT CTGCAGGAGG TGGGGTCTC GAGAGTCCC CAGGAGGGCG AGGGCGACAT GGCGCACAGG									
1910	1920	1930	1940	1950	1960	1970	1980	1990	2000
TTATCACTAA ATGTCATCGA GACTGTCCCC AGACACTCAC AGGGTGCAG GCAGTCTCTC CTTTCACCCCT TGCAAACCCCT CCCCTGGGAG GTGCCCATCT									
2010	2020	2030	2040	2050	2060	2070	2080	2090	2100
GCTCTGCCAG GCAGCAGGAG AGGACTGGCC AATGTCAAAG AGCCAGCCGG GAGCAGACCC CAAATCTCA AGATGCTCT GGGGGCGTCA CCCCTCCACCA									

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**FIG. I(B)**

2110	2120	2130	2140	2150	2160	2170	2180	2190	2200
GGGCTCTGTG GGGCCCCACA TCCCACCCAA GTTGTCCCTC CCGGACCCAG CGGGCCCTG CCTGGGAAGC CAGTGAGCCG AGAGGGGCC AGAAAGAAC									
2210	2220	2230	2240	2250	2260	2270	2280	2290	2300
TGGACCCCTGC AGGGACCGCTG GTCTGCACAG CCGTGCTAAG TTGCTCTCT GTGGTGTCCC CACCCGGCC AACCCCCAAC CCTCTCTTGC TTTCCCCATC									
2310	2320	2330	2340	2350	2360	2370	2380	2390	2400
TCTCACCAAGG CATCAGCAGG TCCCAGAAAG ACCCCGACCC CAAAGGCCCT GTGGCACTTG CGGCCACGA AGCCATGACA GGGCCCCCTA CTACTCCTGT									
2410	2420	2430	2440	2450	2460	2470	2480	2490	2500
CCCCCTCACG TCCACTGCCT GTGGCCCCCT ACTACTCCTG TCCCCTCCAC GTCCACTGCC TGCCCCCAT GGCGCCAGC ACCCCACAGC CACAGGTGGG									
2510	2520	2530	2540	2550	2560	2570	2580	2590	2600
TGCCAGGGTA CAGCGACCCC TGTCACTCCC CCCTCTCCG CCTTCTAGCC TGGGTCCCTG CCTCTCTTGG GGTGGGAGGG TCGAGGGAGG CCCCTGGGCAG									
2610	2620	2630	2640	2650	2660	2670	2680	2690	2700
AGAGCAGGGG CTTGGCTCTT AGAATAGAGA CGCTAGAACC CTAGAGGCTG GGAGCCACAG GCCAAAGGGG CTTGAGGACA CCTGGGTCAA CCTGTTCTG									
2710	2720	2730	2740	2750	2760	2770	2780	2790	2800
AGCCCTGCCA GGGGATTTCAG GGATCAGTTC AGCTTCCAAG ATCGTCTCC TCTGCCCTT CAAGCCATTG CTGGAAAGTG CTCCCAGACC ATTGTGGCCA									
2810	2820	2830	2840	2850	2860	2870	2880	2890	2900
GACGGCTGCA GGAAC TGAGA GCAAGGTGC TGGGGGCAGC GAGGCCATCC TGACATGCAG CAAAGACTG GCCTTATCTC CCAATGGTGC TTCTGCCTCC									
2910	2920	2930	2940	2950	2960	2970	2980	2990	3000
GTGGTCCCTG GAGCCCCGCC CACACCTGT CCCCACCTGC CCCAGGGCC TCTCTGTCT TAGCCCCCTCA GCAGCAACAC CGGTGGGATG GATGGAGCAG									
3010	3020	3030	3040	3050	3060	3070	3080	3090	3100
GGTAGCCCCA GAAAGCAAAT GTCTCTGATC AGCAGGGCAA ACGGAGCCTC TGGAGCTACG TTGGACCCAC CGTGGGCTGC TCGGAATGTGG AGGCTGTGTG									
3110	3120	3130	3140	3150	3160	3170	3180	3190	3200
TGTAGTCAA GGCCAGGCCA GGGCAGACG TCCTGCCCT CAGGGGCTG CCACAGACAG GCATGGAAAC CTGATTCTCG CTCGCCCTCC AACGGAGGGA									
3210	3220	3230	3240	3250	3260	3270	3280	3290	3300
TTACGTGTA TTCAAGGCTG GGGGTGTGA GTGGCCCTCT GCTCTCACCT GGACTCACCT GGGGAGTATC CCACTCTGTG CAGTCCAGGT GCCAGGGGTC									
3310	3320	3330	3340	3350	3360	3370	3380	3390	3400
TGAAAGGATT TATCCTTCCA GAGGGCACCA GGAAGACGAT GACCAAGGGG GAATTCTCC TGGTCCCAGC CACGGAGGGG TGCTCCAATA GCCTGCCACA									
3410	3420	3430	3440	3450	3460	3470	3480	3490	3500
CCCTGTCCCC CGCCACCCCTG CAGGGAGGAC CTGGTGGGA CTCCCTGGCC CTTGGTAGTG CCCTGGCCCT CCATCTCTCT GATCCAAGGA GACCTGCC									
3510	3520	3530	3540	3550	3560	3570	3580	3590	3600
ACTGATCCTT CCCCTGGGT GGCATTCTA AGGCAGAGTC CCTCTACAGC TCTGCTGGC TGTGGCTGGT GGACATCAGG CTCCCAGACA GGCA TAGCTG									
3610	3620	3630	3640	3650	3660	3670	3680	3690	3700
AGAGAAGACC TCCTCTTCC TAGGCCATCC AGAGCAGTC CCTGGGGCAG CACACCCAC CTCTTCTAC ATCCTTCTT TTCTGAGAG CATTACAGG									
3710	3720	3730	3740	3750	3760	3770	3780	3790	3800
AGGCATTTTC TAGCCAAAAG ATTGGAGGAT TTCCGGGAAG CCTCTGTGACC CAGGAATCCT CTTGGGGTG GAAGACATGG GTCACTCTGA GAATTCTGGA									
3810	3820	3830	3840	3850	3860	3870	3880	3890	3900
CTTCAACAT AGGTTGCCCG AGCCACAAAG GACCTGTGCT TTGCTGATGA GCCTGTGGTG GGCAGACAGA AGCAAAACCA CGTGGTGGTG GTGCTGTGCC									
3910	3920	3930	3940	3950	3960	3970	3980	3990	4000
TGTCTCCAAA CAGGGGTTG CCTGGGAGGC CAGATACTCT CCATATCACA TGTGCAAGTG CACACATGCA CACACACACA CACATGCATG CACACACACA									
4010	4020	4030	4040	4050	4060	4070	4080	4090	4100
GGCATGCACA CGCACATGTA CACACACACA CACACAGAGG AATCCATTG CAGAGCTGCT TCTGACTTGG TGCCAGGGCC AGCCGTGGGA GGCTGGGCAG									
4110	4120	4130	4140	4150	4160	4170	4180	4190	4200
ATTGTGCAAG TTGGGATTA AAGAGGAAA GTCAAGAGGCC AGACTGGGAA ATGCAGGGGA GTTGAGGGTC CCCAGGACCC TCAGTGAAGCA GAAGGCACAC									

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**FIG. I(C)**

4210	4220	4230	4240	4250	4260	4270	4280	4290	4300
CCTCTCGGCA AGACAGTGCT GCTCTGCACC TAGCCCTGTA TCAAGAAGCA GGACATTAGG CGAGGGGTG GCTCCAATGT GACAGCCAGT GGCCCCTACA									
4310	4320	4330	4340	4350	4360	4370	4380	4390	4400
GCCACATCTA GGGGCTCCTC CCTCCCTCTC AGCAACTGAA GCCCCTGTCC AGAGCCCCCA TTAATGAAAA CGATCATTCG AGTAGCTGAG GGTGAGTTCT									
4410	4420	4430	4440	4450	4460	4470	4480	4490	4500
CCTGGGCTGT GCTCGTATGA TTGTATCATC ATATCATTGT ATTCTGGGCT CACAGCTCCG TGAGATGGAG GCTGTTATTI TCCTAGTCCC ACAGGTGAGG									
4510	4520	4530	4540	4550	4560	4570	4580	4590	4600
GGATCGAGGC TTAGGAAGAA GCAGCTGGAT TTTATGATAT GTAAAATTAC ACCTCAATCA AGCTGTTCA GAAGAAAAAA GGGGCAGCTG CTCAAGGTCT									
4610	4620	4630	4640	4650	4660	4670	4680	4690	4700
CAGAATTATG GAGAGGCACG GGCAAGGATT GAACTCAGGG CTCGCCAACT CAGCCACCCA AAGCTATTGT CCTGAGGCCT CCAGGGCTA TGAGGTAGAG									
4710	4720	4730	4740	4750	4760	4770	4780	4790	4800
CTATCTTTT TTTTTTTTT TTCAGATGGA GTTTCCCTCT TGTCGCTGAG GCTGGAGTGC AATGGAGCAA TCTCAGCTCA CTGCAACCTC CGCCCCCCCCA									
4810	4820	4830	4840	4850	4860	4870	4880	4890	4900
GGTTCAGCA ATTCTCCTGC CTCAGCCTCC CGAGTAGCTG GGATTACAGG CACCTGTCAC CATGTTCAAGC TACTTTTGT CTTTTAGAG, AGACAGGGTT									
4910	4920	4930	4940	4950	4960	4970	4980	4990	5000
TCACCATGTT GGTCAAGGCTG GTGTTGAAC CCTGACCTCA AGTGATCCAC CGGCCTCAGC CTCCCAAAGT GCTGGGATTC CAGGGGTGAG CCACCGCAC									
5010	5020	5030	5040	5050	5060	5070	5080	5090	5100
CGGCCAACTA GTGCTGCTC CAAGGCCTGG CTTGCAGGGC TTCCCAAGTCA CAAAGGAGCA GACCGGGCTT CCATGGGGCC TTGGCACAGC ACACAGGCCA									
5110	5120	5130	5140	5150	5160	5170	5180	5190	5200
TGGCGAGAAC TTGCTTCCCA CACACCTGAG TGIGTCCCTG GGCAGCCAAA GCCAGGACTC CCTCCCTCCC CAAGACCCCTG GTCCCTGAAA GATCCTGAAT									
5210	5220	5230	5240	5250	5260	5270	5280	5290	5300
ACCCCCGAGT GCCTCCCAAC AGGTGCTTCG GGCTCTTGA ACAGAGTCCA GCTGGGCTC TGAACTCCTG GGCCAGATGT TTCTCCCGCC TGCCAAATGTC									
5310	5320	5330	5340	5350	5360	5370	5380	5390	5400
AAGCTGTCTG GAGGACAGCG CTGGGGCGCG GAAAACGCGC TGGAGACACT AATCCTTCC TGGGCTGGGC ACCGAGGATG GAGGGAGACA GGCTCTGAAG									
5410	5420	5430	5440	5450	5460	5470	5480	5490	5500
CAAATGCCCT CAGGGCTGGC TTTCATGG CTCTAATTAA GCCTGCAATT TGGGCTGGC GCTCATCTTC CCACTGAACA TCATAATTAA AGTCAATTCA									
5510	5520	5530	5540	5550	5560	5570	5580	5590	5600
GTGTCCAAAG CTCCCCGCTC CCAGCTGGAA GTCTTCGAC TTGTTAGCTG GTAGCTTCC TITCTTCCCC ACAGCCACCG TTGTGTATAA TCCCTICAAG									
5610	5620	5630	5640	5650	5660	5670	5680	5690	5700
AAGCGAAAC AGCAGCGCTC CCCTGTCCTC TGGTTGCTCT TTGAAATTG GCACAGGCAG TTCTTGCCTG CCTGCCTTGC TGGCTGTGTG									
5710	5720	5730	5740	5750	5760	5770	5780	5790	5800
TCCCCTAGT CTACGGGCTG AGCGTTGTGT CACTGGTCA TGCTGGGTC CCTGGTGAAA ATGGGCCAGG CCAGGGTCA GGAAGGTAGA AGGGCAGTGA									
5810	5820	5830	5840	5850	5860	5870	5880	5890	5900
TCAGGGAAAGC AGGTCAAGATG CTGGGAAGG CTCCGGTCCC TGGATTGCGG CTGGACAGGA AGGACACCTT CCAGGACACT TCTGGACACA TGIAAGATCT									
5910	5920	5930	5940	5950	5960	5970	5980	5990	6000
TGGCCCGAAG AGATGTCCCA CTTCCGAGCC ATGTAGCCAG AGAGATCAGC TCAGAGAGGT CTGGGCCAG AGGGGGGACC TGGTCGTAGC TCTGTCTTC									
6010	6020	6030	6040	6050	6060	6070	6080	6090	6100
AGTCAGAACG GGGACGGGCA CAGGGAGTGT AGAAGGGTCT CGCTGAAGAA GTATGCAGAT TCTCAGGCAG TGGGTTCAAC TCTCATCTAT CGGGCTTAA									
6110	6120	6130	6140	6150	6160	6170	6180	6190	6200
GTCTGCATGT GGCCTCCACA GGCTAAATAG TGTAGATGCT GCCTATGTAG TAGATTTGGA CCCAATTCTT TTGGCCATGT AGACAGAGCC TCTCCTTATA									
6210	6220	6230	6240	6250	6260	6270	6280	6290	6300
GTGCTGCTGC TTCTAAGGG CCTGTGGGTAA GCGGGGCTGT GATGCCTCAG TATGTACCCA GCTTCCCTCA GCACCACCCC CTCGCATAAC TTGGTTCTT									

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**FIG. I(D)**

6310	6320	6330	6340	6350	6360	6370	6380	6390	6400
CTCTTCTTCC CCCCAAGAGT GGACCAGGCC ATCTACGGCT GCCCCTCTCT CGACCACGTG GTCCCAGGTG CCCTCCCGTG CAGAAGGTAT GGGGGCAAG									
6410	6420	6430	6440	6450	6460	6470	6480	6490	6500
GCCTGTGATG GGCCTGAGAC CCCGGGAAGC GCCCTCTAG ACTCGTAGCC CCTCCCTCTG TAGTGAAGT AGCAGTGTGAG ATGGTGGAC CTAGTGGAG									
6510	6520	6530	6540	6550	6560	6570	6580	6590	6600
GGGGGCCCCA GGAACCAACT GAGGGCACGG TGTAGAATGT CGGTGCCCTGG GGACCTCTAG CCCACAGTGG TGAGGGAGCG CCCTGGTAGA GCAGGTCTAC									
6610	6620	6630	6640	6650	6660	6670	6680	6690	6700
CAGCTCTGCC CCCAAGCTCA CCTGCTCAA GAGGTCCAT GTGGCCACCC CCACGCCAAG CCCTCCACC AGCACTCCCT CCGAGGGCTT CGGAGTCTGG									
6710	6720	6730	6740	6750	6760	6770	6780	6790	6800
TAGAGCCCCG CCTCCCACGA CAGGAACCCC CCTCTCCAGC TGCCCTTGCTCACAGGACAC CTGGGAGTT GCTGGATCAG AGAGTCAGAG GGGGCTTCCT									
6810	6820	6830	6840	6850	6860	6870	6880	6890	6900
GCAGGAGCGG GGGCCATGAG ACCTCGGAGG GTGGACTGTG GTGGGTGAAG GGAGAAGGCA GCACATTCCA GGCCGCAGGC AGCCGGGCA AAGGCTGGC									
6910	6920	6930	6940	6950	6960	6970	6980	6990	7000
AGTGGGATCG CAGGGAGCCT GACAAAGTGG AAAATGTCTG GTTAAAGGA GGGAGGGCG GGTCTGGAA GACACTGACA TCCTCTGCT ACGTGGGAGG									
7010	7020	7030	7040	7050	7060	7070	7080	7090	7100
AGACACAGGG CTCATCTGTA GCCATAGACA GACATGCCAA GGAAACGCCG AGGCCTGCCG GACTCTCCAG AAGGGAAATT GTCCCTGGCC CCAGCTCAC									
7110	7120	7130	7140	7150	7160	7170	7180	7190	7200
AAGCCTGGTC GGGCAATTA GGGCTAGTC TAGGAACAG GTGAGCTGTT CCTTCCAGCTCACATGTTCA AATTCTCTCC AGCCCCAGCT CTGAGCAGCG									
7210	7220	7230	7240	7250	7260	7270	7280	7290	7300
AGCCGGGCTT TGAGGGCCCT CTACTGGCAG GAAGCTCTGG CGCTGGAGC ATGTTAGAG AGGCTCTGAG GCTCGGTTCC TAGAACCTG GAGGACCTGG									
7310	7320	7330	7340	7350	7360	7370	7380	7390	7400
GCCTGGTGTG CTCTGTGGTG ATGGAGACAG AGCTGGGGG AGCCATCGCT TCCCTACCCCT GGGCCAACCA GGGCACCACA GACCCAGAGG GAAGCCAAGG									
7410	7420	7430	7440	7450	7460	7470	7480	7490	7500
TAGTGACGAT CCCGGGACAG TGGCTGTC ACCCACAGAT AGGGCGTTGG GGTCCCAGCG GATTCTGGC AGTGGAGGC AGGTGGCTCC GTGTCCTGG									
7510	7520	7530	7540	7550	7560	7570	7580	7590	7600
CTTGACAGCA CTTGCGAGTG GGACTCCAGG GACAGCGAAG GATTCACTTC GGCTGGAGCA GGAAGAGTGT TTCAGAAAGG AAGGGAGATG CCAAAGTCCT									
7610	7620	7630	7640	7650	7660	7670	7680	7690	7700
TAAATGCCAA GTTTAGTCTC TGGGTTGAT GCTCCAGGAA GTTGGAGAG GCGGTGGGA GAGCAAGAGA CGGGCGTGGT GTGCAATGTG ATGTCATCT									
7710	7720	7730	7740	7750	7760	7770	7780	7790	7800
ATCTAAAAAC AGTTGGCTT CCAAGAAGGT CTTAGCAGGG CGCGGGGGTG TCAGGGTTA CAGAACTCAT TTGAGATTAA TCCCAGCAGA TGTGTCATGT									
7810	7820	7830	7840	7850	7860	7870	7880	7890	7900
CTCAGAGAGG GACCAAGGGC AGGGCTGATT TGCAGCTTG GGATGTGCTG TGTTCCTTC AGAAGGGTCC CACCTCCCTG GGCTCTCGA GGAGAGGGC									
7910	7920	7930	7940	7950	7960	7970	7980	7990	8000
TGTTGATTI GAGGCCAGAG GGGCTCTCC CTCCTCACAC TCTGAGCAGG CGACAACTG CCTGCCCTAG AGCTGGCCCA GGGGGCTCG GAAGCCTTG									
8010	8020	8030	8040	8050	8060	8070	8080	8090	8100
CTGGGCTCTT CCCTGGGGAG TGGGACCATG ACAGACGAAA GAACCTGTT CTCATCTCTC CAAGCTGTGG GCACCCCTGC CGCTGCCCT GCCCTGCCA									
8110	8120	8130	8140	8150	8160	8170	8180	8190	8200
AGGGCTACAA ACTTTCCAG CTCAAGCCCA AATCTCTCA ACTGATGCCT ATTGAAGAT TCCAAGGTAA GAGGATGGAC CTGGGCCCCC ATCAGCCCTC									
8210	8220	8230	8240	8250	8260	8270	8280	8290	8300
CCTGACACCT GTTCCCCATC CGCCGCTGGAA AAAAGACGGT GCAGGATAGA GGACCGATGC CTGGCTCCGA AAACCCCTCT CGAGTAGCTG GGTCAAGGTT									
8310	8320	8330	8340	8350	8360	8370	8380	8390	8400
AAACTGAGTC TCTCTCCCT ACAGGCCCTCC CTCCCCAAGG GACCTGGAG CAGGTATGAG TCAGAAGCCA ACTTGGGCAC AGTGGCTAG GCCACAGAGC									

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**FIG. I(E)**

8410	8420	8430	8440	8450	8460	8470	8480	8490	8500
AGCCAGAGCA GATGCCAGAA ATAGCCCAC CCGGCTTCCC TGGGAGGTGT GGCCCTGGGG CTGGTTGTG TCTAAGCAGA ATCTGGACAC ACGGTACCA									
8510	8520	8530	8540	8550	8560	8570	8580	8590	8600
TGCTGCTCTT GGACACTATA GGATGCCCTCA TCTCTCATT ATCTCTGGAG GGACAAAGTG AAGGGGGCAO GACTAGTGGA CAGTGGATG CCCACCATCT									
8610	8620	8630	8640	8650	8660	8670	8680	8690	8700
CTCCTGGCA CAGGCTGTTT CTCTAGTC CCAATGCCCT TGACCACTGG GTCACTCCCT CATCCCATCA CAAAAGGGAA GCTGGGTCT CTAGAGATAC									
8710	8720	8730	8740	8750	8760	8770	8780	8790	8800
ACAGATGGTG TTTCAAGAGG GTGGCCGTTG TCCTTCCTTG TCGGGGGCA GCCACATGG CTTTCTTGCT GGAGGGTGGG TGGTGGTGT AGTACTGTGT									
8810	8820	8830	8840	8850	8860	8870	8880	8890	8900
CCCTTCGTAG AACATCAAGG ATGCCCCCCC ATTCTTAGGG ATGTGACCTT CCTCACCAAA TCCTCCATTG ACAATGTGGG ATTACCTTCC AATCCCCTGA									
8910	8920	8930	8940	8950	8960	8970	8980	8990	9000
GAGAGCCTGG CCCAGGCAG TCACGGCTT GTCTGGCTT TGGAGGGAG CTGGTTAGGC AGGGGTAGC CTGAGAACCA CGTACGGGTG GGOTGCAGGA									
9010	9020	9030	9040	9050	9060	9070	9080	9090	9100
GGCGGCAGGA CATGGTGGTG GTGGTCTTG GATGAAACC ATGTGCTTCC AGGAGCAGCG AGTCAGAACG CGGGCCAGGA CCAGGGGAGG GCATGCAGGT									
9110	9120	9130	9140	9150	9160	9170	9180	9190	9200
TCCCAAGGGCT CCTGCTTAA AGTGGCACTC ACTCTTAGCA TCCTGCAAAT CAATCAAAT TGACAAAGC TCAGGCTAAT AAGAAAGGGT CTGGCAGGTG									
9210	9220	9230	9240	9250	9260	9270	9280	9290	9300
GGCGTTTCC TCCCAGCCAT CTTCCAAAGC ACCATGGCA GGAGCTCCTG GCCCATTGCA TCTTGTCCAG CGTCCATCCA TGCATTATC TACCGAGGA									
9310	9320	9330	9340	9350	9360	9370	9380	9390	9400
TACCAAGGGC AGCGCCGTGA ACCCAGGGT CGGCTCCCCC AGTGCACAGC CAGGTGGCAT GACCCGTCCC TCCTTGCATG AATCACTTIC TAATCACCCCC									
9410	9420	9430	9440	9450	9460	9470	9480	9490	9500
GGCATGTGGG CATTCCCTCA GCGAGCGTT GGCCTGGTG CCCAGCCAGG CATTAGCAGG AGCTGCCAT GGCCCTGCCT GGTTCCCTGG GGACAGGCAG									
9510	9520	9530	9540	9550	9560	9570	9580	9590	9600
GTGGGAATCC TGGGCTAGCT ACTCAGGTC TCCTCTGGC TCAAACCCAGG GAGGCCTCTC TCTTCTGAA TCCGATGGCA ACGGTGGGAG GCCTAGGGCA									
9610	9620	9630	9640	9650	9660	9670	9680	9690	9700
CCTTCCGTA CCTTTCCAA AGATGCCCTC CTCCGCCCT GCATGACCTG GGGTGAGTCC TTCCCTGCC TGTCCCTCAG TTTCCTGAAT GCTCGCTGAC									
9710	9720	9730	9740	9750	9760	9770	9780	9790	9800
CATGGTATT TCTCCCACTT GGCGGGCCA GACTGCCAAT GCTACGGTCA CTCCAACCGC TGCACTACA TTGACTTCCT TGAATGTGGT GACCTGCGTC									
9810	9820	9830	9840	9850	9860	9870	9880	9890	9900
AGCTGCAAGC ACAACACGCG AGGTCAAGCAC TGCCAGCACT GCGGGCTGGG CTACTACCGC AACGGCTCGG CACAACCTGG AATGAGAAC GTCTGCATTG									
9910	9920	9930	9940	9950	9960	9970	9980	9990	10000
GTTGAGAGGG CACGGACACG GCACAGGGAA CTTGCTGGAA TGGCTGCAGG GTGCACTGCC CTGGCAGGTG GCCTCTGGGG GCCCCCTGCA TCAGAACAC									
10010	10020	10030	10040	10050	10060	10070	10080	10090	10100
CTGGGAGAC TGTGGGAATT CTAACTCCAG GGCCTCTCC AGTTGAGCAT CTCTAAGGAC AGAAAGCTCC AGAAAGCTGT CTATTAGTA CCTACCCCTG									
10110	10120	10130	10140	10150	10160	10170	10180	10190	10200
CGGTCTCCG GTAAGTTTG CACTGGAGTT GCAAAACTTA CCAGTGGCC TICCCCTCTC GGGCAACTGG AGGGGACACT GACCCCTCTG GCTCAAAGAG									
10210	10220	10230	10240	10250	10260	10270	10280	10290	10300
CTGTGACTCT GGCAAGGTGGC AGGGCACTCA TGGCAGAGGC CACTGAGCAT CTGTCTGGGG CTGGTGTGT GGGGGTCCCC CTCCATAGCT CCTTCCAGA									
10310	10320	10330	10340	10350	10360	10370	10380	10390	10400
AAGGTGGAGG ACCAGCCTAT CCCTCCCT GCAGGGGCC AGTTGGGGCC AAAAGATCGC CTTGCTGCCT GCATTGTGC AAGTCCCTTC CCGTTGCTGG									
10410	10420	10430	10440	10450	10460	10470	10480	10490	10500
GCCCTCAGCTT CCTCATTCAT CAAATTGGCA GGCAGATCA ATCAAAGGT TTCAGCTCTT TTTCAGGGCT GAAGCTTTTC TTCAAATGCT TTACCAAGGCC									

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FIG. I(F)

10510 10520 10530 10540 10550 10560 10570 10580 10590 10600  
AGGTCCAGCT ATAAAGCTGC TCTTCACCCC TGGTGGGCAC CCAGTCTGCT TTCTTCCAAG TTGCTACTCA AGGACTGGCT TCTGGGTAGA GAACGAAGTC

10610 10620 10630 10640 10650 10660 10670 10680 10690 10700  
CATCAGGGCC CTGGGCTGGG CAAAGACCCA AAGCCATGAC CCCAACCAA ACCCACAGCT CGAATGGTG CCCGTGCGTC AGTAGAGGCC AGGTCTCGGC

10710 10720 10730 10740 10750 10760 10770 10780 10790 10800  
CTCAGGGGCT GTCCCCAAC CCTGCCCAGC CAGGCCCTTG GGACACCATC ACCCATCCCC CACCCAGCAG OAGGCTCTGG CTGCCCAGAG GAGGGGCTCC

10810 10820 10830 10840 10850 10860 10870 10880 10890 10900  
TGC~~AAAGCTG~~ GAGCTGTCGG TCTGAATTCT GGC~~GGCC~~CATG TCAGATAATT CCATCAACTC TAAGTGTCA AAGCCGCTGA CGTCACAGGG GCCCAGCTGC

10910 10920 10930 10940 10950 10960 10970 10980 10990 11000  
AGGGACAGGG CAGGGCCTT GGATCCAATT AGAGGTGCC ACACCCCTGGC ACCCTCTCC TCTCCCTGGC TCTCCCTGCC TCCACCCCGA GAGCCAGCAC

11010 11020 11030 11040 11050 11060 11070 11080 11090 11100  
TGAGCTGCAA GGT~~TTCT~~CAG GGTGGACGAT ATT~~ACCC~~CTC IC~~CC~~ACAGAG CCCAAGGCA ACCAAGTGGG CCCACCCGGG ACCAGGAATA GGCTGTTCT

11110 11120 11130 11140 11150 11160 11170 11180 11190 11200  
CCACGTCCCC TGCAAAGGAG CTATGGAGGG GGGCCACCCA CAACACAGCA GCCCCAGACA TGCTCAGTGG CCTCTGCTGA GTTCTGCCA CTGTGGAGT

11210 11220 11230 11240 11250 11260 11270 11280 11290 11300  
CATAGCTCTT TGGAGATGGG AAGGACAGCG ACCCTCTAGT TGCC~~CC~~AGAGA GGGGAAGGGG CTGACCAGGC CACACCAGTG CCAGGGGGG GAAAGTGGGG

11310 11320 11330 11340 11350 11360 11370 11380 11390 11400  
CTGGGACGTG TTGATCCC AGGAAGGAAG CCAGAGCTT CTCTCCAGGC CTGGCCACCC TGGGAAGTCC CCACCTGCCG TCCAGCCCGG GGCTCACGTG

11410 11420 11430 11440 11450 11460 11470 11480 11490 11500  
GACCCAGTGT GGGGAGCATC CCCTGGGGAG TGTGGAGATG CTCCCTGCGA GCCCCGGAGA GTGGGGTCC GAGCAAGACG GCGCCACAC GTAGCCCTGA

11510 11520 11530 11540 11550 11560 11570 11580 11590 11600  
CCGCGCGCCC GTGCCCGTGT CCGTCCAGAG TGTAACTGCA ACCAGATAGG CTCCGTGCA GACCGGTGCA ACAGACCCG CTTCTGCGAG TGCCGCCAGG

11610 11620 11630 11640 11650 11660 11670 11680 11690 11700  
GCGCGGCCG CCCCAGTGC GACGACTGCC TCCCACGCAC TACTGGCCA CGGCTGCTAC GTGAGTGCAGC GCCGTCCCCG TGGCGGGCCT CGGAAAGGG

11710 11720 11730 11740 11750 11760 11770 11780 11790 11800  
ACGGGCTAGA CCAGGCATGG CGGCCTATGG CAGGGCAGGC GTGGCATGGC CTAGCAAGCA GGGCAGGCCG GGAATGGTGG GCCTATGGCA GGGCAAGAGG

11810 11820 11830 11840 11850 11860 11870 11880 11890 11900  
CGTGGCGGGG CCTCGCGAGA CGGGGCAGGC CGGGCAATGG TGGGCCTAGT GAGACGGGCA AGGTGGAT AGTTGGCAGG GGCTGGTGA GATGGACCG

11910 11920 11930 11940 11950 11960 11970 11980 11990 12000  
ACCCGGGATC GTGGACGGGA CTCTAGCGAG ACCGAGCTGG CAGGTGGGG GGGACAGGAT OCTGCTGAGG TCCGGGGCAC GGGCCAGGG CGGGGTCCAA

12010 12020 12030 12040 12050 12060 12070 12080 12090 12100  
GAGCTCGGGG CGGGGCCTGA TGGGACCTGA GCACGGTGGT GCCTGGTGG AACTACGAGA AAGACCGAGC TGGGGTTGGA AAGGTATTTG CGGGGACAGA

12110 12120 12130 12140 12150 12160 12170 12180 12190 12200  
GGGAGGGAGG CTGTCCAAGT CGGCCTTACG CGCGGGCACA GGGTCAAAGG AGGCTCCAGG CGCGTGGAAC ACCACGTGCA CAGCTCTGGA GACTGCAGGC

12210 12220 12230 12240 12250 12260 12270 12280 12290 12300  
GCGTCTGAG AACAGCACCG AGGCCAGTGG GGGGGGAGA GAGGGCAGC GGTGGAGGC ACCGGGGGCC AGATCTGCC CGGGCGCCGT CACCCCTCCGA

12310 12320 12330 12340 12350 12360 12370 12380 12390 12400  
GGGGGGACGT TTGCAACCCA GGGCCCTGG AGCCTCTAC ATCCCCGGCC CAGACGGGCC CCCGGGGTC TGGCACACCC TGTTCGAGAG CTGGAGGTT

12410 12420 12430 12440 12450 12460 12470 12480 12490 12500  
GGGGGGGGGA CGGGGCCACC CCCCGTGCAG ACCGCCCCCT CCTGCAGCCA ACGTGTGCGA CGACGACCGAG CTGCTGTGCC AGAACGGAGG CACCTGCC

12510 12520 12530 12540 12550 12560 12570 12580 12590 12600  
CAGAACCCAGC GCTGGCGCTG CCCCGCGCGC TACACGGGG TGGCCTGCCA CGCAGCCCCG CTGCGACCCCC GCGGACGATG ACGGCGTCTG GACTGCC

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**FIG. I(G)**

12610 12620 12630 12640 12650 12660 12670 12680 12690 12700  
GGCGCCCCGG GCGCGCCCCG CGCCCCGCC ACCCTGCTCG GCTGCCTGCT GCTGCTGGG CTGGCCGCCG CCTGGGCCGC TGAGCCCCGC CCCGAGGAGT  
12710 12720 12730 12740 12750 12760 12770 12780 12790 12800  
CCCCGGCACCG GAGGCCGGGG TCCCAGGTCC GGCGGGCCG CAGTCGAGGC CGGGGGTAG AAGGGTCCGG CCCGAGGTGC TCCCAGGTGC TACTCAGCAG  
12810 12820 12830 12840 12850 12860 12870 12880 12890 12900  
GGCCCCCGCCG CGGGCCCGCG CTCCCGCCCG CACTGCCCTC CCCCCGCAGC AGGGGGCCT TGGGACTCCG GTCCCCGCAG CCTGCGATTT GGTTTGGTT  
12910 12920 12930 12940 12950 12960 12970 12980 12990 13000  
TTCTTTGTA TTATCCGCCG CCCAGTCCCT TTTTGTCTT TCTCTCTCTC TCTTTTTTTT TTTTTTTTC TGCGGGTAG CAGAGGTGC GGAGAACGC  
13010 13020 13030 13040 13050 13060 13070 13080 13090 13100  
TGCTCGCCCC ACACCCGTCC TGCCTCCCAG CACACTTACA CACACGGGAC TGTGGCCGAC ACCCTGGCC TGTGCCAGGC TCACGGCGG CGGGGGACCC  
13110 13120 13130 13140 13150 13160 13170 13180 13190 13200  
GAGCTGCAGT TGCCTACAAT TCCCTAGCGCT GACTTGCTT GTTTCTATTTC TTATTTCTT GCAACCCACC ACACCCAGG CCTACCGCAG GCGCCCGTGA  
13210 13220 13230 13240 13250 13260 13270 13280 13290 13300  
CCACGCAACT CACCTCTGGG GAGGAGGAGA GAAGCAAGG GTGGGGGGCC CTGGAAATTTC GCTTCGTAG AGAATCTTT TGTGTTGATTC CACTGCTCTG  
13310 13320 13330 13340 13350 13360 13370 13380 13390 13400  
CAAGGGGAC GGGCAGGACT CGTCAGCCGC GGGGGCCAT GTGGAGAAT CCGAGGAAT AAAGAGGTTT GCTCACTGCT GCCTCCACGG CCTGTTTCT  
13410 13420 13430 13440 13450 13460 13470 13480 13490 13500  
TTCTGTGTTG GGCACGGTGG GCAGGTGTGG GGCTTACAGA GGAATCCACA ACACGCCCTT AAAGAAACGT TTCCTACTGG GGCCACCATT TCCCTGGGCC  
13510 13520 13530 13540 13550 13560 13570 13580 13590 13600  
TTCTGTGGA TTCCAGCAGC AGTCCCCCTT CCCCCCAGGC TTGGCTGGCA GAGTTTCCA CCCCCGGGCC AGGCTGCAGG TGCCCCACCT GTTACGGACCC  
13610 13620 13630 13640 13650 13660 13670 13680 13690 13700  
TCCCCACACT GAAAGGCTGC CTCCCTCCTT TCCCAAAAAA GAAATCCGGA GTGTATTGGC CCTTTCTAC AAGAAGTCCA AGGAAATGA CTCAGGGAGA  
13710 13720 13730 13740 13750 13760 13770 13780 13790 13800  
ATCCTACCGAG AGCTTGAATC CAATGCTCTG ATTATACTG TGTCTCGGTG GCCACCTCCG ATGGATGTGT CATCTCAGAC CTGTTGCAGC CGGAGCCTCA  
13810 13820 13830 13840 13850 13860 13870  
AGTCCAATAT CACATGAAGC TGAAACCCACA ATGTCGGCCA CGCGCTCCTT CGCAGATTTC AGATGGCATG AATTG

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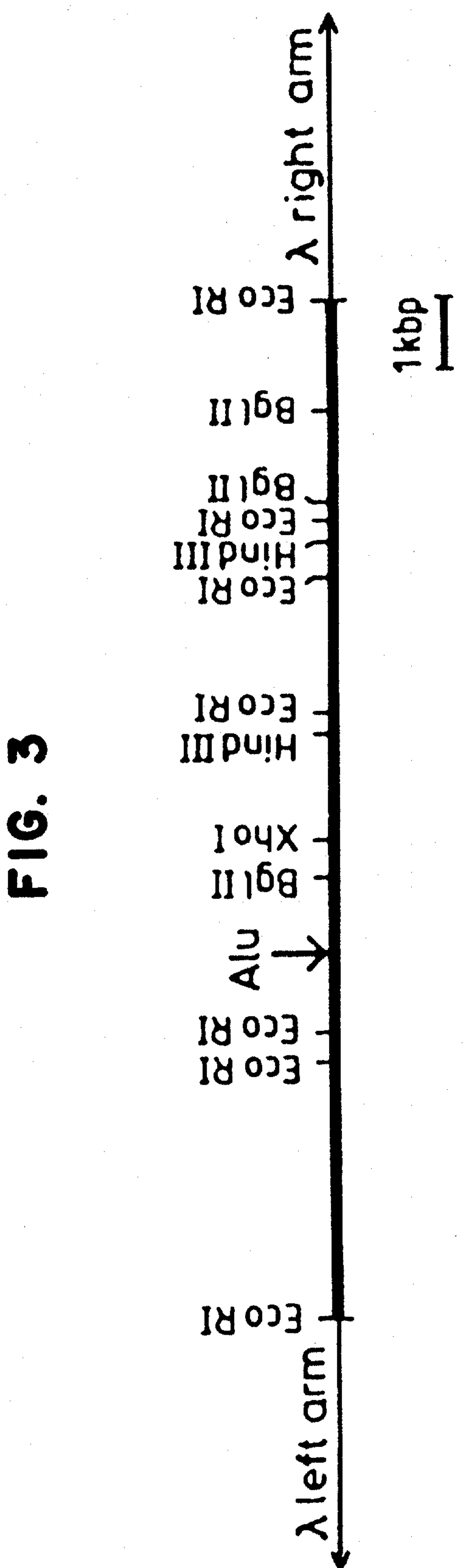


FIG. 3

UNSCANNABLE ITEM

RECEIVED WITH THIS APPLICATION

(ITEM ON THE 10TH FLOOR ZONE 5 IN THE FILE PREPARATION SECTION)

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DOCUMENT REÇU AVEC CETTE DEMANDE

NE POUVANT ÊTRE BALAYÉ

(DOCUMENT AU 10 IÈME ÉTAGE AIRE 5 DANS LA SECTION DE LA  
PRÉPARATION DES DOSSIERS)