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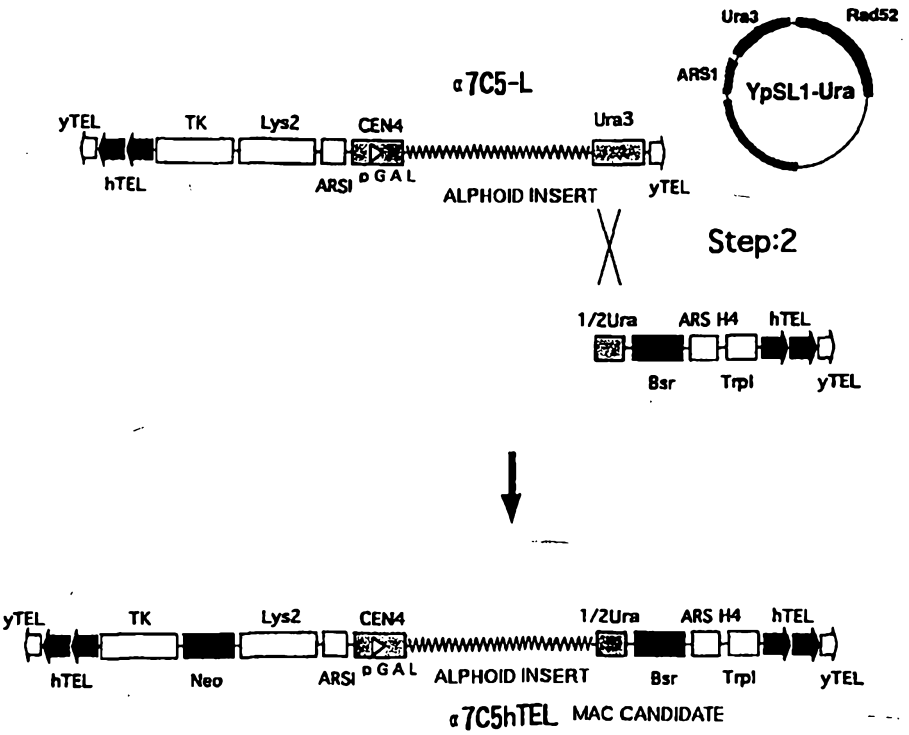

(57) Abstract

A mammalian chromosome is produced according to the method comprising the steps of: introducing a DNA construct comprising a mammalian telomere and a centromere into a mammalian cell, wherein the centromere has a DNA sequence containing some copies of CENP-B box sequence consisting of: 5'-NTTCGNNNNANNCGGGN-3', wherein N is any one of A, T, C and G.

DESCRIPTION

MAMMALIAN ARTIFICIAL CHROMOSOMES

[Technical Field]

This invention relates to mammalian artificial chromosomes (MACs) that can replicate autonomously, be stably maintained extrachromosomally and transmitted efficiently in mammalian cells. The invention includes methods to construct, modify and stably maintain in yeast cells yeast artifiical chromosomes (YACs) which have the potential ability to form MACs when introduced into mammalian cells.

[Background Art]

To construct a mammalian artificial chromosome (MAC) carrying the known functional elements required for chromosome replication , stable extrachromosomal maintenance and segregation in a manipulatable form will be of great value not only for basic studies on organization and function of mammalian chromosomes but also as a vector to introduce DNA segments (genes) of interest to test their functions in mammalian cells or bodies, since the genes carried by MACs will neither be subject to variable expression due to integration position effect nor cause unpredictable insertion mutation on host chromosomes. Further more, MAC will have capacity to accomodate a DNA segment up to megabases wherin an entire large gene or group of genes and regulatory elements could be included. For these reasons, MACs will offer exciting alternative vectors to currently existing vectors for somatic gene therapy, because frequently used infectious vectors derived from viruses are either integrated randomly into host chromosomes or stay extrachromosomally but only trangiently. Besides, these vectors are able to carry only short DNA

segments. A new way to generate transgenic mice will be provided by invention of MACs, if their stability during meiosis is established in mammalian development. However, the construction of a MAC has not yet achieved due to the technical difficulties (Willard, Proc. Natl. Acad. Sci. 93, 6874-6850,1996).

Yeast artificial chromosomes (YACs) has been constructed (Burke et al. , Science, 236, 806-812,1987) with three essential DNA elements from the budding yeast, Succharomyces serevisiae; namely, an origin of replication or autonomously replicating sequence (ORI or ARS) required for initiation of DNA replication, telomere sequences (TEL) required to stabilize and facilitate complete replication of chromosomal ends, and a centromere (CEN) required for faithful segrigation of sister chromatids after replication. Since then, YACs became a major tool for cloning of large gene segments of complex genomes. In analogy to YACs, MACs are believed to be constructable with the three essential elements derived from mammalian genomes. Among the three, telomeres have been isolated from mammalian chromosomes and used for the mammalianchromosome manipulation (Brown et al., Hum. Mol. Genet., 27-1237,1994; Farr et al., EMBO J., 14, 5444-5454,1995), but centromeres and origins of replication of mammalian chromosomes were found to be difficult to isolate because of unavailability of activity assays.

The present investigators have analyzed specific structure of mammalian centromere locus in order to reach information on the essential functional structure of mammalian centromeres. They have found that centromere protein B (CENP-B) , one of the antigens recognized by anti-centromere antibodies (Moroi et al., Proc. Natl. Acad. Sci. USA, 77,1627-1631,1980) at centromeres of various mammalian chromosomes, specifically

recognizes and binds 17bp sequence (CENP-B box) in centromere satellite DNA (alphoid DNA) in human genome (Masumoto et al., J. Cell Biol., 109, 1963-1973, 1989; Muro et al., J. Cell Biol., 116, 585-596, 1992). The recognition sequences of CENP-B were found in centromeric satellite DNA of mouse spieces (Masumoto et al., J. Cell Biol., 109, 1963-1973, 1989; Kipling et al., Mol. Cell. Biol., 15, 4009-4020, 1995) and the consensus sequence of CENP-B box was established to be 5'-NTTCGNNNNANNCGGGN-3' (Masumoto et al., NATO ASI Series, vol.H72, Springer-Verlag, pp31-43, 1993; Yoda et al., Mol. Cell. Biol.,16, 5169-5177, 1996). They have also demonstrated that a pair of CENP-B formed in themselves a dimer at the C-terminal and bound to a CENP-B box located in the centromeric satellite DNA at the N-terminal of each CENP-B polypeptide, so that a stable complex consisting essentially of the dimer protein and the two regions (or strands) of the DNA, was formed (Muro et al., J. Cell Biol.,116,585-596,1992; Yoda et al., J. Cell Biol., 119, 1413-1427,1992; Kitagawa et al., Mol. Cell. Biol. 15, 1602-1612,1995). Further, the investigators studied the location of CENP-B box on the human chromosome 21 (Ikeno et al., Hum. Mol. Genet., 3, 1245-1257,1994), and found that there were two distinct regions in the alphoid DNA array of the chromosome 21 as regards the CENP-B box: one (α21-I) was furnished with a regular series of CENP-B box sequence, and the other (α21-II) was possessed of scarcely any CENP-B box sequene, that the two regions go side by side for a stretch of about several megabases, and that the former was located at the position on the chromosome where the centromere proteins located and the latter was located at a position slightly shifted towards the short arm of the chromosome (Ikeno et al., Hum. Mol. Genet., 3, 1245-1257,1994).

he alphoid The present investigation relates to tDNA region with CENP-B box. It is an object of the investigation to provide artificial chromosomes derived from the above region that can be kept stably in extrachromosomal region of mammalial cells, especially in human cells, and be safely transmitted to cells of succeeding generations. The present investigation includes development of methods to construct, modify and stably maintain the precursors of such artificial chromosomes in yeast cells as YACs, which have potential ability to form mammalian artificial chromosomes when introduced into mammalian cells.

[Disclosure of the Invention]

This invention has been achieved by finding a method of constructing an yeast artificial chromosome construct comprising a DNA sequence including CENP-B box sequences from the alphoid DNA region of human chromosome 21 that can interact with CENP-B or the centromere protein B, and a segment of human telomere sequence by the use of homologous recombination in yeast cell, and finding further that when the construct is introduced into a human cell, the construct is replicated autonomously in a human cell and is stably maintained in lineage.

This invention provides a DNA construct comprising a mammalian telomere and a centromere, wherein the centromere has a DNA sequence containing some copies of CENP-B box sequence consisting of : 5'-NTTCGNNNNANNCGGGN-3', wherein N is any one of A,T,C and G.

In a preferred embodiment of this invention, a DNA construct comprising a mammalian telomere and a centromere, wherein the centromere has a DNA sequence containing some copies of CENP-B box sequence designated as sequence No. 1.

Preferably, the centromere contains spaced repeats of the CENP-B

box sequence.

This invention provides a DNA construct comprising a mammalian telomere and a centromere, wherein the centromere has a DNA sequence containing some copies of sequence designated as sequence No.2 or a sequence derived from the sequence No.2 in which one or more nucleotides are added, deleted and/or replaced.

In a preferred embodiment of this invention, the centromere is derived from human chromosome. The DNA construct further comprises one or more sequences necessary for the DNA construct multiplication in yeast cells. The DNA construct further comprises a sequence encoding a selectable marker gene. The DNA construct is capable of being maintained as a chromosome in a transformed cell with the DNA construct. Preferably, the DNA construct is capable of being maintained as a chromosome in a human cell. Further, the DNA construct is capable of being maintained as a chromosome in a mouse cell.

This invention provides a host cell transformed with the DNA construct. In a preferred embodiment, the host cell is a human cell. Further, the host cell is a yeast cell. Still further, the host cell is a mouse cell.

This invention provides the DNA construct further comprises a sequence of a gene of interest. The DNA construct further comprises a genome DNA sequence containing structural region and its regulatory region.

This invention provides a method of homologous recombination comprising the steps of:

(i) producing a recombinant DNA construct from two or more linear and/or circular DNA sequences partially homologous in a DNA recombination

deficient host cell with a plasmid for DNA recombination,
(ii) collecting cells carrying the recombinant DNA construct without the plasmid. This invention, allowing a highly efficient intracellular homologous recombination can simplify the conventional in vitro recombination based on the use of restriction enzymes, and thus bring in a new recombination method whereby a given DNA segment can be formed. Preferably, the host cell is a yeast cell. Further, the recombinant DNA construct is of a yeast artificial chromosome. Still further, one of the DNA sequences is of a yeast artificial chromosome. Additionally, one of the DNA sequences is of a yeast artificial chromosome which has a repetitive DNA sequence.

This invention provides a method of establishing a yeast artificial chromosome construct comprising the steps of:
(i) producing a first recombinant yeast artificial chromosome with a mammalian telomere and a centromere in a DNA recombination deficient host cell with a plasmid for DNA recombination, wherein the centromere has a DNA sequence containing some copies of CENP-P box sequence consisting of :
5'-NTTCGNNNNANNCGGGN-3',
wherein N is any one of A,T,C and G.
(ii) selecting cells carrying the first recombinant yeast artificial chromosome without the plasmid.
(iii) Producing a second recombinant yeast artificial chromosome with the telomeres and the centromere from the first recombinant yeast artificial chromosome in the host cell with a plasmid for DNA recombination,
(vi) selecting cells carrying the second recombinant yeast artificial chromosome without the plasmid.

This invention provides a method of producing a mammalian artificial chromosome comprising the step of :
introducing a DNA construct comprising a mammalian telomere and a centromere into a mammalian cell, wherein the centromere has a DNA sequence containing some copies of CENP-B box sequence consisting of :
5'-NTTCGNNNNANNCGGGN-3',
wherein N is any one of A,T,C and G.

Further, the mammalian cell is a human cell. Further, the mammalian cell is a mouse cell. Still further the DNA construct is derived from Saccharomyces cerevisiae α7C5hTEL designated as FERM BP- 5625.

This invention provides a mammalian artificial chromosome which is produced according to the method of producing a mammalin artificial chromosome of this invention.

This invention provides a method of fragmentation of a chromosome comprising of step of :
introducing a DNA construct comprising a mammalian telomere, a centromere and a DNA sequence partially homologous to the chromosome and a DNA costruct comprising a mammlian telomere and a DNA sequence homologous to the chromosome into the mammalian cell, wherein the centromere has a DNA sequence containing some copies of CENP-B box sequence consisting of :
5'-NTTCGNNNNANNCGGGN-3',
wherein N is any one of A,T,C and G.

Brief Descripion of Drawings

Fig.1 shows schematic representation of the human chromosome 21 centromere region. The α21-I locus is composed of 11 monomer order repeat units containing five CENP-B boxes. α21-II locus on the short arm

side of α21-I is composed of diverged alphoid families and may contain other repetitive DNA sequences. CENP-B boxes are distributed regularly in the α21-I locus. On the other hand, the α-21-II locus contains only rare CENP-B boxes.

Fig.2 shows construction of pYAC55pkc used for Alphoid DNA cloning.

Fig.3 shows alphoids DNA inserts which were cloned to YAC55pkc from WAV17 containing the human chromosome 21.

Fig.4(a) shows cloned alphoid DNA inserts by PFGE(stained with EtBr).

Fig.4(b) shows analysis of cloned alphoid DNA inserts by Southern hybridization using α21-I probe.

Fig.4(c) shows analysis of cloned alphoid DNA inserts by Southern hybridization using α21-II probe.

Fig.5 shows alphoid length and detection of other repetitive sequences in alphoid YAC clones by southern and dot hybridization.

Fig.6 shows CENP-B box immunoprecipitation -competition analysis. PCR generated alphoid DNA from alphoid YAC clones were mixed with end labeled CENP-B box DNA and CENP-B , then immunoprecipitation reactions were carried out . The ratio (%) of immunoprecipitated probe was plotted against the amount of the competitor.

Fig.7 schematically shows construction of pMegaΔ using pCGS990.

Fig.8 schematically shows construction of pMega SV-neo using pMegaΔ.

Fig.9 schematically shows construction of pMCU using pJS89.

Fig.10 schematically shows construction of MCU-bsr using pMCU.

Fig.11 shows YpSL1 and construction of YpSL1-Ura using YpSL1.

Fig.12 shows schematically the procedure used for constructing α7C5-left arm.

Fig.13 shows a schematic diagram of homologous recombination of the YAC left arm(step1).

Fig.14 schematically shows the procedure used for constructing α7C5hTEL

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Fig.15 shows a schematic diagram of the homologous recombination of the YAC right arm(step2), resulting in construction of α7C5hTEL.

Fig.16 shows recombined alphoid YACs digested with BamHI by hybridized with human telomere sequence.

Fig.17 shows the modified YACs after recombination processes analyzed by PFGE.

Fig.18(a) shows merged image of FISH analysis of cell lines transformed with recombinant YAC. Metaphase chromosomes from cell line 7C5HT1 was hybridized with α21-I probe (green signals) and YAC arm probe (red signals) . The chromosome was counterstained with DAPI.

Fig.18(b) shows merged image of FISH analysis of cell lines transformed with recombinant YAC. Metaphase chromosomes from cell line B13HT1 was hybridized with α21-II probe (green signals) and YAC arm probe (red signals) . The chromosome was counterstained with DAPI.

Fig.18(c) shows FISH analysis of cell lines transformed with recombinant YAC. Metaphase chromosomes from cell line H7C5HTm1was hybridized with α21-I probe (green signals) and YAC arm probe (red signals) . The chromosome was counterstained with DAPI.

Fig.19 shows efficiency of the minichromosome formation by alphoid YAC DNA transfections.

Fig.20 shows stability of the alphoid YAC-derived minichromosome as determined by plating efficiency and by FISH analysis.

Fig.21 shows stability of the alphoid YAC -derived minichromosome(or

integration site) of cell lines recloned from 7C5HT1.

Fig.22(a) shows merged image of detection of centromere antigens on minichromosomes and chromosomes. Metaphase chromosomes from cell lines 7C5HT2 was analyzed simultaneously by FISH with α21-I probe (green signals in panel) and YAC arm probe (red signals) .

Fig.22(b) shows merged image of detection of centromere antigens on minichromosomes and chromosomes. Metaphase chromosomes from cell lines 7C5HT2 was analyzed simultaneously by FISH with YAC arm probe (red signals) and by indirect immunofluorescence with anti CENP-B antibody (green signals in panel) .

Fig.22(c) shows merged image of detection of centromere antigens on minichromosomes and chromosomes. Metaphase chromosomes from cell lines 7C5HT2 was analyzed simultaneously by FISH with YAC arm probe (red signals) and by indirect immunofluorescence with anti CENP-C antibody (green signals) .

Fig.23(a) shows merged image of detection of centromere antigens on minichromosomes and chromosomes. Metaphase chromosomes from cell lines 7C5HT1-2 was analyzed simultaneously by FISH with α21-I probe (green signals in panel) and YAC arm probe (red signals) .

Fig.23(b) shows merged image of detection of centromere antigens on minichromosomes and chromosomes. Metaphase chromosomes from cell lines 7C5HT1-2 was analyzed simultaneously by FISH with YAC arm probe (red signals) and by indirect immunofluorescence with anti CENP-B antibody (green signals in panel) .

Fig.23(c) shows merged image of detection of centromere antigens on minichromosomes and chromosomes. Metaphase chromosomes from cell lines 7C5HT1-2 was analyzed simultaneously by FISH with YAC arm

probe (red signals) and by indirect immunofluorescence with anti CENP-C antibody (green signals) .

Fig.24 shows metaphase chromosomes from cell line 7C5HT1 detection by indirect immunofluorescence both with anti CENP-B antibody (red signals) and anti CENP-C antibody (green signals). The chromosomes were counterstained with DAPI .

Fig.25 shows mitotic segregation of minichromosome in 7C5HT12.

Fig.25(a) shows metaphase cells of 7C5HT1-2 cell line hybridized with α21-I probe (green signals) and YAC arm probe (red signals).

Fig.25(b) shows anaphase cells of 7C5HT1-2 cell line hybridized with α21-I probe (green signals) and YAC arm probe (red signals).

[Detailed Description of the Invention]

In the following description, reference will be made to various methodologies well known to those skill in the art of recombinant genetics. Publications and other materials setting forth such well known methodologies will be referred to in the course of this description, and are incorporated herein by reference.

This invention provides a DNA construct that contains a centromere and a mammalian telomere . The DNA construct can be maintained as a chromosome. Even a long DNA fragment, by being inserted into the DNA construct of this invention, can be cloned in mammalian cells or other cells, and allowed to express itself stably.

(A Centromere)

The centromere of this invention has a DNA sequences containing some copies of CENP-B box sequence consisting of :

5'-NTTCGNNNNANNCGGGN-3',wherein N is any one of A,T,C and G(Masumoto et al., NATO ASI Series, vol.H72, Springer-Verlag, pp31-

43, 1993; Yoda et al., Mol. Cell Biol., 16, 5169-5177, 1996). Further, CENP-B box sequence is a 17bp sequence designated as sequence No. 1 . The sequence is derived from human chromosome 21(Ikeno et al , Human Mol. Genet., 3, 1245-1257).

The centromere of this invention has spaced repeats of CENP-B box sequence. The centromere has copies or repeats of CENP-B box sequence in sufficient quantity to provide a centromere property to a DNA construct in a host cell. Preferably, the centromere has a region where CENP-B box sequence is contained at high frequency.

A DNA sequence with regular repeats of the CENP-B box sequence which is located at centromere region of human chromosome contains giant repeats with 11 MER as a unit. Each 11 MER body has a size of approximately 1900bp, and each monomer (approximately 170bp) contains five spaced repeats of CENP-B box sequence (Ikeno et al , Human Mol. Genet., 3, 1245-1257).

One of the 11 MER body on human chromosome 21 has been isolated, sequenced and designated as sequence No. 2 (Ikeno et al , Human Mol. Genet., 3, 1245-1257).

It is to be noted that the centromere has a DNA sequence containing some copies of the sequence designated as sequence No.2 or a sequence derived from the sequence No.2 in which one or more nucleotides are added, deleted and/or replaced.

Further, dimers comprising a part of this 11 MER body or another 11 MER body were isolated, sequenced and designated as sequence No. 3 , No.4, No.5, No.6 and No.7(Ikeno et al , Human Mol. Genet., 3, 1245-1257).

(Cloning of centromere)

The centromere with CENP-B box sequences is prepared from human cells, from hybrid cells containing only specific human chromosomes such as WAV 17, or other mammalian cells.

In a preferred embodiment, suspension of those cells in PBS is mixed with LMP agarose solution in PBS, to solidified the cells as plugs. The plugs are treated with a proteinase, EDTA and lauroyl -sarcosine, and purified by dialysis to produce agarose plugs with concentrated DNAs of human DNA. Then, the plugs are treated with restriction enzymes such as Bgl I, Bgl II and Bam HI, and submitted to pulsed field gel electrophoresis (to be referred to as PFGE hereinafter) to remove DNA fragments of 50kb or less. The remaining plugs are recovered, treated further with Msp I, and dissolved to make a solution. The resulting DNA solution is submitted to dialysis for purification.

After dialysis, the concentrated DNAs are inserted into YAC vectors each with a marker, the resulting YACs are fractionated by PFGE, and agarose blocks containing DNA fragments of 50kb or more are picked up. Other methodologies are also useful in the DNA extraction and purification procedure.

These DNA fragments (YACs) extracted from the agarose blocks are mixed with yeast cells lacking DNA recombination enzymes that have been converted to spheroplasts so that the YACs may be introduced into the yeast cells. The yeast cells are grown on culture under a condition that allows transformed cells to be selected. The use of yeast strains lacking DNA recombination enzymes ensures stable maintenance of YACs in the transformed cells.

For selecting the centromere DNA sequence with CENP-B box sequences from among the transformed cells, CENP-B box sequence, the

whole or a part of 11 MER body, or the whole or a part of 2 MER body is made as a probe and hybridized with colonies or the DNAs extracted from transformed cells. This maneuver enables isolation of the transformed cells with CENP-B box sequences of human chromosome.

The centromere DNA sequences which are hybridized with the probes form a part of the alphoid region of human chromosome 21which is designated by the inventors as α21-I region or other alphoid region of mammalian chromosomes.

The α21-I region essentially consists of repeats of 11 MER body, the length of which is approximately 1.3Mbp. This region can be readily extracted from the genome DNAs of WAV 17, a mouse-human hybrid cell containing only human chromosome 21 as genetic materials of human origin.

Other alphoid region of the human chromosome 21 contains very small number of CENP-B box sequence or no CENP-B box sequence. The DNA sequence of this region can be selected in the similar manner described above: human genomes are treated with appropriate restriction enzymes, desired DNA fragments are collected by PFGE and introduced into YACs, the YACs are introduced into yeast cell strains lacking DNA recombination enzymes, α(Y)a and α(Y)b sequences are used as primers and multiplied by PCR, and the PCR products are hybridized with DNA extracted from the yeast cells so that the DNA sequence of interest is detected and cloned. The DNA sequence thus cloned does not contain above DNA sequence with repeats of CENP-B box sequence or the DNA segment which can be detected by 11 MER body or 2 MER body acting as a probe.

The sequence constitutes a part of the alphoid region of human

chromosome 21, and is designated by the inventors as α21-II. This is located close to α21-I region but more towards the short arm of the chromosome (Ikeno et al , Human Mol. Genet., 3, 1245-1257).

The α21-II region can be readily extracted from WAV17 cells like α21-I.

(Mammalian telomere)

A mammalian telomere sequence signifies here the DNA sequence which contains the repetitive sequences located in telomeres or terminal sequences of mammalian chromosomes. To construct a DNA construct which act as mammalian chromosomes, it is preferable to use a DNA sequence with repeats of 5'-TTAGGG-3' sequence or a repetitive unit of human telomeres.

(DNA construct)

The DNA construct containing a centromere with CENP-B box sequences and a mammalian telomere can be maintained stably as a chromosome in transformed mammalian cell with the construct. In other words, it can replicate autonomously in mammalian cells and be maintained stably extrachromosomally and transmitted to the progeny cells. CENP-B box sequence is preserved among human and other mammals containing mouse and apes (T. Haaf et al J. Mol. Evol., 41: 487-491). In additionally, a human chromosome 21 is maintained stably in a mouse-human hybrid cell WAV17. Accordingly, when the DNA construct with a centromere with CENP-B box sequence of the invention is introduced into a mammalian cell, it is maintained as a chromosome in the mammalian cell regardless of the centromere's origin.

(Preparing DNA construct)

To prepare a DNA construct by homologous recombination in a host

cell, it is necessary that the host cell is competent for homologous recombination and simultaneously able to maintain the recombinant DNA stably. The latter requires conditions defective in DNA recombination. Thus, the homologous recombination in this case consists of the following processes: to allow a plasmid carrying the gene for DNA recombination maintained temporarily in the DNA recombination deficient host cell (The plasmid expresses the DNA recombination enzyme while it stays in the host cell), thereby producing a recombinant DNA through transient homologous recombination, and then to select cells carrying the recombinant DNA without the plasmid.

(DNA recombination deficient host cell)

DNA recombination deficient host cells of the invention include eukaryotic cells containing mammalian cells and yeast cells, and bacterial cells. Preferably, the cells are yeast cells. Further, the cells lacking one or more DNA recombination enzymes are to be used in this invention as the host cell. The host cell with a plasmid carrying the gene for DNA recombination is a host cell of homologous recombination, and the cell without the plasmid is a host cell of maintaining the recombinant DNA construct, which is a stable provider of the recombinant DNA.

The yeast cells lacking DNA recombination enzyme to be used in this invention include the variant lacking the gene rad 51 or rad 52, or the gene coding for the expression of DNA recombination enzymes, or other variants that lacks the genes responsible for the expression of DNA recombination enzymes. Particularly, Saccharomyces cervisiae EPY305-5b strain (provided by Dr. Resnick, NIEHS, USA) or the strain with rad 52^- can be mentioned as a recommended material for this invention.

(The plasmid for carrying the gene for DNA recombination)

The plasmid expresses DNA recombination enzyme for the homologous recombination in the host cell. When the plasmid responsible for DNA recombination is not evenly distributed to daughter cells in cell division, it is capable expressing DNA recombination enzyme temporarily.

The plasmid includes, for example, YpSPL1 (Adzuma et al., Mol. Cell. Biol. 4, 2735-2744, 1984) (provided by Dr. T. Ogawa National Institute of Genetics, Japan) or the plasmid responsible for the expression of RAD52, and YpSL1-Ura. The latter is produced in the following manner: YpSL1 is cut with Eco RV, 0.57kbp Eco RV fragment (containing a part of TRP1 gene) is replaced with Sal I-Xho I fragment (containing URA 3 gene) of pYAC 55 which has undergone a blunt end treatment. Other plasmids with the same functions in the host cell can be used for this invention.

The plasmid to be used in this invention should preferably contain a selectable marker depending on which one can recognize whether a given cell contains the plasmid or not.

The plasmid of this invention signifies a genetic factor which can replicate autonomously extrachromosomally in the host cell.

(Recombinant DNA)

The recombinant DNA constructed through homologous recombination should be autonomously replicated and maintained stably in extrachromosomally in the host cells. Consequently, when the recombinant DNAs are constructed in yeast cells through homologous recombination, the recombinant DNA should act as a yeast artificial chromosome which is autonomously replicated and maintained stably in yeast cells.

The recombinant DNAs contain preferably a selectable marker that allows transformed cells to be selected under a specific condition.

(DNA sequence for recombination)

One DNA sequence to be introduced into host cells should be partially homologous to one of the other DNA sequences to be introduced. Homologous recombination takes place between the two sequences.

DNA sequence for recombination can be linear and / or circular. In each form, DNA sequences can be recombined homologously.

Each DNA sequence for recombination is designed such that the recombinant DNA formed by homologous recombination in cells are replicated autonomously in the host cells and are maintained stably extrachromaomally. In the case of that cells are yeast, the recombinant DNA should have a telomere sequences, an autonomously replicating sequence(ARS) and a centromere(CEN). Thus, the DNA sequence to produce the recombination DNA should contain these segments, so that after the homologous recombination, the recombinant DNA is produced to function as a yeast artificial chromosome.

For example, in order to produce yeast artificial choromosome from two DNA sequence, yeast artificial chromosome is used as one DNA sequence and the other is produced having telomere sequences at 3'-terminal or 5'-terminal and homologous sequences to the yeast artificial choromosome. Then, 3'-terminal or 5'-terminal of the yeast artificial chromosome replaced with the other DNA sequence.

Further, the 3'-terminal and the 5'-terminal of a yeast artificial chromosome may be replaced with a DNA sequence having telomere sequences at 3'-terminal and a DNA sequence having telomere sequences at 5'-terminal, respectively.

The DNA sequence for recombination has a DNA sequence of a gene of interest. After the homologous recombination, the recombinant DNA comes

to contain the DNA sequence of a gene of interest.

Particularly, when the DNA sequence for recombination has a large-sized DNA sequence of a gene of interest, this homologous recombination would be beneficial. Because while with the conventional method it is very difficult to construct a recombinant DNA with a large-sized DNA sequence in vitro using restriction enzymes, this intracellular homologous recombination can readily produce a recombinant DNA with a large-sized DNA, and allows the recombinant DNA to be stably maintained in host cells. For example, the large-sized DNA sequences include a mammalian genome DNA sequence containing structural gene and its regulatory gene, and a repetitive DNA sequence.

More preferably, the DNA sequence for recombination has a repetitive DNA sequence of a gene of interest. When the DNA sequence contains such a repetitive sequence, the repetitive sequence is readily modified in the host cell which express functional DNA recombination enzyme. However, this intracellular homologous recombination makes it possible for the DNA sequence with such repetitive sequence to be recombined appropriately. The resulting recombinant DNA can be maintained stably in the yeast cell without recombination enzyme. Such a repetitive DNA sequence is located on mammalian chromosomes.

Further, by this homologous recombination method, resulting recombinant DNA with a large-sized DNA sequence or a repetitive DNA sequence can be modified appropriately and maintained stably in the host cell.

The DNA sequence should preferably contain a selectable marker by which cells with the recombinant DNA is selectively recovered.

(Yeast artificial chromosome)

Yeast artificial chromosome (YAC) used as a DNA sequence for recombination and YAC constructed through homologous recombination in this invention signify DNA fragment which can satisfy at least following requirements; to replicate autonomously in yeast cells and be maintained in their extra chromosomal space. Accordingly, YAC contains telomeres, an autonomously replicating sequence, a centromere, and a sequence necessary for initiating replication, and has these elements arranged in an effective way. Besides, various other YACs well known among those skilled in the art can be utilized with profits.

(YAC for DNA sequence for recombination)

YAC is a useful tool for DNA recombination. YAC containing a DNA sequence of a gene of interest is maintained stably in a yeast cell. Preferably, YAC is used as a DNA sequence for recombination with a large-sized DNA or a repetitive DNA sequence. YAC should be preferably furnished with a selectable marker which allows selective collection of the transformed yeast cells containing the YAC undergoing homologous recombination.

The YAC vector plasmid particularly useful in this invention is pYAC 55pkc or the derivative of pYAC 55 (provided by Dr. Olson, University of Washington). pYAC 55pkc can be prepared as shown in Fig. 6. pYAC 55 is cleaved at Cla I with Cla I and is allowed to have blunt ends. Then, it is allowed to undergo self- ligation in the presence of T4 DNA polymerase. A DNA sequence having Not I sticky ends, and Pst 1(Bst XI), Kpn I, and Cla I positions is inserted into above plasmid at its Not I positions, to produce pYAC 55pkc. This plasmid has cloning sites at Not I, Pst I(Bst XI), Kpn I and Cla I positions. This plasmid, after being completely cut with Bam HI, produces the YAC that is used in this invention. Or, this

plasmid, after being completely cut with Bam HI and CIa I, provides the left and right arms of YAC. These left and right arms are utilized for preparation of YAC to be used for cloning a DNA segment with a gene of interest.

(Homologous recombination in cells)

The DNA recombination deficient host cell which has, extrachromosomaly, the plasmid carrying the gene for DNA recombination and not evenly distributed to daughter cells after cell division should be preferably prepared by introducing a plasmid into the host cell. Using YAC as the DNA sequence for the recombination , the host cell for homologous recombination should be prepared preferably as follows: the yeast host cells are transformed by introduction of YAC , and then the plasmid is introduced into the transformed cell.

Particularly, when the plasmid is introduced, a DNA sequence for recombination should preferably be introduced concurrently. Such concurrent introduction of the two DNA sequences dispenses with the need for the procedure so that host cells having DNA recombination enzymes expressed are selectively collected, thus making homologous recombination more efficient.

Introduction of the plasmid, or the plasmid and the DNA sequence into yeast cells can take place by various known methods including the usage of $LiCl_2$ or $Li(CH_3COO)_2$.

As described above , homologous recombination takes place between the DNA sequences in cells containing the plasmid to produce the recombinant DNA.

(Selection of cells capable of stably maintaining the recombinant DNA)

For the recombinant DNA to be maintained stably in cells, it is

necessary to select the cells carrying the recombinant DNA without the plasmid. This selection takes place by appropriately combining multiple selection markers: the markers contained in the recombinant DNA, the markers removed by the removal of the plasmid and the markers removed from the DNA sequence by the homologous recombination. In the cells selected through above processes, the recombinant DNA can be maintained stably.

(Modifications added to the DNA construct)

Various modifications can be added to the DNA construct having a minimum necessary length of DNA segments, and the resulting construct can be used as a vector to be applied for mammalian cells and other cells.

For example, such DNA construct is made to contain a certain DNA sequence encoding a marker which allows transformed cells to be selected under a specific condition. The selectable marker includes DNA sequences relating to a certain drug resistance or certain nutritive requirements. When the DNA construct is made to contain a DNA segment with such selectable marker, the transformed cells containing the DNA construct can be selectively collected under conditions that a selectively acting chemical or chemicals are present or absent. Such selectable markers are well known among those skilled in the art, and they can choose appropriate markers according to the DNA constructs to be used, based on knowledge commonly shared.

It should be noted here that because the DNA construct of this invention is grown in mammalian cells, the marker must function in mammalian cells.

Further, the DNA construct can contain one or more DNA sequences which allow the DNA construct to replicate autonomously in cells other

than mammalian cells. Such DNA sequences allow the DNA construct to act as a chromosome in the cell. Accordingly, the DNA construct with such DNA sequence can act as a shuttle vector for mammalian cells and other than mammalian cells including yeast cell and bacterial cell. The DNA sequence that is necessary for a chromosome to be autonomously replicated in yeast cells or bacterial cells is well known among those skilled in the art.

When cells which are not mammalian cells and which have been transformed with the DNA construct are selectively collected, the selectable marker must function in those cells which are other than mammalian cells. Such markers are well known among those skilled in the art, and they can be used appropriately by such those skilled in the art.

Further, the DNA construct can contain DNA sequences which are necessary for expression of foreign genes inserted. Such DNA sequences allow the artificial chromosome to act as a vector that is necessary for expression of the gene of a interest in mammalian cells.

The DNA sequences which are necessary for expression of the gene of interest in various types of cells are well known among those skilled in the art, but they should preferably include at least a promoter sequence and a poly-A sequence provided that the gene of interest should be expressed in mammalian cells.

Further, the DNA construct can contain a genome DNA sequence comprising structural region and its regulatory region. In this case, a product derived from the structural region is expressed appropriately in transformed cell according to its regulatory region.

(Preparation of a DNA construct with the centromere)

Particularly, to produce the DNA construct comprising a mammalian

telomere and a centromere having a DNA sequence containing some copies of CENP-B box sequences through homologous recombination, a YAC having the DNA sequence with CENP-B box sequences is used as a DNA sequence for recombination. This invention comprises two steps of recombination process.

Another DNA sequence (the first arm) necessary for homologous recombination is allowed to contain at least a DNA sequence which is homologous to a DNA sequence located on one end of above YAC, a mammalian telomere, and one or more DNA sequences that are necessary for proper functioning of YAC on one end of the YAC.

The mammalian telomere here has the same meaning with that defined for a newly invented DNA construct.

Further, the DNA sequences necessary for the proper functioning of YAC on one end of the YAC should contain at least a telomere function in yeast cells. If One or more of a centromere, ARS and ORI, exist on one arm of the YAC which is to be replaced with the first DNA arm, the sequences should be provided in addition to a telomere .

The first arm should be preferably furnished with a selectable marker by which the yeast cells having the first recombinant YAC with this DNA arm inserted can be selectively recovered. Further, the first arm should be preferably furnished with a selectable marker by which the mammalian cell having the recombinant YAC construct with this arm inserted can be selectively recovered.

When such first arm is allowed to undergo homologous recombination with the yeast cell with the YAC, one arm (one end) of YAC is replaced with this arm, to produce a first recombinant YAC comprising the centromere containing CENP-B box sequences, and a mammalian telomere

on one end.

(Replacement of the first recombinant YAC with a second arm)

In this process , homologous recombination takes place between the first recombinant YAC and a second DNA sequence(a second arm).

When the yeast cell with the first recombinant YAC divides itself, the plasmid replicates once at each cell cycle, but it is not distributed evenly to daughter cells. Therefore, appropriate combination use of markers on the YAC and the first arm, and a marker on the plasmid allows selective recovery of yeast cells which contain the first recombinant YAC but not the plasmid.

According to the procedure, the resulting yeast cell without genes for DNA recombination can maintain extrachromosomally the first recombinant YAC stably. Next, the plasmid is introduced into the YAC thus collected.

A second arm to be used for the second recombination is to replace the other end of the YAC that remains untouched during the first recombination. The second arm, similarly to the first DNA fsequence, should be allowed to contain at least a DNA sequence homologous to a DNA sequence located on the other end of the YAC, a mammalian telomere, and DNA sequences that are necessary for proper functioning of the YAC on the other end of the YAC.

The second arm should be preferably furnished with a selectable marker by which the yeast cells having the second recombinant YAC with this arm inserted can be selectively recovered. Further, the second arm should be preferably furnished with another selectable marker by which the mammalian cells having the second recombinant YAC with this arm inserted can be selectively recovered.

When homologous recombination is allowed to take place in the yeast cells thus prepared, a second recombinant YAC is obtained wherein the other end of the first recombinant YAC is replaced with the second arm, a DNA sequence with CENP-B box sequences is present, and mammalian telomeres are present at both ends.

The second recombinant YAC produced as above through homologous recombination is furnished also with DNA sequences necessary for it to function in yeast cells. However, if it is inserted into mammalian cells to be maintained in them, those DNA sequences are not always necessary, and can be eliminated.

Furthermore, the recombinant YACs and recombinant DNA construct thus prepared through homologous recombination can be submitted to the same recombination process repeatedly to produce further altered recombinant YACs and DNA constructs.

(The recombinant DNA construct transfection into mammalian cells)

Purified YAC or DNA may be introduced into mammalian cells by the following several methods known in the art. For example, DNA transfections into mammalian cell using lepofectamine(Gibco. BRL) were carried out basically according to the manufactures instruction. Then, MAC transfection was carried out by microinjection. Other methodologies are also useful in the present invention.

YAC or DNA constructs introduced into the cell are maintained stably without integrating into host chromosomes, autonomously replicated, and transmitted to their progeny cells.

In other words, a centromere with some copies of CENP-B box sequence which is located on the DNA construct of the invention is a functional centromere apparently. As a result, it is possible to construct

vectors for fragmentation of choromosome. The one of the vectors is constructed to combine the centromere of the invention, a homologous sequence to the chromosome for recombination, and a mammalian telomere on one end. The other is constructed to combine a homologous sequence to the chromosome, and a telomere on one end. The vectors can be designed to be able to cut the chromosome at a desired position and obtain a fragment of the chromosome which has a desired size of length. If such designed vectors are used for homologous recombination with the host chromosome in the host cell, a recombinant vector comes to be a minichromosome which has a desired fragment of host chromosome, maintained stably in the host cell. Further, this technique of fragmentation of chromosome and YAC technique make possible to clone a YAC which comprise such a vector for fragmentation with a fragment of mammalian chromosome (minichromosome) into a yeast cell. Additionally, the minichromosome can be introduced into cells or individuals. These technique can develop chromosome engineering.

[Industrial applicability]

The mammalian artificial chromosome of this invention, because of being replicated autonomously in mammalian cells and maintained there, can act as a vector when a foreign gene is to be introduced in the mammalian cells, and a vector when a foreign gene is to be expressed in the mammalian cells.

This artificial chromosome makes it unnecessary for a gene of interest to be introduced into a native chromosome, that is, mere introduction of the chromosome into the cell, the cell leads to transformation. When this artificial chromosome is used as a vector, the gene of interest can be cloned stably; it does not undergo digestion within the transformed cells, and

transformation proceeds without the position effect by the gene integration into the host chromosomes. Particularly, this would be beneficial when used as a vector in gene therapy in which it is necessary to carry normal genes to affected cells, or genes which control the expression of abnormal genes in such affected cells.

Best Mode for Carrying Out the Invention

Having now fully described the present invention, the same will be more clearly understood by reference to certain specific examples which are included herewith for purposes of illustration only, and not intended to be limiting of the invention, unless specified.

Mammalian Cell lines

WAV17, a mouse-human somatic hybrid cell containing the human chromosome 21 with two or three copies per cell as the only human component, was obtained from Dr.F.Ruddle (Yale University, USA) and HT1080, a human male fibroblast cell was obtained from Dr.D.Broccoli (The Rockefeller University). WAV17 and HT1080 were maintained in DME medium (Nissui,Japan) supplemented with 10% fetal calf serum (Bio Whittaker)at 37°C under 5% CO_2.

Antibodies

Anticentromere antibodies (ACA) -positive serum from a scleroderma CREST patient,K.G., which recognize three major centromere antigens, CENP-A, B and C by immunoblotting analysis with Hela nuclear extract, was obtained from Dr.Y.Muro (Nagoya University,Japan). The polyclonal antibody against the NH_2-terminal region of human CENP-B (BN1) was described previously (Kitagawa et al Mol. Cell. Biol. 15,1602-1612, 1995). Two polyclonal antibodies CGp2 and CRa2 against the COOH-terminal region of human CENP-C were raised by immunizing a guinea pig

and a rabbit, respectively, with polypeptides containing the residues 630-943 of CENP-C . A plasmid expressing the COOH- terminal region of CENP-C, pETCC630-C, was constructed by cloning a Xbal-BamHl fragment of CENP-C cDNA derived from pCNPCC1, a clone screened from Okayama Berg library (Okayama et al., Mol. Cell. Biol. 3; 280-289, 1983), to Ndel-BamHl site of pET3c using a Ndel-Xbal linkers (5'-TATGAATCTTGATTGTT - 3'). The polypeptides were expressed in E.coli cells using pETCC630-C by the T7 expression system (Studier et al., Methods Enz. 185; 60-89, 1990) and purified by the same procedure described in Kitagawa et al 1995.

Yeast strains and media

The Saccharomyces cerevisiae strain EPY305-5b (MATα, rad52-Δ2000, leu2- Δ1, lys2, ade2-101, his3-Δ200, trp1:: HisG,ura3-52) was obtained from Dr.M.Resnick (NIEHS, USA) and grown in a rich medium YPD or in a synthetic minimal SD medium. Media and solutions used in examples are described as follows:

YPD: 1% yeast extract, 2% polypeptone,2% glucose

SCE (for transformation): 1M sorbitol, 0.1M sodium citrate pH5.8, 10mM EDTA pH8.0

SCE (for agarose plug): 1M sorbitol, 0.1M sodium citrate pH7.0, 50mM EDTA pH8.0

STC: 1M sorbitol, 10mM Tris-HCl pH7.4, 10mM $CaCl_2$

PEG: 20% PEG 8000, 10mM Tris-HCl pH 7.4, 10mM $CaCl_2$

SOS (10ml): 5ml of 2M sorbitol, 2.5ml of YPD, 2.4ml of DW,
70μl of 1M $CaCl_2$, 100μl of 100xUracil

SORB plate: 1M Sorbitol, 0.67% yeast nitrogen base without amino acids,
2% glucose, 1.7% bacto agar, 1x amino acids

Top agar: 1M sorbitol, 0.67% yeast nitrogen base without amino acids, 2% glucose, 17% bacto agar, 1x amino acids

SD: 0.67% yeast nitrogen base without amino acids, 2% glucose, 1x amino acids

10x amino acids: 400mg/l arginine-HCl, 200mg/l histidine-HCl, 600mg/l isoleucine, 600mg/l leucine, 500mg/l lysine-HCl, 200mg/l methionine, 500mg/ l phenylalanine, 500mg/ l tyrosine, 100mg/ l adenine sulfate, 2000mg/ l threonone, 400mg/ l tryptophan, 200mg/ l uracil

100x tryptophan: 4.0g/l tryptophan

100x uracil: 2.0g/l uracil

100x lysine: 5.0g/l lysine

Solution I :SCE (for agarose plug) supplemented with 2mg/ml zymolyase 100T (Seikagaku Corporation) and 33mM DTT

Solution II :0.45M EDTA pH9.0, 10mM Tris-HCl pH8.0, 50mM DTT

Solution III :0.45M EDTA pH9.0, 10mM Tris-HCl pH8.0, 1% rauroyl-sarcosine, 1mg/ml proteinase K

YAC vectors and plasmids

Construction of YAC cloning vectors

YAC vector and pYAC55 pkc used for Alphoid DNA cloning are derivatives of pYAC55 (gifted by Dr.M.V. Olson, U. Washington). A Cla I site of pYAC55 was disrupted by digestion with Cla I and self-ligation after the blunt ends creation with T4 DNA polymerase. Then, the derivative plasmid was digested with Not I and oligonucleotides containing Pst I(Bst XI), Kpn I, Cla I sites flanked by Not I sites (YL1 and Y L2) were inserted into the Not I site of the derivative plasmid, resulting in the construction of pYAC55 pkc creating Not I, Pst I (Bst XI), Kpn I, Cla I cloning sites (Fig.2).

Rad52 expressing plasmids

Rad 52 expressing ARS plasmid YpSL1(Adzuma et al., Mol. Cell. Biol. 4, 2735- 2744, 1984) was a gift from Dr.T.Ogawa (National Institute of Genetics, Japan). YpSL 1 was digested with EcoRV, and a 0.57kb EcoRV fragment containing a part of TRP 1 gene was replaced with a 2.6 kb Sal I-Xho I fragment containing URA3 gene derived from pYAC 55 after the blunt ends creation with T4 DNA polymerase. The resulting plasmid YpSL1-Ura contained URA3 gene instead of TRP1 gene. Oligonucleotides used in this procedure were described as follows:

YL1 5'GGCCGCCCAATGCATTGGTACCATCGATGC 3'

YL2 5'GGCCGCATCGATGGTACCAATGCATTGGGC 3'

Preparation of agarose plug

WAV17 cells at a concentration of $8X10^7$cells/ml in PBS were mixed with an equal volume of 1% solution of Low Melting Point (LMP) agarose (Seaplaque GTG,FMC) in PBS and then distributed into 100µl plug former (molds). Solidified agarose plugs were treated with 0.5 M EDTA, 1% lauroyl sarcosine and 10 mg of proteinase K per ml for 24 hrs at 50°C. Just before restriction endonuclease digestion, agarose plugs were dialyzed against TE (10 mM Tris-HCl pH 7.4 and 0.1 mM EDTA) containing 1 mM PMSF and then against TE. For Southernhybridization sliced agarose plugs about 8µl in volumes were digested with 20-50 units of restriction endonuclease in the 50µl mixture (Takara,Japan or NEB) as specified in each experiment.

An yeast strain containing a YAC was inoculated into 50ml of SD medium (-Ura ,-Trp or-Lys,-Trp) and was grown in a flask to late log phase (4 days, at 30°C). The cells were transferred into a 50ml Falcon tube and collected by centrifugation at 2000g for 5min, and then

resuspended and washed with 50ml of 50mM EDTA. After the centrifugation and removal of all the supernatant, the cell pellet (about 330µl) was resuspended with the same volume of Solution I supplemented with 33mM DTT and 4mg/ml zymolyase 100T (Seikagaku Corporation) and incubated at 37°C for 15 min. Then, the cell suspension was mixed thoroughly with the same volume (about 330µl) of 2% LMP agarose (Seaplaque GTG.) in 0.15M EDTA by pipetting with cut off tip. Using a cut off yellow tip 100µl aliquots of the mixture was poured into plug formers kept on ice. The agarose plugs were transferred into Solution II and incubated at 37°C overnight. Then, the buffer was replaced with Solution III and the plugs were incubated at 50° C overnight. The plugs were stored at 4 °C until use.

Pulse-field gel electrophoresis (PFGE)

PFGE was performed using a pulsaphor electrophoresis unit (Pharmacia) in 1%LE agarose (FMC) and 0.5xTBE (50 mM Tris-HCl, 50 mM Boric Acid and 1 mM EDTA) at 10° C . After ethidium bromide staining and photography, gels were treated with 0.25M HCl for 20min and transferred to nylon membranes (Hybond N , Amersham) in 0.4 M NaOH overnight.

Southern hybridization

Hybridization probes were prepared by labeling gel-purified insert fragments of plasmid DNA or PCR ^{32}P-dCTP using the random primer method. Nylon membranes to which DNA was transferred from the gel of PFGE, were prehybridized and hybridized under conditions of 50% formamide, 4xSSPE, 1% SDS, 0.5% skim milk and 500 µg/ml sonicated salmon sperm DNA and supplemented with or without probe DNA at 42°C. Final washing was performed in 0.1 x SSC and 0.1% SDS at 68° C.

Immunoprecipitation-competition assay

Complementary oligonucleotides containing the CENP-B box sequence were synthesized chemically.

CB29a : 5'TCAGAGGCCTTCGTTGGAAACGGGATTTC 3'

CB29b : 3'CTCCGGAAGCAACCTTTGCCCTAAAGAGT 5'

End-labeled probe DNA was prepared from annealed CB29a and CB29b DNA by repairing the single-stranded ends with Klenow Fragments. End-labeled oligonucleotides (0.5 ng) were mixed with varying amounts of unlabeled DNA and incubated with HeLa 0.5 M NaCl extract (1.5 $x10^5$ nuclei) in 100 μl of binding buffer (10 mM Tris-HCl pH 8.0, 10% glycerol, 1 mM EDTA, 2 mM DTT, 150 mM NaCl, 0.05% NP-40, 100 μg/ml poly(dI•dC),poly(dI•dC)) for 1 h at RT. Then, 1 μl of anticentromere serum (K.G.) diluted (1:5) with binding buffer was added to the mixture and incubated for 30 min on ice, After 25 μl of protein A-Sepharose (Pharmacia) was added, the mixture was incubated for 30 min on ice and washed three times with 0.5 ml of binding buffer containing 0.5% NP-40. Finally, radioactivity of the pellet was determined using a liquid scintillation counter (Beckman).

Preparation of 0.5 M NaCl extracts from interphase nuclei was performed as described by Masumoto et al. (1989, J. Cell Biol. 109, 1963-1973).

PCR

PCR was carried out to amplify various subfamilies of alphoid DNA specific for human α21-I or α21-II using complementary strand primers which were chosen from consensus sequence in alphoid monomers. In case of tandemly repeated sequences, when complementary strand primers of a conserved motif were used for PCR, various sequences

between the motives in the repeats were amplified. α(1)a/b primers were used to amplify alphoid sequence cloned in α7C5 YAC, and α(Y)a/b primers were used to amplify alphoid sequence cloned in αB13 YAC.
primer sequences:
α(1)a 5'ACAGAAGCATTCTCAGAA 3'
α(1)b 5'TTCTGAGAATGCTTCTGT 3'
α(Y)a 5'-AGAAACTTCTTTGTGATG-3'
α(Y)b 5'-CATCACAAAGAAGTTTCT-3'

All PCR reactions were carried out in a 50 ml reaction mixture containing 10 mM Tris-HCL pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM dNTPs and 1 unit of Taq polymerase (Perkin-Elmer) using 1 mM of primers and 1 ng of WAV17 genomic DNA or yeast genomic DNA containing a YAC. 30 reaction cycles were performed, consisting of 30 sec denaturation at 94° C, 90 sec annealing at 55°C or 57°C and 60 sec extension at 73°C. PCR products were precipitated with ethanol to remove primers and used as probes.

Fluorescence in situ hybridization

Metaphase cells of transformants arrested by colcemid or TN16 (Wako Pure chemical) and interphase cells of HT1080 and derivatives were fixed in methanol/acetate (3 : 1) and spread on coverslips with conventional procedure. FISH was carried out according to the method described by Masumoto et al 1989, Exp. Cell Res., 181, 181-196. Biotin-labeled probe was detected with FITC conjugated avidin (1 :250 dilution with 4× SSC, 1% skim milk, Vector) and digoxigenin-labeled probe was detected with TRITC conjugated anti-digoxigenin (1 : 20 dilution with 4 × SSC, 1% skim milk, Boehringer Mannheim) by incubation at 37°C for 1 hr. Chromosomes and nuclei were counterstained

with DAPI.

Simultaneous detection of FISH and indirect immunofluorescence

The simultaneous detection method described by Masumoto et al 1989, Exp. Cell Res., 181, 181-196 was modified as follows. Swollen and PLP fixed cells of transformants were incubated with an anti-CENP-B (BN1) or anti-CENP-C (CGp2) antibody at 37°C for 1 hr and then washed and incubated with the 2nd antibody, FITC-conjugated anti-rabbit and anti-guinea pig IgG (Bethy Labolatry), respectively. The cells were washed and fixed again with PLP fixative on ice for 30 min and then with methanol/acetate (3 : 1). After dehydration and air drying, cells on coverslips were denatured with 70% formamide at 70°C for 30 min and hybridized under the same condition as described in the following section. Coverslips were washed twice with 50% formamide/2 × SSC at 37°C for 7 min, twice with 2× SSC for 7 min at 37°C, once with 1× SSC at room temperature for 10 min and blocked in 5% skim milk /4× SSC for 1hr at RT. Signals from YAC arms were simultaneously detected with the signals from CENP-B or CENP-C and TRITC-conjugated anti-digoxigenin, respectively.

Plating efficiency

The passage of the transformed cells in the condition without selection was analyzed by plating efficiency up to 60 days (about 60 generations). Every 4 days, the cells were plated with new medium without BS at 1 : 16 dilution. After 20, 40 and 60 days from the start, 1-2 × 10^3 cells were duplicated and plated into medium with and without BS. Colonies were counted after 7-8 days. Non resistance cells died within 3-5 days.

DNA Probes for FISH

Probes were prepared by the random primer method or nick translation using labeled dUTP as a substrate. Gel purified 11 mer body described as sequence NO.2 (11-4 alphoid DNA) (for detection of the α21-I locus and clones,),α(Y)a/b primed PCR products (for detection of α21- II locus and clones), YAC vectors (telomere sequences were removed by Cla I•Kpn I digestion or Cla I - Xho I digestion from the vectors) and a telomere sequence (TTAGGG)n were labeled with biotin-11-dUTP (Enzo Biochem) or digoxigenin-11-dUTP(Boehringer Mannheim). After removal of free nucleotides in the reaction mixture by a Sephadex G-50 spun column, probes were precipitated with ethanol and dissolved in formamide.

EXAMPLE 1

Cloning of alphoid DNA Arrays in the Human Chromosome 21 into YAC

We tried to clone the centromeric alphoid DNA regions into YAC using rad 52⁻ yeast host, EPY305-5b.

One agarose plug (100μl) containing WAV17 genomic DNA was divided into four pieces and digested completely with Bgl I (60U), Bgl II (60U) or Bam HI (72U) at 37°C overnight. The reaction was stopped by adding final 50m M EDTA and solution was replaced with NDS (0.45M EDTA, 1% rauroyl-sarcosine, 10mM Tris- HCl pH7.4) and stored at 4°C. The plugs were equilibrated with 1xTBE for 2hr and loaded on 1% pre-cooled LMT agarose gel. After removing short DNA fragments less than 50 kb by PFGE (90v,30 sec.pulse,for 70 to 90 min.), the agarose plugs were recovered from the separation gel. Through these steps, alphoid DNA from the human chromosome 21 was concentrated up to 8 to 10 folds as compared with the starting fraction of bulk genomic DNA. The

recovered plugs (total 100μl) were incubated with Msp I digestion buffer for 2hr to equilibrate the buffer and put in a new buffer. The DNA in plugs were digested partially with MspI (120U) at 37° C for 1hr and then the reaction was stopped by adding final 5mM EDTA. After changing the buffer in the gel with 1xTAE supplemented with 50mM NaCl for 2hr, all the remaining buffer was removed and the agarose of the plugs was melted at 68°C for 3 min, then cooled to 42°. 20U of agarase (Sigma) was added to the melted solution (100μl), and the solution was incubated at 42°C overnight. The DNA in the solution was then concentrated up to 20 folds in the colodion bag (pore size 8 nm, cut off MW 12000, sartorius) by vacuum dialysis against the buffer containing 10mM Tris-HCl pH7.4, 0.5mM EDTA pH8.0, 50mM NaCl, and the concentrated DNA solution was dialyzed again against the same buffer for 4hr. The concentrated genomic DNA (about 35μg from 17 initial agarose plugs) was ligated with the same amount of YAC55 pkc right and left arms (42 μl solution and 3360U ligase). The ligated DNA solution was mixed with gel loading buffer, loaded on pre-cooled 1.2% LMP agarose gel and size fractionated with PFGE at 170V, 8 sec. pulse, for 2hr and then at 120v, 8 sec. pulse for 18 hr. The agarose block containing the DNA molecules greater than 50kb in size, estimated by the staining of control marker lanes, were dissected, and the DNA in the gel was used for yeast transformation after the buffer exchange with 1xTAE supplemented with 50mM NaCl and agarase treatment.

Yeast Transformation with Spheroplast

Yeast colonies (EPY305-5b) on YPD plate were inoculated to 50ml YPD medium at OD660 was 0.02, and grown at 30°C for 14-20hr until OD660 was reached to 1.6-1.8. The cells were transferred to a 50ml tube,

pelleted at 1500rpm for 5min at room temperature, and washed once with 25ml of water and then washed with 25ml of 1M sorbitol, and spinned again. The cells were resuspended with 20ml of SCE and mixed with 40μl of β-mercaptoethanol. 100 μl of the samples was removed from the suspension and the starting OD800 was determined after diluting with 900 μl of water. The cell suspension was mixed with 100μl of 2mg/ml Zymolyase and incubated at 30°C. The incubation was stopped when the OD800 of the sample decreased to 75-80% of the starting value. Then the spheroplasted cells were pelleted at 950rpm for 5min, resuspended and washed gently with 15ml of STC twice. The cells were pelleted again and resuspended in 1ml of STC. 100μl each of the spheroplasted cells were mixed gently with 10μl of the transforming YAC DNA prepared in Example 1, incubated at room temperature for 10min, then mixed with PEG solution and incubated at room temperature for 10min. The spheroplasts were pelleted at 950rpm for 5min, and the supernatant was removed as much as possible, then resuspended with 200μl of SOS and incubated at 30°C for 30min. The cells were pelleted and resuspended with SD(-ura -trp). The suspension was mixed with 7ml of melted at 50°C TOP agar and poured onto five prewarmed at 42°C SORB plates (-ura -trp). The plates were incubated at 25°C for 5-7 days.

YAC libraries were constructed with genomic DNA of human and mouse somatic cell hybrid (WAV17) containing chromosome 21 as an only human component and about 10000 colonies of the resulting YAC libraries were screened with alphoid DNA probes (an alphoid 11 monomer higher order repeating unit of α21-I and the PCR degenerated α21-II (Ikeno et al Hum. Mol. Genet. 3, 1245-1257, 1994). We obtained 4 and 7 stable alphoid YAC clones containing α21-I and α21-II arrays, respectively.

Fig.3 shows that YAC clones, α7C5 and αB13 from α21-I and α21-II array respectively (Fig.2), containing the longest insert sizes estimated from PFGE analyses though the sizes of insert were about 100 - 110 kb (Fig.4). These two clones were analyzed by dot hybridization using repetitive DNA probes, restriction digestion and Southern hybridization, PCR analysis of cloned ends and by DNA immunoprecipitation-competition assay with CENP-B proteins (Figs.4, 5and 6). The results indicated that α7C5 was composed of rigid tandem repeats of the 11 mer repeating unit and contained CENP-B boxes at very high frequency with every other alphoid monomer units (Fig.1). In contrast to this clone, αB13 was mostly composed of diverged alphoid DNA and contained about 2kb fragment of satellite III sequence on its one of cloning ends, but contained no CENP-B box. αB13 also may contain a small copy of Alu sequence. Thus, these two YAC clones from α21-I and α21-II regions represent characteristic alphoid arrays in the chromosome 21 centromere, and they will provide good controls for each other to assay whether these two alphoid sequences have a centromere function or not in human cells.

EXAMPLE 2

Construction of replacement vectors

To construct replacement YAC arm vectors, a left arm and a right arm YAC vectors were modified with human telomere sequences. Also, yeast and mammalian selection maker genes (Blastisidine S resistant gene: Bsr, and / or Neomycin resistant gene: Neo) were cloned into the vectors.

A Not I - Cla I fragment containing 600 bp of mammalian TTAGGG telomere repeats (0.6 kb element) from the vector pEND2R (a derivative plasmid of pHUTEL2 [Thesis, Edinburgh University, U.K.]) was subcloned in a head to tail arrangement with the 0.8kb element of pgb4g7

(Thesis, Edinburgh University, U.K.), which contains 0.5 kb of mammalian telomere repeats flanked by 0.3 kb of yeast TG1-3 telomere repeats. An oligonucleotide containing the 18 bp Isce I recognition site (Boehringer) flanked by Bam Hl compatible ends was inserted into a Bam Hl site located at the junction of the 0.6 kb and 0.8 kb elements. Only one Bam Hl site was retained, immediately downstream of the Isce I site. The 0.6 kb element followed by an Isce I site then the 0.8 kb element constitutes the omega cassette.

The left arm replacement vectors are modified versions of pCGS990 described previously (Smith et al 1993; Mammalian Genome, 4, 141-147). pCG990 was a gift from D. T. Moir, Collaborative Research, Inc. A 2.6 kb Sal I-Cla I fragment which contains a neomycin resistance gene derived from pMC1polA (Stratagene) and a copy of the omega cassette was cloned into Sal I-Cla I digested pCGS990, resulting in the construction of pMeganeo (Fig.7). Tetra Hymena telomere sequence and the neomycin gene in pMeganeo were removed by Not I complete digestion and Xhol partial digestion and the plasmid was circularized with ligation after the creation blunt ends with T4 DNA polymerase treatment. Then, oligonucleotides containing Sal I, Cla I and Not I sites flanked by Cla I compatible sites(YL3 and YL4) were inserted into the Cla I site of the derivative plasmid, resulting in the construction of pMegaΔ retaining only one Sal I, Cla I and Not I site (Fig. 8). A 3.6 kb ApaLl - EcoRI fragment containing a neomycin resistance gene derived from pSV2-neo was treated with T4 DNA polymerase and cloned into the partial digested EcoRI site between Lys2 and TK genes of pMega Δ, resulting in the construction of pMegaSV-Neo (Fig. 8). pMega Δ or pMegaSV-Neo were used for the replacement of the alphoid YAC left arms by homologous recombination

after the linearization with Sal I and Not I digestion.

The right arm replacement vectors are derivatives of pJS89 described previously(Shero et al 1991, Genomics, 10, 505-508). pJS89 was digested with Bam HI and Cla I to delete the Y'a element. Insertion of the omega cassette into Bam HI-Cla I site resulted in the construction of pJS89mega (Fig. 9). A 1.1 kb Sal I - Eco RV fragment (1/2 ura element) from pYAC 4 containing a truncated (0.42 kb), non functional copy of URA3 gene from the Eco RV site flanked by 0.7 kb of YAC4 sequences up to the Sal I site (Burke et al 1987, Science, 236, 806-812), was subcloned into pBluescript to create pblue 1/2 ura. The 1/2 ura element was then subcloned as a 1.1 kb Sal I-Bam HI fragment into Sal I-Bgl II digested pJS89mega resulting in pMega Conversion Ura (pMCU) (Fig.9). The Not I and Sal I sites of pMCU were disrupted by Not I or Sal I digestion and self ligation after the creation of blunt ends with T4 DNA polymerase treatment. Then, the plasmid was digested with EcoRV partially and a 2.6 kb Pvu II-EcoRI fragment containing brasticidine S resistance gene derived from pSV2-Bsr (Kaken seiyaku) was cloned into the one of EcoRV sites between 1/2 URA and ARS H4 genes of pMCU derivative. Finally, oligonucleotides containing Sal I, Cla I and Not I sites flanked by Cla I compatible sites (YL3 AND YL4) were inserted into the Cla I site of the derivative plasmid, resulting in the construction of pMCU-Bsr retaining only one Sal I, Cla I and Not I site (Fig.10). pMCU-Bsr was used for the replacement of the alphoid YAC right arms by homologous recombination after the with Sal I and Not I digestion.

Oligonucleotides used in this example were described as follows:

YL3 5'CGTCGACCATCGATACCAATGCATTGGCGGCCGC 3'

YL4 5'CGGCGGCCGCCAATGCATTGGTATCGATGGTCGA 3'

EXAMPLE 3

RAD52 plasmid mediated transient homologous recombination in rad52⁻ host

YAC strains and homologous recombination procedure

For the centromere functional assay in vivo, the terminals of the linear DNA fragment should be stabilized to avoid integration, degradation from the ends and end replication problems. Therefore we replaced the left and right arms of these two YAC clones into human telomere sequences. YAC can be modified very easily using yeast homologous recombination systems (Pachnis et al 1990, Proc. Natl. Acad. Sci., 87, 5109-5113, Pavan, 1990, Proc. Natl. Acad. Sci., 87, 1300-1304), but in this case, a dilemma is that we used rad52⁻ host to stabilize the repetitive DNA in the YAC . To overcome this problem, we developed a retrofitting method transiently inducible in rad52- host.

The alphoid YAC arms were replaced with the modified YAC vectors by homologous recombination mediated by transient expression of RAD52 gene in rad 52⁻ host. In the first step, α7C5 or αB13 yeast cells were co-transfected with the linealized left arm replacement vector (MegaΔ or MegaSV-Neo respectively) and a RAD52 expressing plasmid containing TRP1 gene (YpSL1) (Fig.11 and 12). Yeast colony showing +LYS, +URA and -trp phenotype indicates that the left arm replacement was successfully carried out (Fig.13). The RAD52 expressing plasmid, YpSL1, contains yeast ARS fragment but no yeast CEN sequence. Therefore, the plasmid rapidly disappeared from the transfected cells in the absence of TRP selection. 14 to 30% of colonies showed -trp phenotype among the colonies showing +LYS, +URA phenotype. The insert size and the replaced YAC arms of the clones were certified by PFGE and Southern

analysis (Fig.17). The clones which showed intact insert size were used for the second retrofitting with the linealized right arm replacement YAC vector (MCU-Bsr) and a RAD52 expressing plasmid containing URA3 gene (YpSL1-URA)(Figs.11and 14). In the second retrofitting, 23 to 42% of the colonies showed -ura phenotype among the colonies showing +LYS, +TRP phenotype. Finally, we have obtained aimed YAC clones (MAC candidates, α7C5hTEL and αB13hTEL) whose arms were replaced with new left and right YAC arms containing the human telomeres and the selection maker without the rearrangement of the insert alphoid DNAs (Fig. 15, 16 and 17). These telomere modified YAC DNA were purified from the yeast cells using PFGE separation, agarase treatment and dialysis.

Detailed experimental procedure of this two steps homologous recombination (Figs. 12 and 14) is described bellow.

Two yeast rad52$^-$ strains containing YACs which have human chromosome 21 alphoid DNA insert called α7C5 (100kb) and αB13 (110kb) were used forRAD52 plasmid mediated transient homologous recombination. Each yeast strain was inoculated into 10ml liquid selection medium, SD (1st; -ura -trp, 2nd; -lys -ura) and grow overnight to 1-2x10^7cells/ml (OD600=0.5–1.0). The cell culture was diluted to 2X10^6 cell/ml with the fresh medium and regrowed to 1x10^7cell/ml. Then the cells were harvested and washed with sterile water, resuspended with 1.0ml water and transferred to 1.5ml microfuge tubes. The cells were washed with 1.0ml of LiAc/TE and resuspended at 2X10^8-2X10^9cells/ml with LiAc/TE (50-500μl).

The 50μl of the yeast suspension was mixed with 500ng linealized YAC arm replacement vector DNA (1st step: MegaΔ for α7C5 or MegaSV-Neo for αB13, 2nd step:MCU-Bsr), 500ng an ARS plasmid

containing RAD52 gene (1st:YpSL1 and 2nd:YpSL1-Ura) and 50μg of single stranded salmon sperm carrier DNA, and then mixed with 300 μl of 40% PEG 4000 solution. The yeast and DNA mixture was incubated at 30°C for 30min with agitation and heat shocked at 42°C for 15min. The yeast cells were collected by spinning down for 5 sec. at 7000rpm, resuspended with 1ml YPD and incubated at 30°C with agitation for 2hr. incubation. The yeast cells were collected and washed with the selection medium SD(1st: -lys -ura, 2nd: -trp -lys) and placed on the appropriate selection SORB plate. After 4-5 days incubation at 25°C, colonies were replicated on with or without Trp SORB plate (1st: +/-trp, 2nd: +/-ura) and incubated at 25°C. After 3-4 days incubation, colonies showing appropriate phenotype (1st: +LYS +URA -trp, 2nd: +TRP +LYS -ura) were picked up and were analyzed the insert size and the existence of human telomere by PFGE and Southern Hybridization. In the first homologous recombination step, YAC55 pkc left arm was replaced with linealized Mega Δ or MegaSV-Neo. The frequency of colonies with a -trp/ +LYS +URA phenotype for the 1st recombination were 14-30%. Yeast strains containing a correctly modified left arm YAC were used for the 2nd recombination of the right arm with linealized MCU-Bsr replacement vector. The frequency of colonies with a -ura/ +TRP +LYS phenotype for the 2nd recombination were 23-42%. Yeast strains correctly modified both arms were maintained on -lys -trp plates and used for the materials for YAC DNA purification and YAC DNA transfection into human cells.

The recombinant YAC containing α7C5insert was designated α7C5 h TEL.The recombinant YAC containing αB13 insert was designated αB13 h TEL. Saccharomyces cerevisiae containing α7C5 h TEL (Saccaromyces cerevisiae EPY305-5b α7C5 h TEL) was deposited to Agency of Industrial

Science and Technology, National Institute of Bioscience and Human technology (NIBH) on August 14, 1996, its deposit number is FERM BP-5625. LiAc/TE and 40% PEG solution used in this Example were described as follows:

LiA/TE ;

(0.1M Li-Acetate pH7.5/10mM Tris-HCL pH7.5/1mM EDTA) freshly made from 10xLi/Ac (1M LiAc pH7.5 adjusted with acetic acid), TE

40%PEG solution ;

40%PEG 4000/0.1Li-Acetate pH7.5/10mM Tris-HCI pH7.5/1mM EDTA freshly made from 50% PEG4000, 10xLiAc, TE

EXAMPLE 4

Purification of intact YAC DNA

Ten agarose plugs prepared from each yeast strain containing a retrofitted YAC (α7C5 h TEL, αB13 h TEL) were equilibrated with 1xTAE (40mM Tris / 40mM Acetic Acid / 1mMEDTA), loaded on 1.2%LMP agarose (Seaplaque, FMC) in 1xTAE and carried out PFGE at the condition at 200v 4°C with pulse time 15sec. for 18-24hr. A gel slice containing only YAC DNA from the preparative lanes was excised according to the size estimation of the EtBr staining of control lanes on either side. The gel slice containing YAC DNA was positioned vertically in the middle of gel tray and 3%LMP agarose were casted around it. About 0.1cm^3 of gel cube was removed from the bottom area in contact with the gel slice containing YAC DNA, and to trap the DNA, a small piece of dialysis membrane was inserted at the position which is 0.5cm apart from the gel slice and then gaps were filled with 1.5%LMP agarose. After the convention gel electrophoresis for 2 hr at 8V/cm, a small piece of 1.5% agarose area was recovered from just in front of the dialysis membrane in

which YAC DNA was concentrated, equilibrated with 1xTAE supplemented 100mM NaCl and all the fluid was removed. Agarose of the gel was then melted at 68°C for 10min. incubation and digested with 50U agarase (Sigma) per 1ml of gel slice at 42° for 4hr. The resulting YAC DNA solution was dialyzed (concentrate) using Ultra Free C3 (Millipore) or Microcon 100 (Amicon) against the buffer containing (100mM NaCl, 10mM Tris pH7.5, 1mM EDTA), and the concentration and the integrity of the DNA was checked by conventional gel and PFGE. Between 50 to 100ng purified DNA were obtained from this scale of experiments. Then the purified DNA was used as a material for YAC transfection into human culture cells by lipofection and microinjection.

EXAMPLE 5

YAC transfection into human cells by lipofection and by microinjection

Our modified YAC constructs have total 1.1 kb human telomere sequences flanked by 0.3 kb yeast telomere sequences on both chromosome ends. However, to minimize the possibility that introduced YAC DNA is integrated into host chromosome by low telomerase activity of the host cells, we chose human HT1080 cells reported as high Telomere Associated Chromosome Fragmentation (TACF) activity (Barnett et al ., 1993, Nucleic Acids Research, 21, 27-36) as a host. The purified YAC DNAs were introduced into human HT1080 cultured cells using two different methods, lipofection and microinjection into nuclei.

YAC transfection by lipofection

YAC transfections into HT1080 cells using lipofectamine (Gibco BRL) were carried out basically according to the manufacturer's instruction. Purified YAC DNA solution (30-100 ng DNA in 300μl

solution) was combined with a premixed lipofectamine (10 μl) and serum free medium (100 μl of Opti-MEM, Gibco BRL) at 30-40 min before in a polystyrene tube, mixed gently and stored for 30 min at RT to allow DNA-liposome complex to form. HT1080 cells (9×10^5 cells) seeded in a 25 cm^2 flask were incubated overnight until 70-80 % confluent and washed twice with 2ml of Opti-MEM, and then all the medium was completely removed. The DNA-liposome complex solution was mixed gently with 1.6 ml of Opti-MEM (2 ml of total vol) and overlaid onto the rinsed cells. After 16 hr incubation at 37°C in 5% CO_2, the DNA-liposome complex solution was replaced with DME supplemented with 10 % FCS, and the cells were allowed to incubate for 24 hr. Then, the cells were transferred into two 10 cm dishes, and selection with 4 mg/ml Blasticidine S(BS, Kaken Seiyaku) was started. Between 50 to 580 BS resistant colonies were obtained per μg of YAC DNA (α7C5 h TEL and αB13 h TEL, 110-120 Kb) transfections using lipofectamine and 410 to 470 BS resistant colonies were obtained per μg of linealized MCU-Bsr (11.2kb) right arm vector (11.2 Kb) transfections.

YAC transfection by microinjection

YAC microinjection was carried out using Zeiss Axiovert 135M equipped with Eppendorf 5242 microinjector, 5170 micromanipulator and a stage incubator and controller settled at 37°C (zeiss). HT1080 cells were grown on poly-D-lysine coated etched grid coverslip (Bellco biotechnology) settled in a 35 mm circular petri dish for 2 days. Immediately before injection, 3 ml of liquid paraffin oil (Boots company) was added over the medium. After centrifugation at 12000 rpm for 5 min, 1.5 μl supernatant of purified YAC DNA solution was inserted into a microinjection needle (Eppendorf Femtotip) using an Eppendorf microloader tip, and

microinjection was performed with a condition of P1; 5000 hPa, P2; 30-60 hPa, P2; 30-60 hPa, P3; 15-30 hPa. After the microinjection, the liquid paraffin oil and the mdeium were removed from the dish, and the fresh medium were added, and the cells were incubated at 37°C for 36 hr in 5% CO_2. Blasticidine S selection (4 mg/ml) was started at this point. In several experiments, the injected cells were continued to grow on the etched cover slips, and derived BS resistant colonies were counted per injected cells on the grid and picked up. In the rest experiments, the injected cells were transferred into a 10 cm dish and started the BS selection. A BS resistant colony was obtained per every 100 to 300 YAC DNA injected cells.

EXAMPLE 6

FATE of Alphoid YACs in HT1080 cells

If these MAC candidates retain all the cis elements required for a mammalian chromosome stability for example, telomeres, a functional centromere/kinetochore structure and a replication origin etc , YAC DNA introduced into the human cells may possibly be maintained without integrating into host chromosomes even after BS selections. To examine this possibility, we analyzed the distributions of the YAC DNA in 24, 20 and 5 cell lines of BS resistant colonies obtained from α7C5hTEL , αB13hTEL and the linealized MCU-Bsr vector introduced cells, respectively, by fluorescent in situ hybridization (FISH) (Fig. 18). Though the number of cells containing a minichromosome which is detectable as an extra chromosomal overlapping signals of α21-I probe and YAC vector probe varied from cell line to cell line, the cells containing such a minichromosome were observed in the most cell lines (11/13 cell lines or 10/11 cell lines) derived from α7C5hTEL YAC transfection

(7C5HT cell lines), regardless of the introducing methods, lipofection or microinjection, respectively. The sizes of the minichromosomes detected by FISH varied in each clones and seemed to gain the size to mega bp order from the initial YAC constructs (~110 kb) through the establishment as a BS resistant cell line. The total 30% of the analyzed metaphase cells from these cell lines have minichromosome signals(Fig. 19). Also, integration signals to the centromere and telomere regions of host chromosomes were observed at about 40% of cells. However, the presence of a minichromosome and an integration event in the same cell was very rare (only 2.3% cells of two clones in total 610 analyzed cells of 24 clones), and the integration of the YAC into the host chromosome arms was also very rare (only 1.8% cells).

In contrast to these results, we could not obtained any signal indicating a minichromosome from total 400 and 100 metaphase cells derived from αB13hTEL YAC and the linealized MCU-Bsr vector introduction (Fig.19). The results obtained from the cell lines derived from αB13hTEL YAC introduction (B13 HT cell lines) showed that the majority of the YAC integration occurred at the telomere region in 75% of cells (Fig.19), and the centromere integrations were 14%, and integration into the host chromosome arms was also very rare (only 1.3% cells). However, though the results of the cell lines derived from the linealized MCU-Bsr vector introduction (MCUHT clones) showed that the telomere integration of the vector were 56%, and no integration into the centromere region and 10 times higher score of the host arm integration (19%) were observed.

Interestingly, though the integration sites of the α7C5hTEL YAC into the centromere and telomere regions varied into several different

chromosomes, cell by cell , in the same cell line, the most of the integrations of αB13hTEL YAC or the linealized MCU-Bsr vector in the same cell line were derived from a single site. These results indicate that the integrations occurred as multiple events and independently through the establishment of 7C5HT cell lines. However, the integration occurred only once through the establishment of many B13HT or MCUHT cell lines.

All these results clearly indicate that α7C5hTEL YAC retains the elements required for de novo formation of a human minichromosome very efficiently, but, αB13hTEL does not.

EXAMPLE 7

Stability of Artificial Minichromosome without selection

Previous reports indicated that extrachromosomal elements without centromere function rapidly disappeared when the drug for the selection was excluded from the medium (Huxley). The stability of the minichromosome in three 7C5HT cell lines obtained by lipofection (7C5HT1, 2 and 3) and two 7C5HT cell lines obtained by microinjection (7C5HTm1 and 7C5HTm3) were analyzed both by FISH and colony formation efficiency on plate (plating efficiency: number of colonies on +BS selection against that on non-selective media) after 20, 40, 60 days of passage on non-selective media (Fig.20). Plating efficiency data from these 5 cell lines indicated that in all 5 cell lines more than 50% of population kept BS resistancy even after the 60 days of passage on non-selective media, and 97% of 7C5HT1 and 7C5HT3 cells kept BS resistancy after the off selection. Corresponding to these data, the data from FISH analysis also indicated that the minichromosomes in 7C5HT1 and 7C5HT3 cell lines were very stable even after the passage on non-selective media for 60 days, and the minichromosomes in 7C5HT2,

7C5HTm1 and 7C5HTm3 cell lines were gradually lost through the passage of non-selective media. Interestingly, the sizes of minichromosomes in 7C5HT1 and 7C5HT3 cell lines were bigger than those in 7C5HT2, 7C5HTm1 and 7C5HTm3 cell lines. All these cell lines containing minichromosomes also have some integration events when each cell line was established after about 30-40 days from the YAC DNA introduction. Therefore, the cells of 7C5HT1 were recloned (7C5HT1-1, 2, 4 to 9 and 19), and the stability of the minichromosomes in each cell lines were analyzed by FISH and the plating efficiency (Fig.21). Ten colonies were isolated from 7C5HT1 cells, but one of them was lost during the establishment. Seven out of 9 established cell lines showed only the minichromosome signals without any integration signal into the host chromosome, and the minichromosomes in 7C5HT1-1 cells were very stable (100%) with 1 or 2 copies through the passage on non-selective media from the both FISH and plating efficiency data. 7C5HT1-9 cells showed a integration signal at the centromere of a host chromosome and 7C5HT1-19 cells showed a telomere integration signal. BS resistancies and the integration sites of B13HT cell lines and MCUHT cell lines were very stable and not changed after the passage through the off selection for 60 days (data not shown).

These data from plating efficiency and FISH analyses of 7C5HT cell lines clearly indicate that the minichromosomes derived from α7C5hTEL YAC were segregated stably through the cell divisions even in the condition without BS selection.

EXAMPLE 8

Functional centromere/kinetochore structure on the minichromosome

We have already shown in this application that an alphoid YAC

containing the CENP-B boxes in high frequency (α7C5hTEL YAC) could form minichromosomes at high frequency, although an alphoid YAC containing no CENP-B box (αB13hTEL YAC) could not. This result indicates that CENP-B must be an essentially important factor for de novo formation of functional centromere structure. CENP-C has reported to be localized at the inner layer of kinetochore structure and also reported to be localized only at the functional side of the centromeres in dicentric chromosomes (Saitoh et al. 1992, Cell, 70, 115-125, Sullivan, 1995, Hum. Mol. Gent., 4, 2189-2197). In order to clarify the molecular bases required for the functional centromere/kinetochore structure of human chromosomes, we analyzed distributions of these two centromere proteins on more than 20 each of minichromosomes in 7C5HT1, 1-1, 1-2, and 7C5HT2 cell lines derived from α7C5hTEL YAC introduction (Figs.22, 23 and 24) using simultaneous staining with indirect immunofluorescence and FISH. Clear CENP-B and CENP-C double dot signals were observed on all the minichromosomes which were detected simultaneously by the YAC vector probe, indicating that the minichromosomes contained essentially important protein factors as centromere/kinetochore components. We could not obtain any positive signals of CENP-B staining on the integration sites of αB13hTEL YAC in B13HT1 cell line.

Multiple previous reports which introduced the human alphoid DNA into mammalian cultured cells showed that the anaphase lagging chromosomes were observed according to alphoid DNA integration to the host chromosomes and this phenomenon might be one of functions of alphoid DNA. Therefore, we analyzed more than 50 each of metaphase and anaphase cells each from 7C5HT1-1,1-2, 1-19 cell lines whether the minichromosome is properly aligned at metaphase plate or not, and the

sister chromatides of the minichromosome (or containing the integration) are segregated properly into each spindle poles or not. All the minichromosomal signals from 7C5HT1-1 and 1-2 metaphase cells were aligned at the metaphase plate in parallel with pole to pole direction, and all the sister chromatids signals of the minichromosomes from 7C5HT1-1and 1-2 anaphase cells were segregated properly and located at the nearest edges of the chromatids to each poles (Fig. 25). No metaphase and anaphase lagging minichromosome (or lagging chromosome with the integrated signals) was observed from any 7C5HT1-1, 1-2 or even from 7C5HT1-19 cells. Our results indicate that the minichromosomes derived from α7C5hTEL YAC introduction retained proper centromere/kinetochore function and anaphase lagging is not a common phenomenon caused by alphoid DNA integration to the chromosome.

SEQUENCE LISTING

SEQ ID NO:1
SEQUENCE LENGTH :17
SEQUENCE TYPE :nucleic acid
STRANDEDNESS :double
TOPOLOGY :linear
MOLECULE TYPE :Genomic DNA
ORIGINAL SOURCE :Homo sapiens
SEQUENCE DESCRIPTION:SEQ ID NO:1:

NTTCGTTGGA AACGGGA

SEQ ID NO:2
SEQUENCE LENGTH :1868
SEQUENCE TYPE :nucleic acid
STRANDEDNESS :double
TOPOLOGY :linear
MOLECULE TYPE :Genomic DNA
ORIGINAL SOURCE:Homo sapiens,Chromosome21,Centromeric region,WAV17 cell
SEQUENCE DESCRIPTION:SEQ ID NO:2:

AATTCAAATA AAAGGTAGAC AGCAGCATTC TCAGAAATTT CTTTCTGATG TCTGCATTCA
ACTCATAGAG TTGAAGATTG CCTTTCATAG AGCAGGTTTG AAACACTCTT TCTGGAGTAT
CTGGATGTGG ACATTTGGAG CGCTTTGATG CCTACGGTGG AAAAGTAAAT ATCTTCCATA
AAAACGAGAC AGAAGGATTC TCAGAAACAA GTTTGTGATG TGTGTACTCA GCTAACAGAG
TGGAACCTTT CTTTTTACAG AGCAGCTTTG AAACTCTATT TTTGTGGATT CTGCAAATTG
ATATTTAGAT TGCTTTAACG ATATCGTTGG AAAAGGGAAT ATCGTCATAC AAAATCTAGA
CAGAAGCATT CTCACAAACT TCTTTGTGAT GTGTGTCCTC AACTAACAGA GTTGAACCTT
TCTTTTGATG CAGCAGTTTG GAAACACTCT TTTTGTAGAA ACTGTAAGTG GATATTTGGA
TAGCTCTAAC GATTTCGTTG GAAACGGGAA TATCATCATC TAAAATCTAG ACAGAAGCAC
TATTAGAAAC TACTTGGTGA TATCTGCATT CAAGTCACAG AGTTGAACAT TCCCTTACTT
TGAGCACGTT TGAAACACTC TTTTGGAAGA ATCTGGAAGT GGACATTTGG AGCGCTTTGA
CTGCCTTTGT TGAAAAGGAA ACGTCTTCCA ATAAAAGCCA GACAGAAGCA TTCTCAGAAA
CTTGTTCGTG ATGTGTGTAC TCAACTAAAA GAGTTGAACC TTTCTATTGA TAGAGCAGTT
TTGAAACACT CTTTTTGTGG ATTCTGCAAG TGGATATTTG GATTGCTTTG AGGATTTCGT
TGGAAGCGGG AATTCGTATA AAAACTAGAC AGCAGCATTC CCAGAAATTT CTTTCGGATA
TTTCCATTCA ACTCATAGAG ATGAACATGG CCTTTCATAG AGCAGGTTTG AAACACTCTT
TTTGTAGTTT GTGGAAGTGG ACATTTCGAT CGCCTTGACG CCTACGGTGA AAAAGGAAAT
ATCTTCCCAT AAAAATAGAC AGAAGCATTC TCAGAAACTT GTTGGTGATA TGTGTCTCAA
CTAACAGAGT TGAACTTTGC CATTGATAGA GAGCAGTTTT GAAACACTCT TTTTGTGGAA
TCTGCAAGTG GATATTTGGA TAGCTTGGAG GATTTCGTTG GAAGCGGGAA TTCAAATAAA
AGGTAGACAG CAGCATTCTC AGAAATTTCT TTCTGATGAC TGCATTCAAC TCATAGAGTT
GAACATTCCC TTTCATAGAG CAGGTTTGAA ACACTCTTTC TGGAGTATCT GGATGTGGAC
ATTTGGAGCG CTTTGATGCC TATGGTGAAA AAGTAAATAT CTTCCCATAA AAACGAGACA
GAAGGATTCT GAGAAACAAG TTTGTGATGT GTGTACTCAG CTAACAGAGT GGAACCTCTC
TTTTGATGCA GCAGTTTGGA AACACTCTTT TTGTAGAAAC TGTAAGTGGA TATTTGGATA
GCTCTAATGA TTTCGTTGGA AACGGGAATA TCATCATCTA AAATCTAGAC AGAAGCCCTC
TCAGAAACTA CTTTGTGATA TCTGCATTCA AGTCACAGAG TTGAACATTC GCTTTCTTAG
AGCACGTTGG AAACACTCTT TTTGTAGTGT CTGGAAGTGG ACATTTGGAG CGCTTTGATG
CCTTTGGTGA AAAAGGGAAT GTCTTCCCAT AAAAACTAGA CAGAAGCATT CTCAGAAACT
TGTTTTTGAT GTGTGTACCC AGCCAAAGGA GTTGAACATT TCTATTGATA GAGCAGTTTT
GAAACACTCT TTTTGTGGAA AATGCAGGTG GATATTTGGA TAGCTTGGAG GATTTCGTTG
GAAGCGGG

SEQ ID NO:3
SEQUENCE LENGTH:338
SEQUENCE TYPE :nucleicacid
STRANDEDNESS :double
TOPOLOGY :linear
MOLECULE TYPE :Genomic DNA
ORIGINAL SOURCE:Homo sapiens,Chromosome21,Centromeric region,WAV17 cell
SEQUENCE DESCRIPTION:SEQ ID NO:3:

AATTCGTATA AACACTAGAC AGCAGCATTC CCAGAAATTT CTTTCGGATA TTTCCATTCA
ACTCATAGAG ATGAACATGG CCTTTCATAG AGCAGGTTTG AAACACTCTT TTTGTAGTTT
GTGGAAGTGG ACATTTCGAT CGCCTTGACG CTACGGTGAA AAAGGAAATA TCTTCCCATA
AAAAATAGAC AGAAGCATTC TCAGAAACTT GTTGGTATAT GTGTACTCAA CTAACAGAGT
TGAACTTTGC CATTGATAGA GAGCAGTTTT GAAACACTCT TTTCGTGGAA TCTGCAAGTG
GATATTTGGA TAGCTTGGAG GATTTCGTTG GAAGCGGG

SEQ ID NO:4
SEQUENCE LENGTH:339
SEQUENCE TYPE :nucleic acid
STRANDEDNESS :double
TOPOLOGY :linear
MOLECULE TYPE :Genomic DNA
ORIGINAL SOURCE:Homo sapiems,Chromosome21,Centromeric region,WAV17-cell
SEQUENCE DESCRIPTION:SEQ ID NO:4:

AATTCGTATA AACACTAGAC AGCAGCATTC CCAGAAATTT CTTTCGGATA TTTCCATTCA
ACTCATAGAG ATGAACATGG CCTTTCATAG AGCAGGTTTG AAACACTCTT TTTGTAGTTT
GTGGAAGTGG ACATTTCGAT CGCCTTGACG CCTACGGTGA AAAAGGAAAT ATCTTCCCAT
AAAAAATAGA CAGAAGCATT CTCAGAAACT TGTTGGTGAT ATGTGTCCTC AACTAACAGA
GTTGAACTTT GCCATTGATA GAGAGCAGTT TTGAAACACT CTTTTTGTGG AATCTGCAAG
TGATATTTGA ATAGTTTGGA GGATTTCGTT GGAAGCGGG

SEQ ID NO:5
SEQUENCE LENGTH:339
SEQUENCE TYPE :nucleic acid
STRANDEDNESS :double
TOPOLOGY :linear
MOLECULE TYPE :Genomic DNA
ORIGINAL SOURCE:Homo sapiens,Chromosome21,Centromeric region,WAV17 cell
SEQUENCE DESCRIPTION:SEQ ID NO:5:

AATTCGTATA AACACTAGAC AGCAGCATTC CCAGAAATTT CTTTCGGATA TTTCCATTCA
ACTCATAGAG ATGAACATGG CCTTTCATAG AGCAGGTTTG AAACACTCTT TTTGTAGTTT
GTGGAAGTGG ACATTTCGAT CGCCTTGACG GCTACGGTGA AAAAGGAAAT ATCTTCCCAT
AAAAAATAGA CAGAAGCATT CTCAGAAACT TGTTGGTGAT ATGTGTCCTC AACTAACAGA
GTTAAACTTT GCCATTGATA GAGAGCAGTT TTGAAACACT CTTTTTTTGG AATCTGCAAG
TGATATTTGA ATAGTTTGGA GGATTTCGTT GGAAGCGGG

SEQ ID NO:6
SEQUENCE LENGTH:340
SEQUENCE TYPE :uncleic acid
STRANDEDNESS :double
TOPOLOGY :linear
MOLECULE TYPE :Genomic DNA
ORIGINAL SOURCE:Homo sapiens,Chromosome21,Centromeric region,WAV17 cell
SEQUENCE DESCRIPTION:SEQ ID NO:6:

AATTCGTATA AAAACTAGAC AGCAGCATTC CCAGAAATTT CTTTCGGATA TTTCCATTCA
ACTCATAGAG ATGAACATGG CCTTTCATAG AGCAGGTTTG AAACCCTCTT TTTGTAGTTT
GTGGACAGTG GACATTTCGA TCGTCTTGAC GCCTACGGTG AAAAAGGAAA TATCTTCCCA
TAAAAAATAG ACAGAAGCAT TCTCAGAAAC TTGTTGGTGA TATGTGTCCT CAACTAACAG
AGTTGAACTT TGCCATTGAT AGAGAGCAGT TTTGAAACAC TCTTTTTGTG GAATCTGCAA
GTGATATTTG AATAGCTTGG AGGATTTCGT TGGAAGCGGG

SEQ ID NO:7
SEQUENCE LENGTH:338
SEQUENCE TYPE :nucleic acid
STRANDEDNESS :double
TOPOLOGY :linear
MOLECULE TYPE :Genomic DNA
ORIGINAL SOURCE:Homo sapiens,Chromosome21,Centromeric region,WAV17 cell
SEQUENCE DESCRIPTION:SEQ ID NO:7:

AATTCGTATA AAAACTAGAC AGCAGCATTC CCAGAAATTT CTTTCGGATA TTTCCATTCA
ACTCATAGAG ATGAACATGG CCTTTCATAG AGCAGGTTTG AAACACTCTT TTTGTAGTTT
GTGGAAGTGG ACATTTCGAT CGCCTTGACG CCTACGGTGA AAAAGGAAAT ATCTTCCCAT
AAAAATAGAC AGAAGCATTC TCAGAAACTT GTTGGTGATA TGTGTCTCAA CTAACAGAGT
TGAACTTTGC CATTGATAGA GAGCAGTTTT GAAACACTCT TTTTGTGGAA TCTGCAAGTG
GATATTTGGA TAGTTTGGAG GATTTCGTTG GAAGCGGG

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A recombinant DNA construct comprising:

a mammalian telomere and

a DNA sequence of a mammalian centromere,

wherein the DNA construct forms an artificial chromosome when introduced into mammalian cells, autonomously replicates in mammalian cells, is maintained extra chromosomally and is transmitted to progeny cells thereof, and wherein said DNA sequence contains a plurality of copies of the sequence:

5'-NTTCGNNNNANNCGGGN-3',

wherein N is selected from the group consisting of A, T, C and G.

2. A recombinant DNA construct comprising:

a mammalian telomere,

a mammalian centromere comprising spaced repeats of the sequence:

5'-NTTCGNNNNANNCGGGN-3',

wherein N is any one of A, T, C and G and wherein the sequence is provided in a quantity that causes the DNA construct to form a mammalian artificial chromosome when introduced into a mammalian cell.

3. A recombinant DNA construct comprising:

a mammalian telomere,

a mammalian centromere comprising tandem repeats of the sequence designated as Seq. No. 2, and wherein the sequence is provided in a quantity that causes the DNA construct to form a mammalian artificial chromosome when introduced into a mammalian cell.

4. A recombinant DNA construct comprising:

a mammalian telomere,

a mammalian centromere comprising a sequence that (1) contains 5'-NTTCGNNNNANNCGGGN-3', wherein N is selected from the group consisting of A, T, C and G, and (2) is capable of hybridizing under selective conditions with a homologous sequence selected from the group consisting of Seq. Nos. 2 - 7, wherein said sequence is provided in a quantity that causes the DNA construct to form a mammalian artificial chromosome

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when introduced into a mammalian cell.

5. The recombinant DNA construct of Claims 1, 2, 3 or 4, further comprising a sequence encoding a selectable marker gene.

6. The recombinant DNA construct of any one of Claims 1-5, which further comprises an exogenous DNA sequence encoding a gene product and a regulatory sequence for expressing the gene product.

7. The recombinant DNA construct of any one of Claims 1-6, wherein said mammalian cell is a mouse cell.

8. The recombinant DNA construct of any one of Claims 1-6, wherein said mammalian cell is a human cell.

9. The recombinant DNA construct of any one of Claims 1-8, further comprising at least one DNA sequence that permits said DNA construct to multiply in yeast or bacterial cells.

10. The recombinant DNA construct of any one of Claims 1-9, wherein said sequence is a cloned human alphoid DNA of about 100 kb and is capable of hybridizing under selective conditions with Seq. No. 2.

11. A cell line deposited at the Agency of Industrial Science and Technology, National Institute of Bioscience and Human Technology and designated as FERM BP-5625.

12. A DNA sequence comprising α7C5 h TEL isolated from the cell line of Claim 11.

13. A mammalian cell having a mammalian artificial chromosome that comprises the recombinant DNA construct of any one of Claims 1-10.

14. The mammalian cell of Claim 13, wherein said mammalian cell is a mouse cell.

15. The mammalian cell of Claim 13, wherein said mammalian cell is a human cell.

16. A method of making a mammalian artificial chromosome comprising:

introducing the DNA construct of any one of Claims 1-10 into a mammalian cell and

permitting the DNA construct to form a mammalian artificial chromosome within the mammalian cell.

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17. A method of making an exogenous mammalian gene product, comprising:

introducing the DNA construct of any one of Claims 1-10 into a mammalian cell, wherein the DNA construct of any one of Claims 1-10 further comprises an exogenous DNA sequence encoding the mammalian gene product and

expressing the exogenous mammalian gene product.

18. The recombinant DNA construct of Claim 1, wherein said DNA sequence is selected from the group consisting of Seq. Nos. 2 - 7.

19. The recombinant DNA construct of any one of Claims 1-10, further comprising:

a yeast telomere,

a yeast centromere and

a yeast replication origin, wherein said DNA construct also forms an artificial chromosome when introduced into yeast cells, autonomously replicates in yeast cells, is maintained extra chromosomally and is transmitted to progeny cells thereof.

20. The recombinant DNA construct of Claim 1, wherein said DNA sequence is derived from human chromosome 21.

21. A mammalian cell comprising the DNA construct of Claim 19.

22. A yeast cell comprising the DNA construct of Claim 19.

23. A method of producing the recombinant DNA construct of any one of Claims 1-10 by performing homologous recombination comprising:

transfecting a DNA construct into a host cell that does not possess a gene for DNA recombination within its DNA, wherein the recombinant DNA construct may be carried during mitotic division of the host cell within a plasmid having a gene for DNA recombination, which plasmid is capable of being eliminated during mitotic division of the host cell, and

collecting host cells that possess the recombinant DNA construct, but do not possess the plasmid.

24. The method of Claim 23, wherein said host cell is a yeast cell.

25. An isolated DNA when used for constructing an artificial chromosome, said DNA comprising sufficient repeats of a sequence

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containing 5'-NTTCGNNNNANNCGGGN-3' such that the isolated DNA functions as a mammalian centromere when introduced into a mammalian cell, wherein N is selected from the group consisting of A, T, C and G.

26. The isolated DNA of Claim 25, wherein said DNA sequence is selected from the group consisting of Seq. No. 2, Seq. No. 3, Seq. No. 4, Seq. No. 5, Seq. No. 6 and Seq. No. 7.

27. The isolated DNA of Claim 25, wherein said DNA sequence derives from the cell line of Claim 11.

28. An isolated DNA comprising the alphoid region of α7C5 h TEL derived from the cell line of Claim 11.

Dated this 17th day of October 2001

Tsuneko Okazaki, Hiroshi Masumoto and Masashi Ikeno

By their Patent Attorneys

CULLEN & CO.

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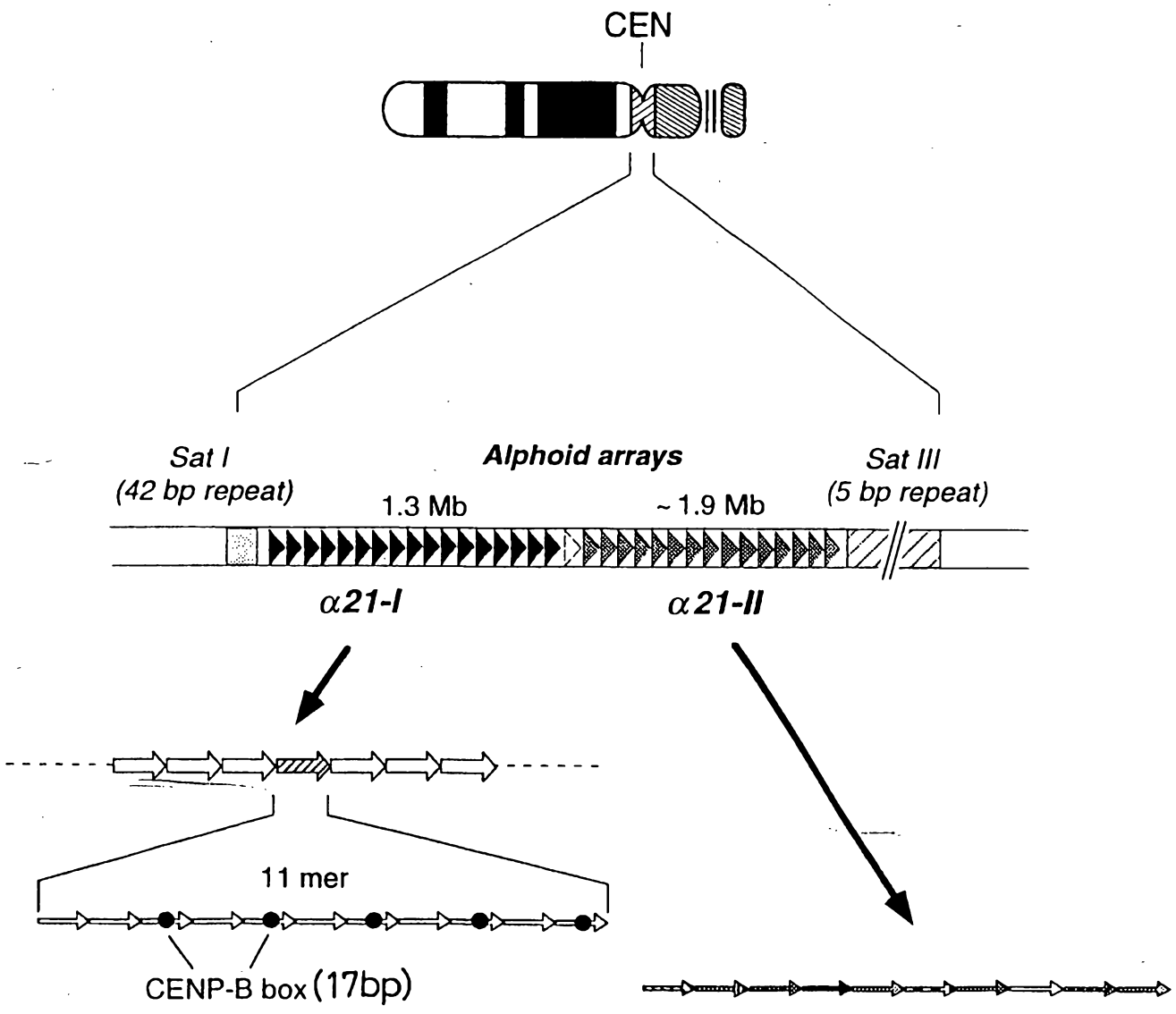

Chromosome 21 Centromere Region
CEN
Sat I
(42 bp repeat)
Alphoid arrays
Sat III
(5 bp repeat)
1.3 Mb
~ 1.9 Mb
α21-I
α21-II
11 mer
CENP-B box (17bp)

FIG.1

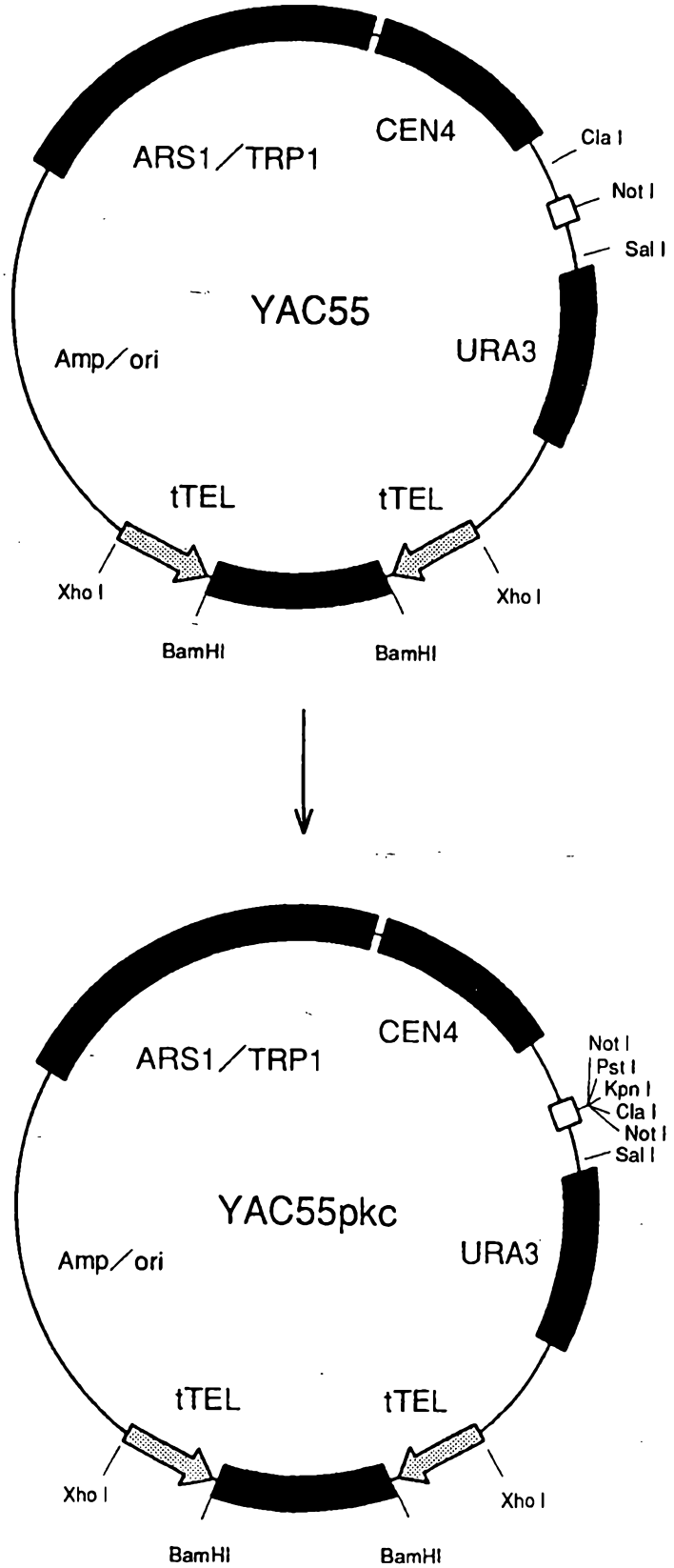
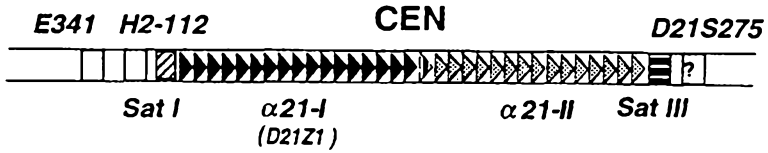

CEN4
ARS1/TRP1
Cla I
Not I
Sal I
YAC55
Amp/ori
URA3
tTEL
tTEL
Xho I
Xho I
BamHI
BamHI
CEN4
ARS1/TRP1
Not I
Pst I
Kpn I
Cla I
Not I
Sal I
YAC55pkc
Amp/ori
URA3
tTEL
tTEL
Xho I
Xho I
BamHI
BamHI

FIG.2

Centromere region of chromosome 21



CEN21 YACs	Insert (kb)	α21-I (kb)
α7C5	100	100
α24C9	70	70
αB12a	40	40
αB13	110	-
α55G4	60	-
α52D2	50	-
αB21	20	-
αB10	15	-

FIG.3

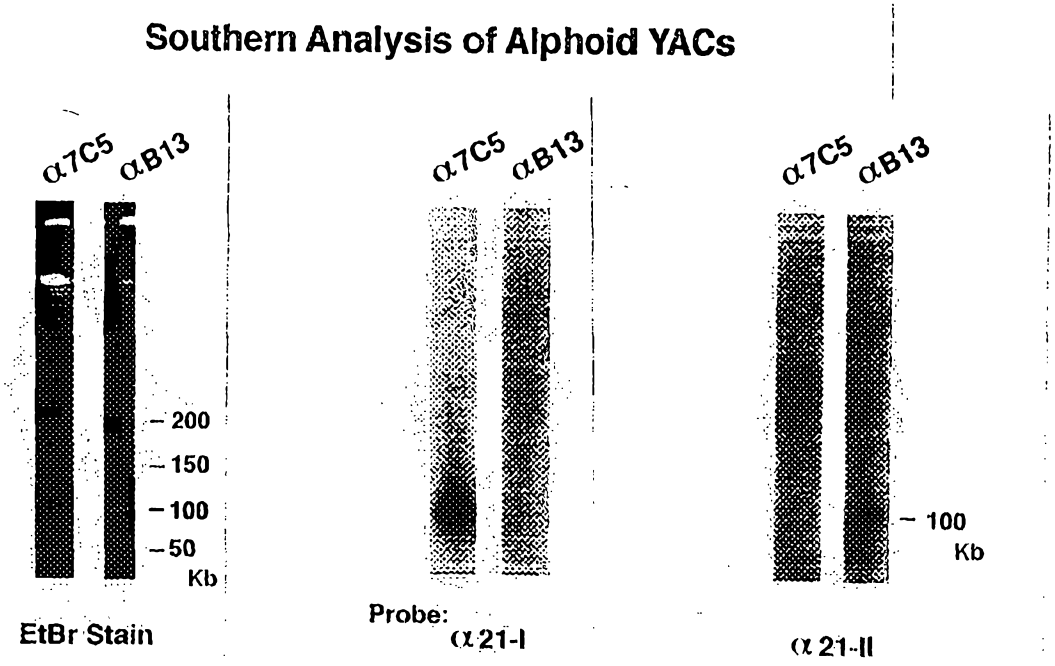


FIG.4(a) FIG.4(b) FIG.4(c)

YAC clone	α7C5	α B13
Insert[a]	100 kb	110 kb
Satellite I	-	-
α21-I[b]	103.0 ± 0.3 kb	-
α21-II[b]	-	104.5 ± 0.5 kb
Satellite III[b]	-	1.89 ± o.o1 kb
Alu	-	±[c]
CENP-B box[d]	275 copies	0 copies

FIG.5

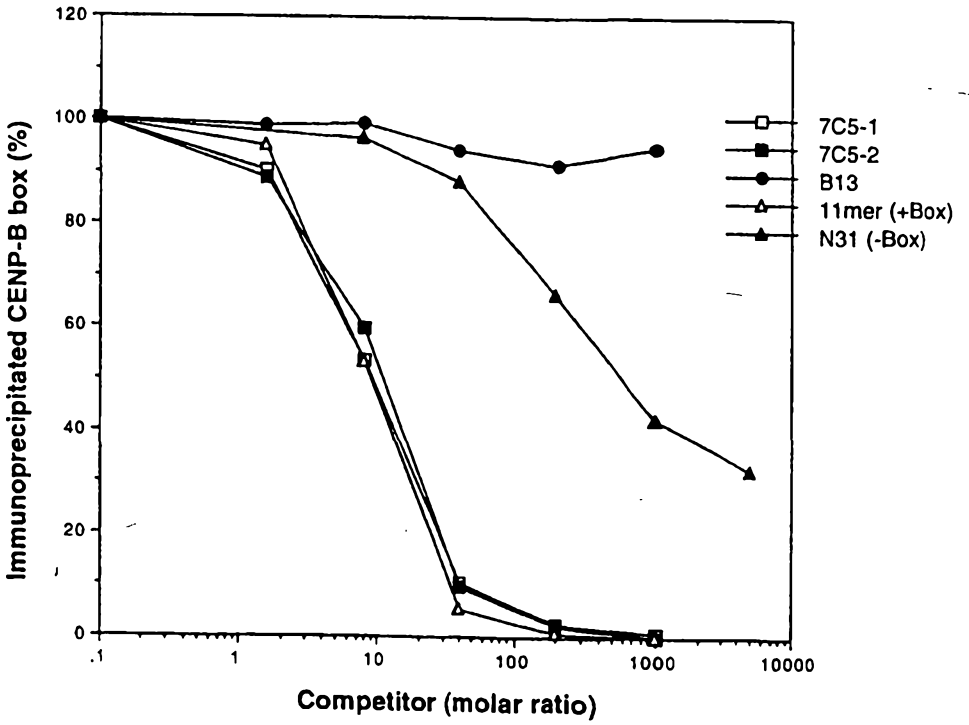


FIG.6

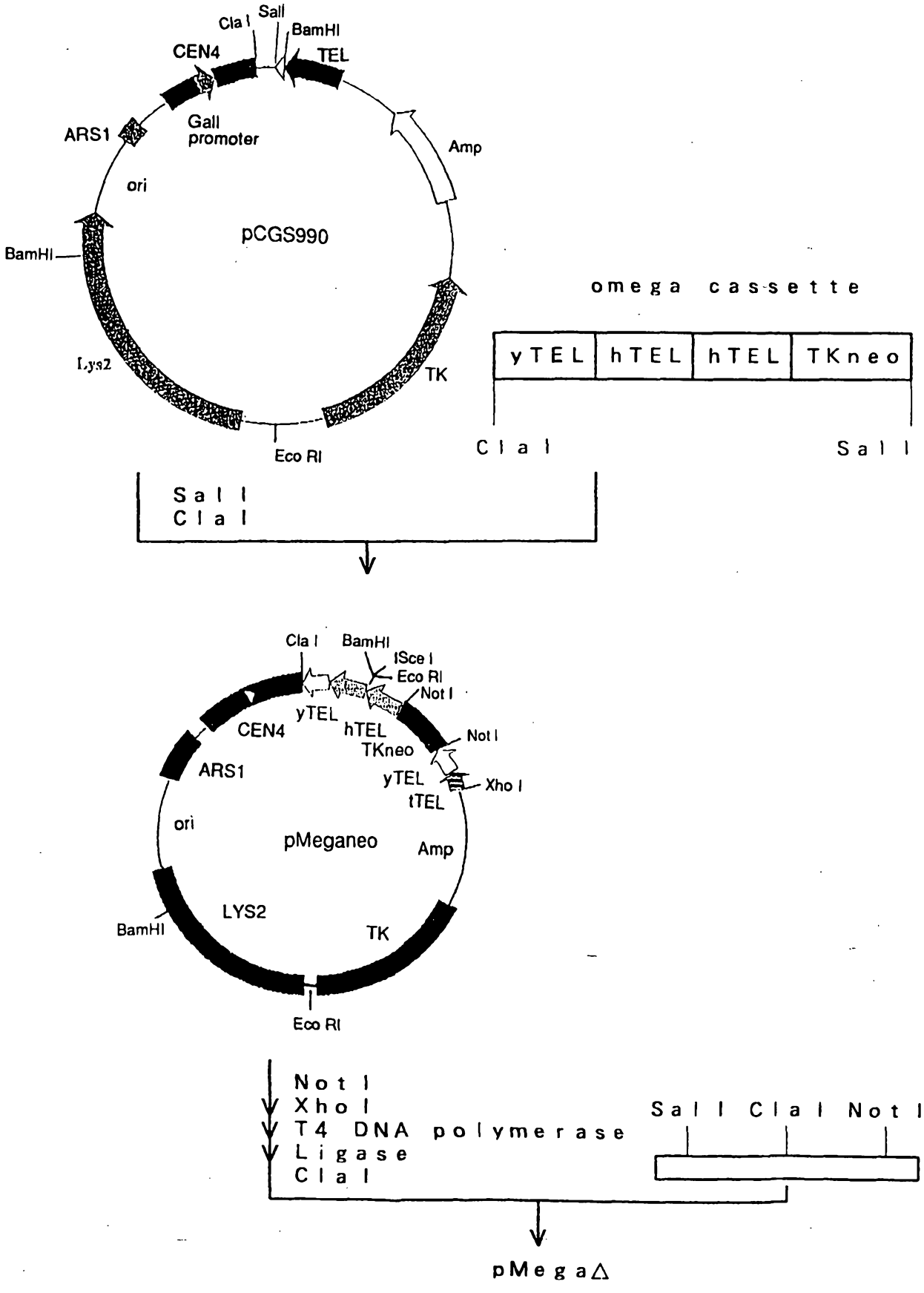

Cla I
Sal I
BamHI
CEN4
TEL
Gal1
promoter
ARS1
Amp
ori
pCGS990
BamHI
Lys2
TK
Eco RI
omega cassette
yTEL
hTEL
hTEL
TKneo
Cla I
Sal I
Sal I
Cla I
Cla I
BamHI
ISce I
Eco RI
Not I
yTEL
CEN4
hTEL
TKneo
Not I
ARS1
yTEL
Xho I
tTEL
ori
pMeganeo
Amp
LYS2
BamHI
TK
Eco RI
Not I
Xho I
T4 DNA polymerase
Ligase
Cla I
Sal I Cla I Not I
pMega△

FIG.7

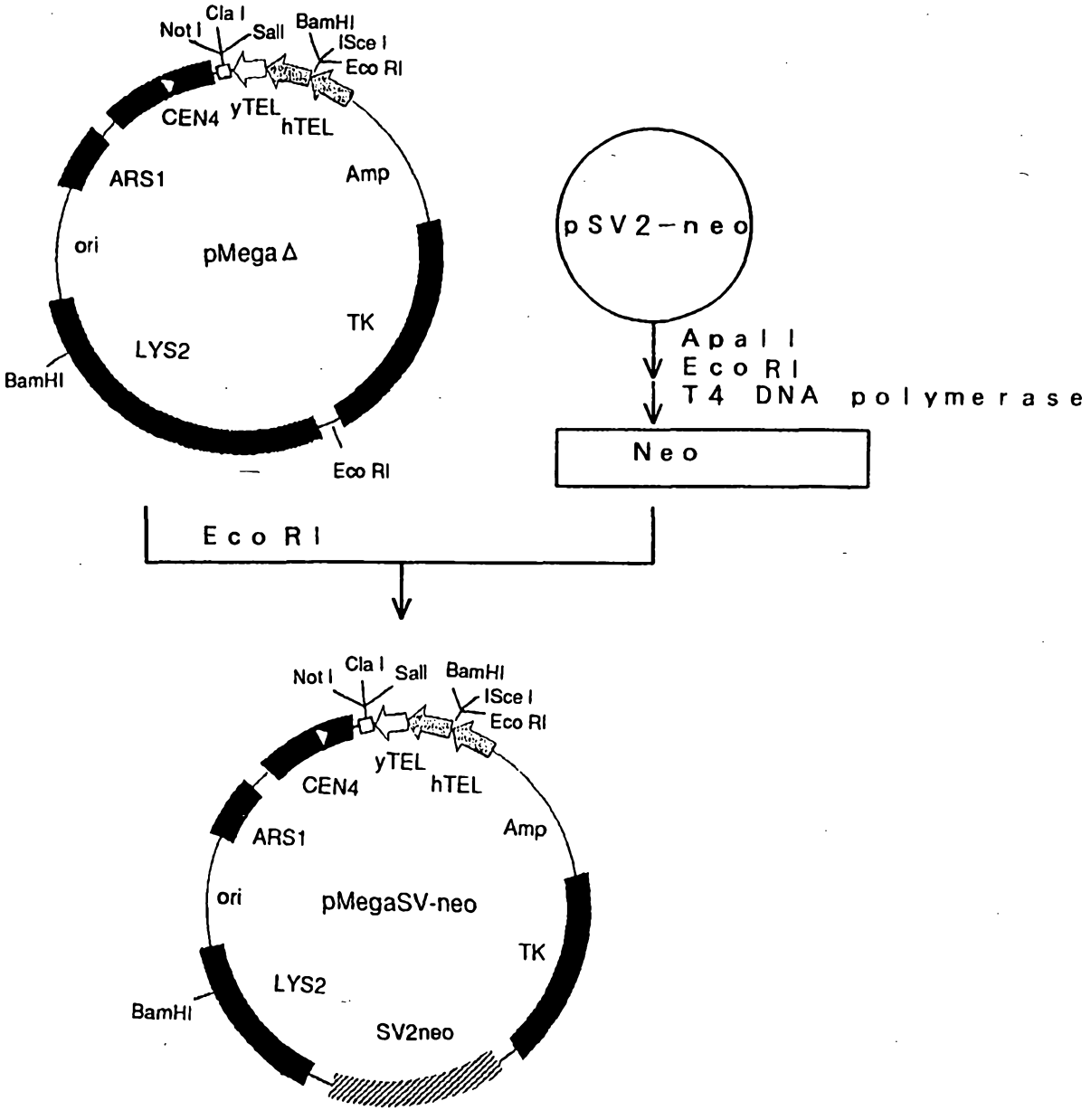

Not I
Cla I
Sall
BamHI
ISce I
Eco RI
CEN4
yTEL
hTEL
ARS1
Amp
ori
pMega Δ
TK
LYS2
BamHI
Eco RI
pSV2−neo
ApaI I
Eco RI
T4 DNA polymerase
Neo
Eco R I
Not I
Cla I
Sall
BamHI
ISce I
Eco RI
yTEL
CEN4
hTEL
ARS1
Amp
ori
pMegaSV-neo
TK
LYS2
BamHI
SV2neo

FIG.8

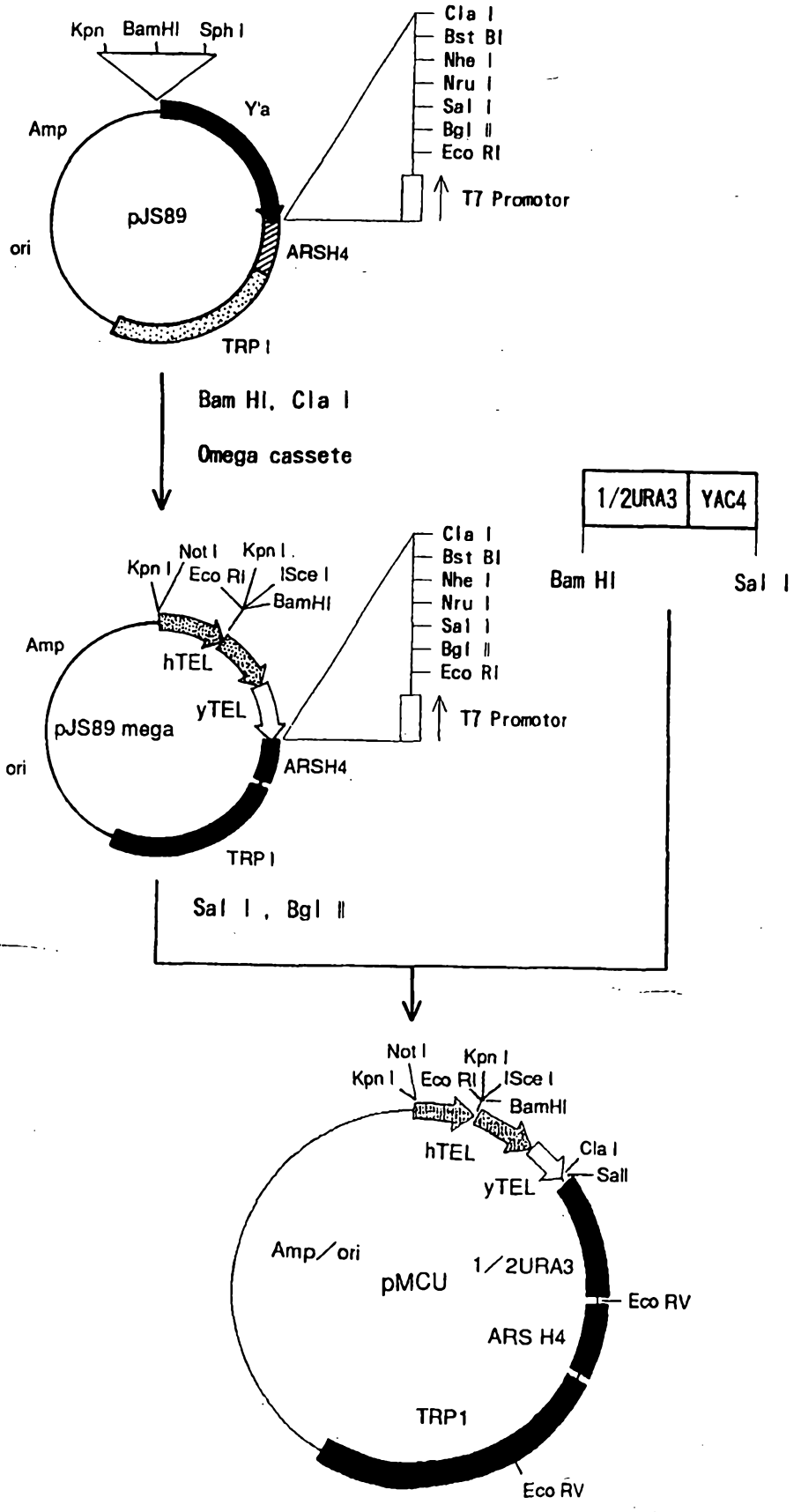

Kpn BamHI Sph I
Y'a
Amp
pJS89
ori
ARSH4
TRP I
Cla I
Bst BI
Nhe I
Nru I
Sal I
Bgl II
Eco RI
T7 Promotor
Bam HI, Cla I
Omega cassete
1/2URA3
YAC4
Bam HI
Sal I
Not I
Kpn I
Kpn I
Eco RI
ISce I
BamHI
hTEL
yTEL
pJS89 mega
Sal I , Bgl II
Cla I
Sall
Amp/ori
pMCU
1/2URA3
Eco RV
ARS H4
TRP1
Eco RV

FIG.9

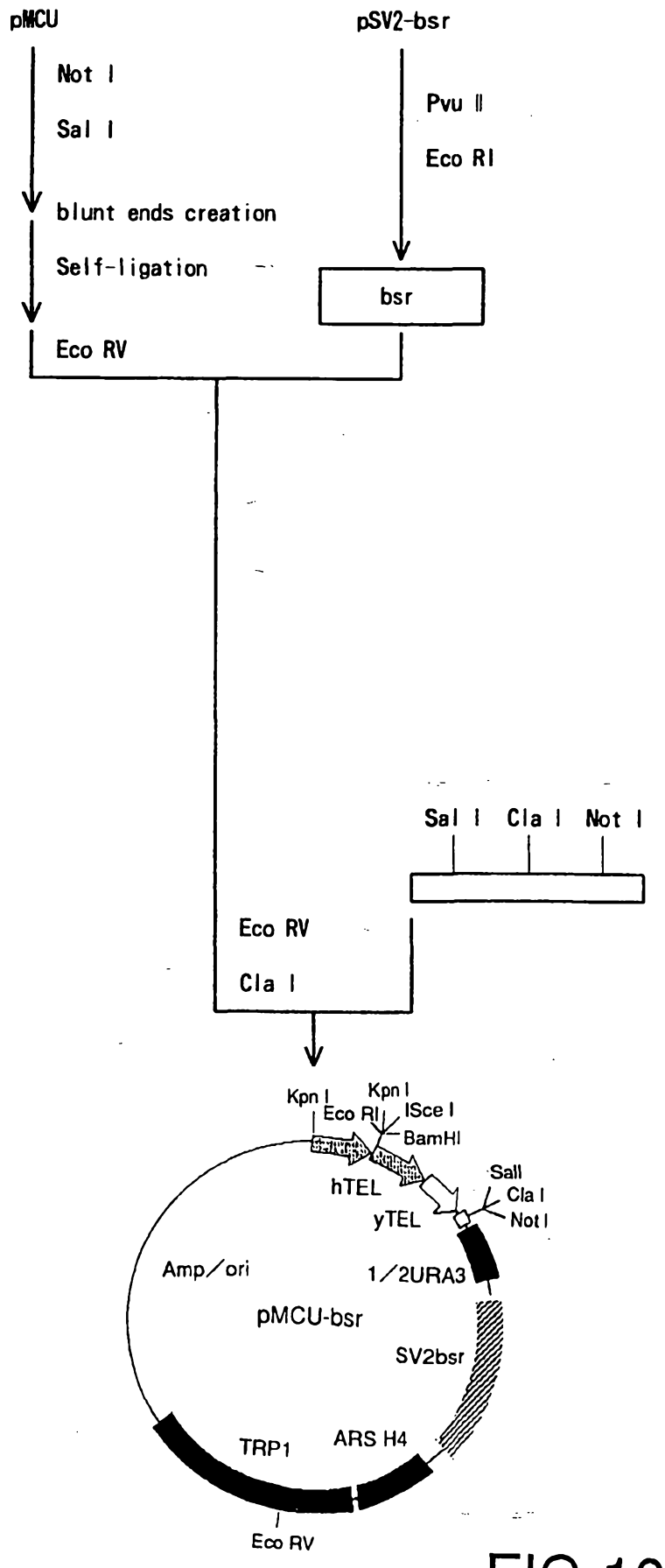

pMCU
pSV2-bsr
Not I
Sal I
Pvu II
Eco RI
blunt ends creation
Self-ligation
bsr
Eco RV
Sal I Cla I Not I
Eco RV
Cla I
Kpn I
Kpn I
Eco RI
ISce I
BamHI
hTEL
SalI
Cla I
yTEL
Not I
Amp/ori
1/2URA3
pMCU-bsr
SV2bsr
TRP1
ARS H4
Eco RV

FIG.10

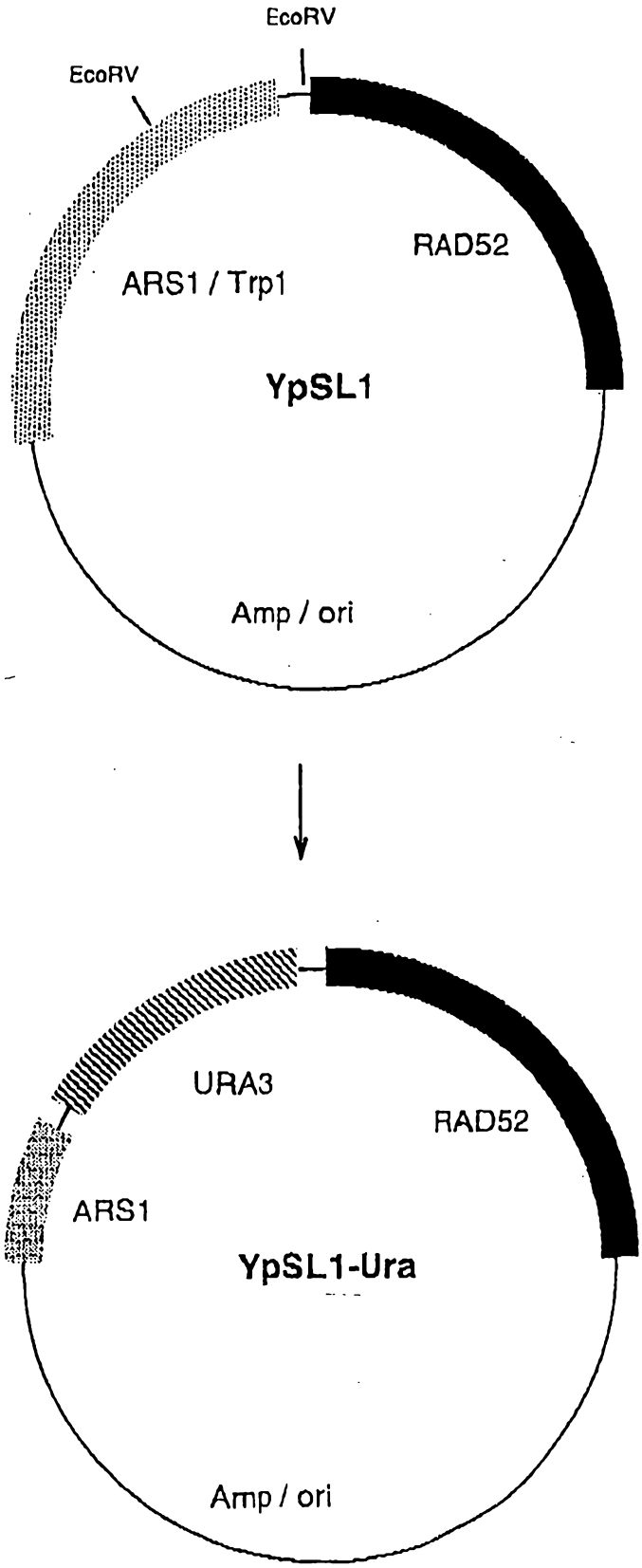

EcoRV
EcoRV
RAD52
ARS1 / Trp1
YpSL1
Amp / ori
URA3
RAD52
ARS1
YpSL1-Ura
Amp / ori

FIG.11

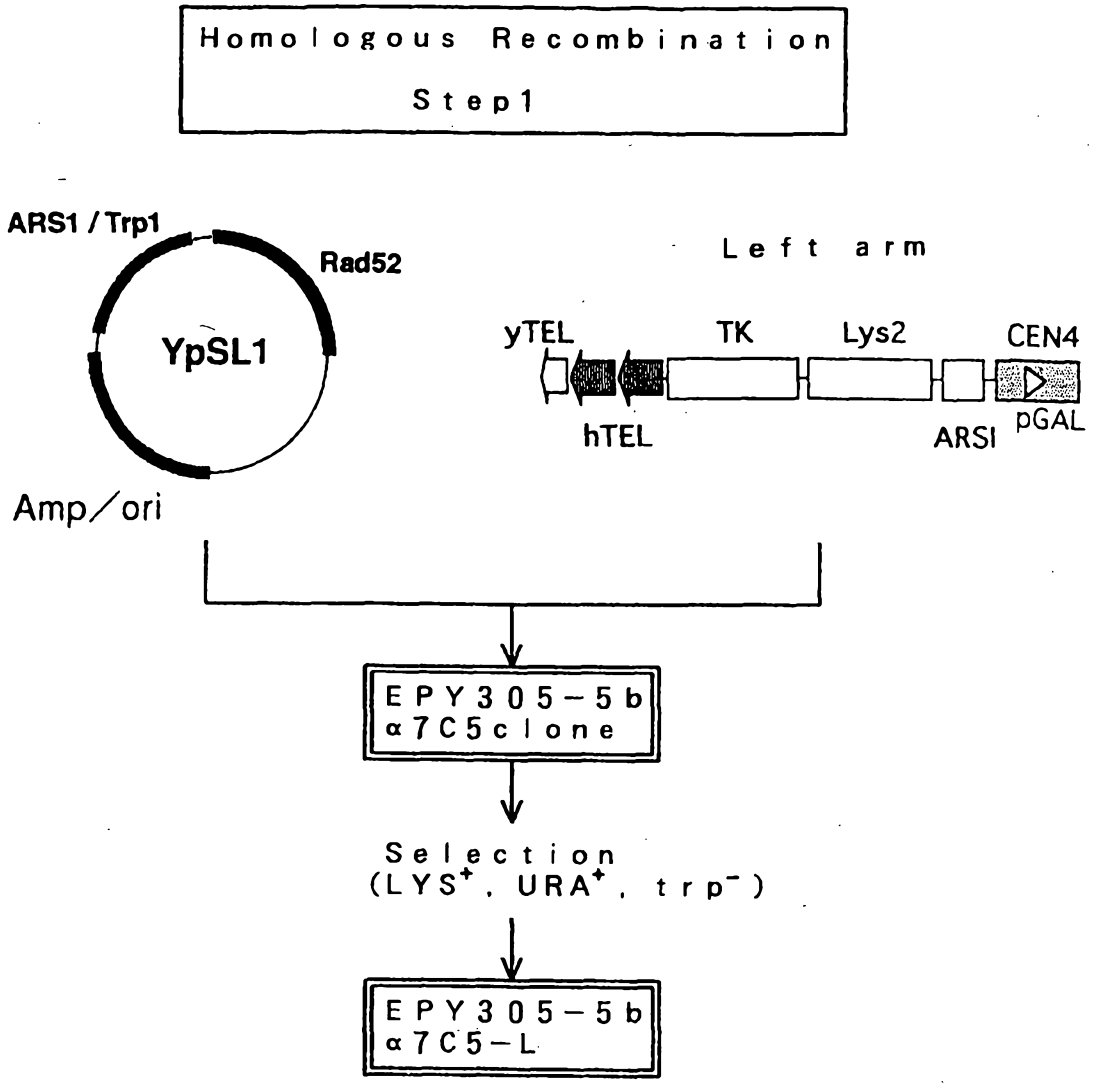

Homologous Recombination
Step1
ARS1 / Trp1
Rad52
YpSL1
Amp/ori
Left arm
yTEL
TK
Lys2
CEN4
hTEL
ARSI
pGAL
EPY305-5b
α7C5clone
Selection
(LYS+, URA+, trp-)
EPY305-5b
α7C5-L

FIG.12

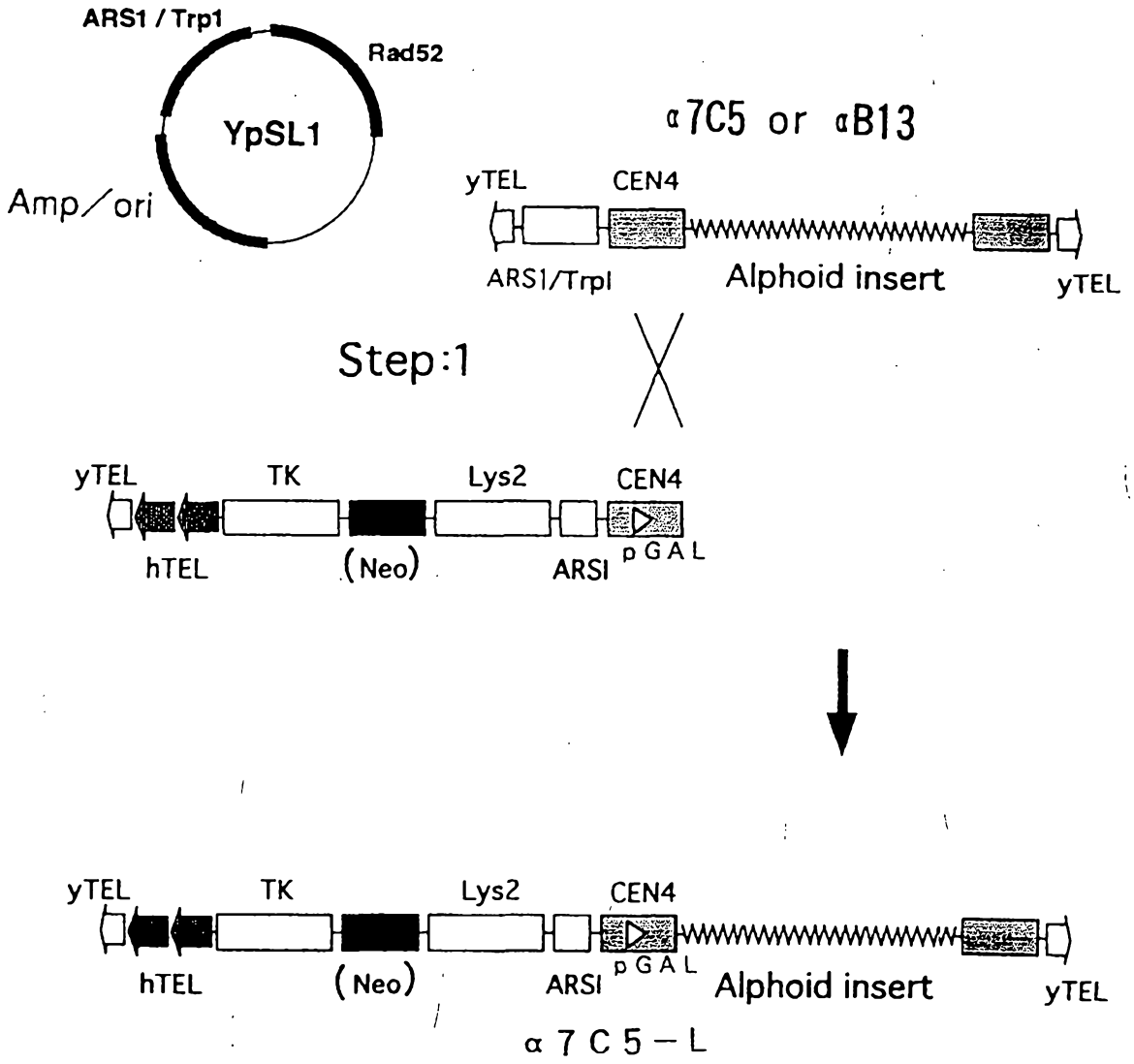

ARS1 / Trp1
Rad52
YpSL1
Amp/ori
αTC5 or αB13
yTEL
CEN4
ARS1/TrpI
Alphoid insert
yTEL
Step:1
yTEL
TK
Lys2
CEN4
hTEL
(Neo)
ARSI
pGAL
yTEL
TK
Lys2
CEN4
hTEL
(Neo)
ARSI
pGAL
Alphoid insert
yTEL
α7C5−L

FIG.13

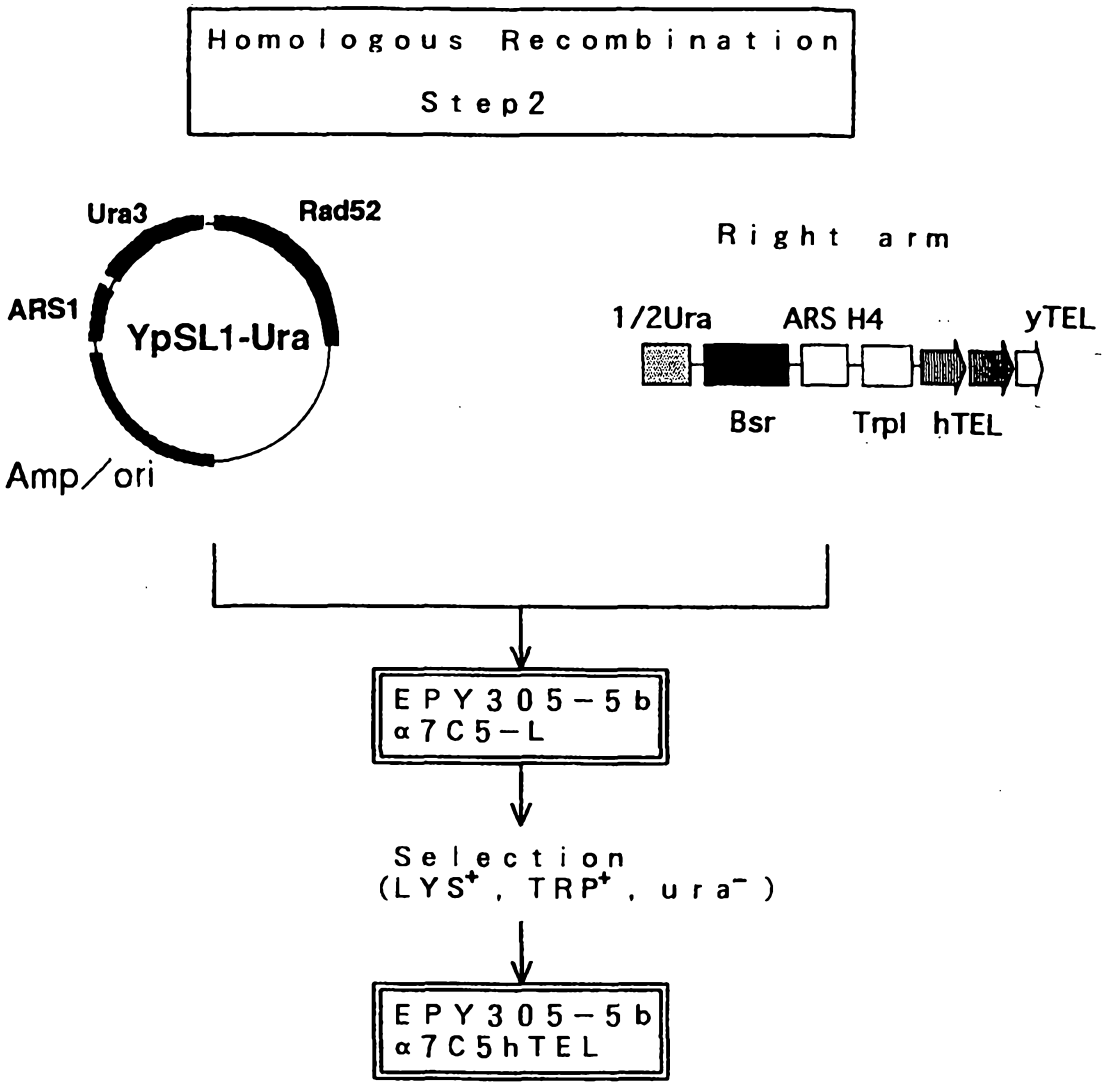

Homologous Recombination
Step2
Ura3
Rad52
ARS1
YpSL1-Ura
Amp/ori
Right arm
1/2Ura
ARS H4
yTEL
Bsr
TrpI
hTEL
EPY305-5b
α7C5-L
Selection
(LYS+, TRP+, ura-)
EPY305-5b
α7C5hTEL

FIG.14

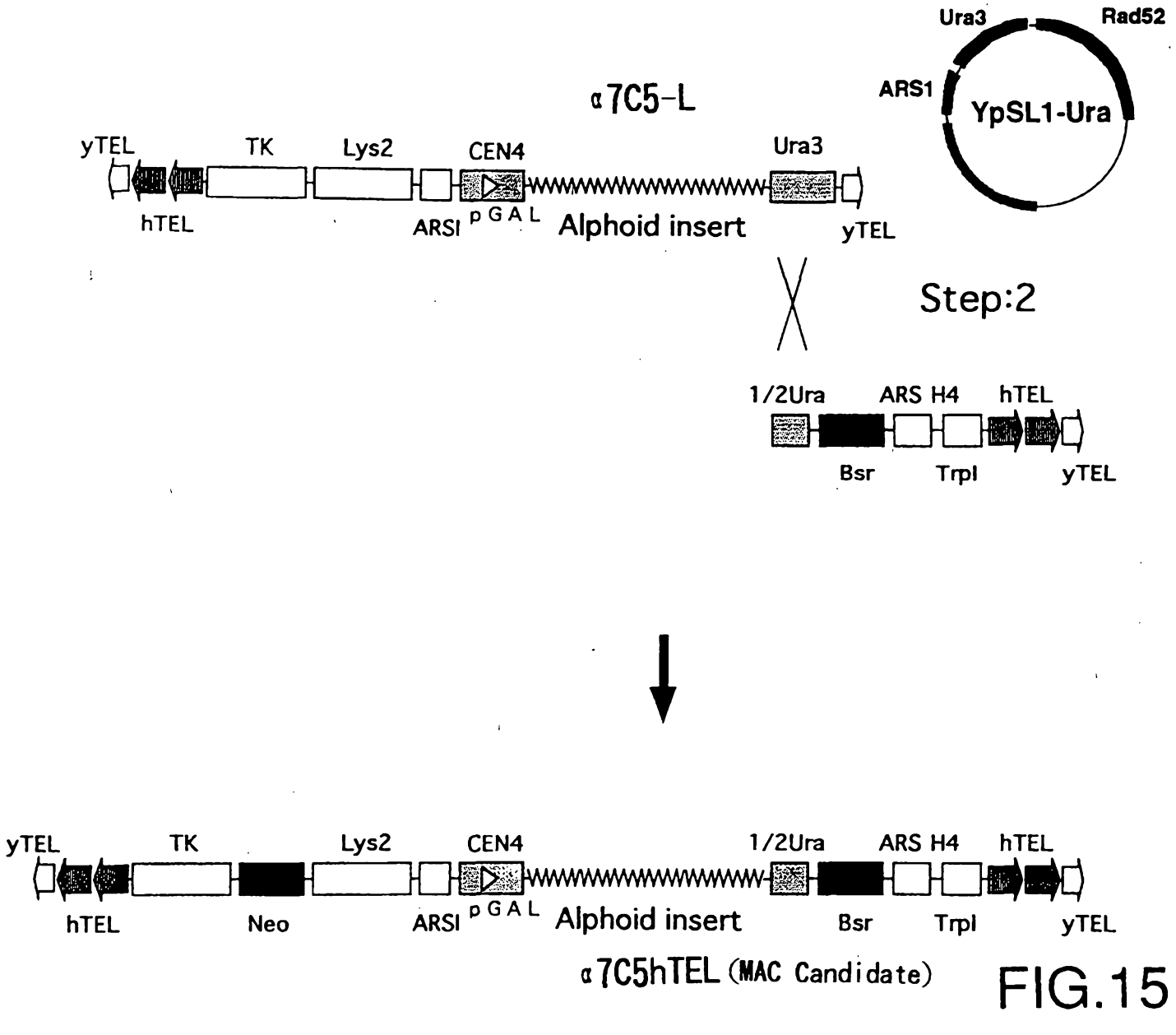

αTC5-L
yTEL
TK
Lys2
CEN4
Ura3
hTEL
ARSI
pGAL
Alphoid insert
yTEL
Ura3
Rad52
ARS1
YpSL1-Ura
Step:2
1/2Ura
ARS H4
hTEL
Bsr
TrpI
yTEL
yTEL
TK
Lys2
CEN4
1/2Ura
ARS H4
hTEL
hTEL
Neo
ARSI
pGAL
Alphoid insert
Bsr
TrpI
yTEL
αTC5hTEL (MAC Candidate)

FIG.15

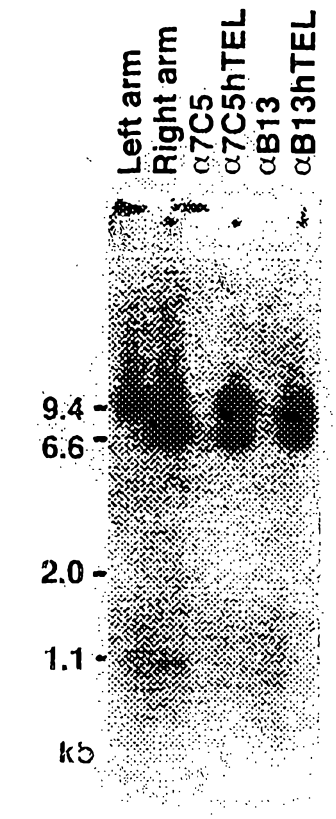

Left arm
Right arm
α7C5
α7C5hTEL
αB13
αB13hTEL
9.4
6.6
2.0
1.1
kb
Probe: human TEL

FIG.16

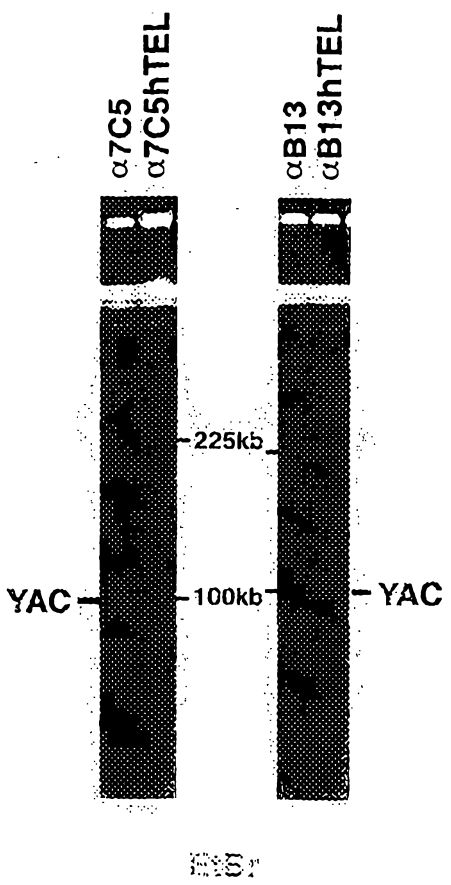

α7C5
α7C5hTEL
αB13
αB13hTEL
225kb
YAC
100kb
YAC

FIG.17

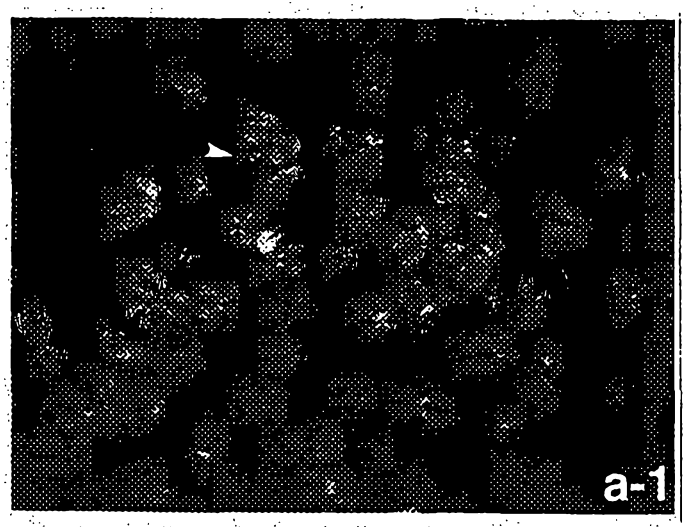


FIG.18(a)

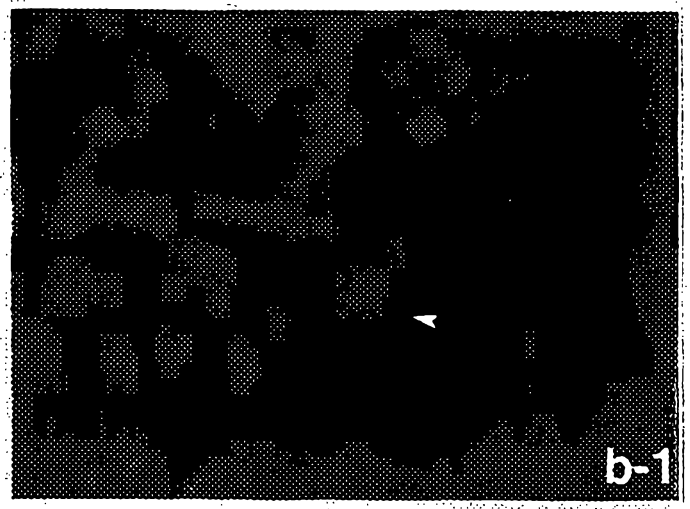


FIG.18(b)

FIG.18(c)

Efficiency of the minichromosome formation by the alphoid YAC DNA transfections

Introduced DNA	Introducing method	No. of mitotic cells analyzed by FISH	FISH analysis % of the cells showing the signals				
			Extrachromosomal	Host chromosomal			No signal
				Tel	Cen	Arm	
α7C5hTEL	Lipofection	350 (13)	30.6 (11)	14.0 (9)	26.0 (11)	0.9 (1)	28.6 (11)
	Microinjection	260# (11)	27.3 (10)	17.7 (6)	27.7 (7)	3.1 (4)	29.6 (9)
	Total	610 (24)	29.2 (21)	15.6 (15)	26.7 (18)	1.8 (5)	29.0 (20)
αB13hTEL	Lipofection	220 (11)	0	83.2 (11)	8.2 (2)	0.9 (1)	7.7 (9)
	Microinjection	180$ (9)	0	63.9 (8)	21.7 (3)	1.7 (2)	13.3 (9)
	Total	400 (20)	0	74.5 (19)	14.3 (5)	1.3 (3)	10.3 (18)
MCU-Bsr	Lipofection	40 (2)	0	58 (2)	0	5 (1)	38 (2)
	Microinjection	60 (3)	0	55 (3)	0	28 (2)	17 (3)
	Total	100 (5)	0	56 (5)	0	19 (3)	25 (5)

Number of cell lines showing the signals are indicated in parentheses.
#, 14 cells in 2 cell lines obtained from microinjection contained both the minichromosome and the integration in a single cell.
$, 1 cell obtained from microinjection contained two integration sites in a single cell.

FIG.19

Stability of the alphoid YAC-derived minichromosome as determined by plating efficiency and by FISH analysis

Cell line	Days off selection (generations)	Plating efficiency Colony No. on +BS selection / that of off selection	FISH analysis Cell No. showing the signals							
			Total M cells	Minichromosome/cell 1	2	3	Integration site Tel	Cen	Arm	No signal
7C5HT1	0	-	50 (%)	29 (58)	4 (8)	1 (2)	3 (6)	13 (26)	0	0
	20	630 / 627 (100.4%)	-							
	40	634 / 642 (98.7%)	-							
	60	655 / 672 (97.4%)	50	30 (60)	0	0	1 (2)	17 (34)	0	2 (4)
7C5HT2	0	-	50	9 (18)	0	0	14 (28)	0	0	27 (54)
7C5HT3	0	-	50	26 (52)	6 (12)	1 (2)	2 (4)	15 (30)	0	0
	20	610 / 614 (99.3%)	-							
	40	658 / 668 (99.5%)	-							
	60	631 / 652 (96.8%)	50	25 (50)	0	0	2 (4)	17 (34)	0	6 (12)
7C5HTm1	0	-	40	14 (35)	5 (13)	0	1 (3)	0	0	20 (50)
7C5HTm3	0	-	40	2 (5)	0	0	36 (90)	0	0	2 (5)

Day 0 is the time on which each cell line was established and already had passed 30 to 40 days from the YAC DNA transfections.

FIG.20

Stability of the alphoid YAC-derived minichromosome (or the integration site) of cell lines recloned from 7C5HT1

Cell line	Days off selection (generations)	Plating efficiency: Colony No. on +BS selection / that of off selection	FISH analysis: Cell No. showing the signals							
			Total M cells	Minichromosome/cell 1	2	3	Integration site Tel	Cen	Arm	No signal
7C5HT1-1	0	-	50	15 (30)	27 (54)	0	0	0	0	8 (16)
	20	633 / 634 (99.8%)	-							
	40	1061 / 1031 (103%)	40	6 (15)	31 (78)	1 (3)	0	0	0	2 (5)
	60	#	-							
7C5HT1-2	0	-	50	46 (92)	0	0	0	0	0	4 (8)
7C5HT1-4	0	-	50	41 (82)	0	0	0	0	0	9 (18)
7C5HT1-5	0	-	40	32 (80)	0	0	0	0	0	8 (20)
7C5HT1-6	0	-	40	31 (78)	0	0	0	0	0	9 (23)
7C5HT1-7	0	-	48	39 (81)	2 (4)	0	0	0	0	7 (16)
7C5HT1-8	0	-	40	32 (80)	0	0	0	0	0	8 (20)
7C5HT1-9	0	-	34	0	0	0	0	13 (38)	0	21 (62)
7C5HT1-19	0	-	30	0	0	0	29 (97)	0	0	1 (3)

FIG.21

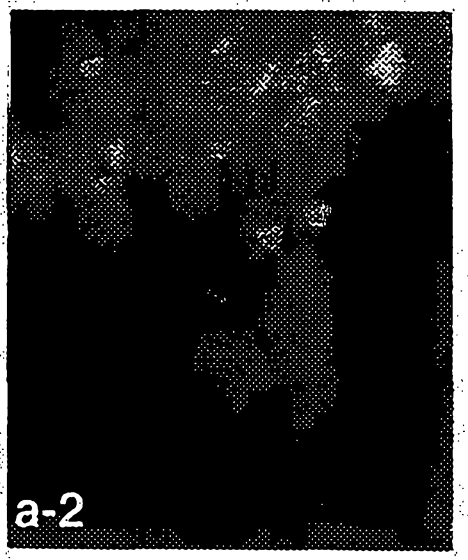


FIG.22(a)

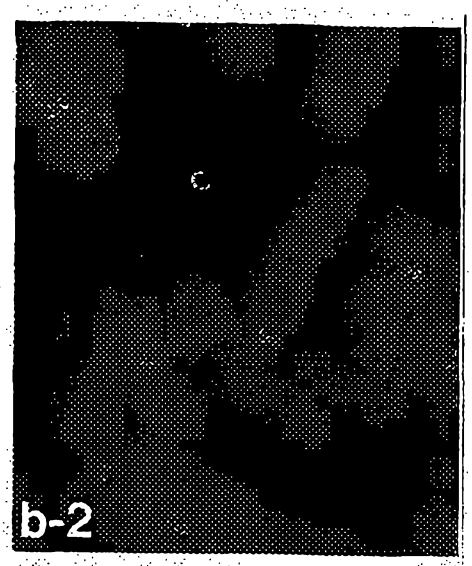


FIG.22(b)

FIG.22(c)

FIG.23(a)

FIG.23(b)

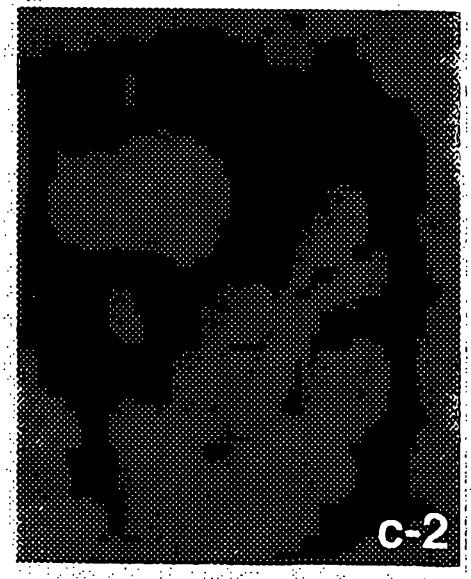


FIG.23(c)

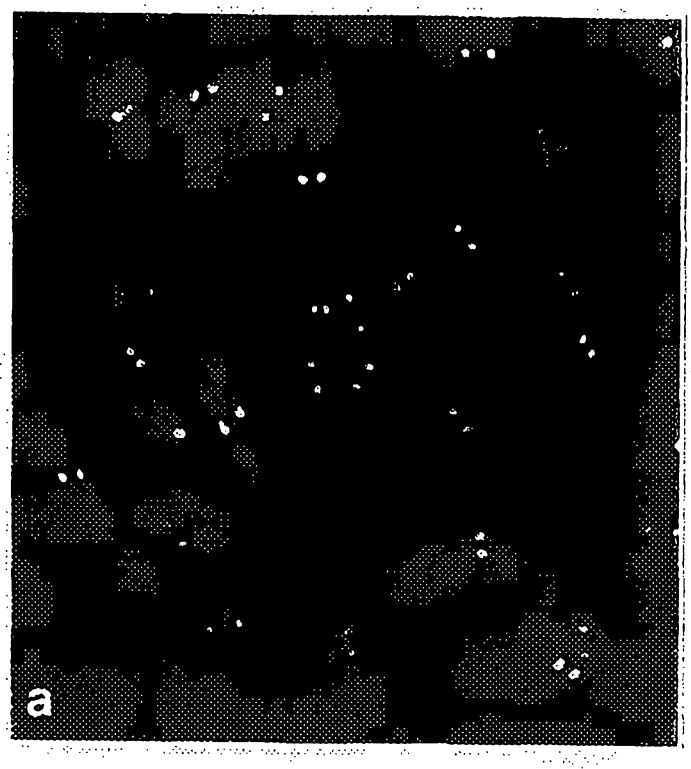


FIG.24

FIG.25(a)

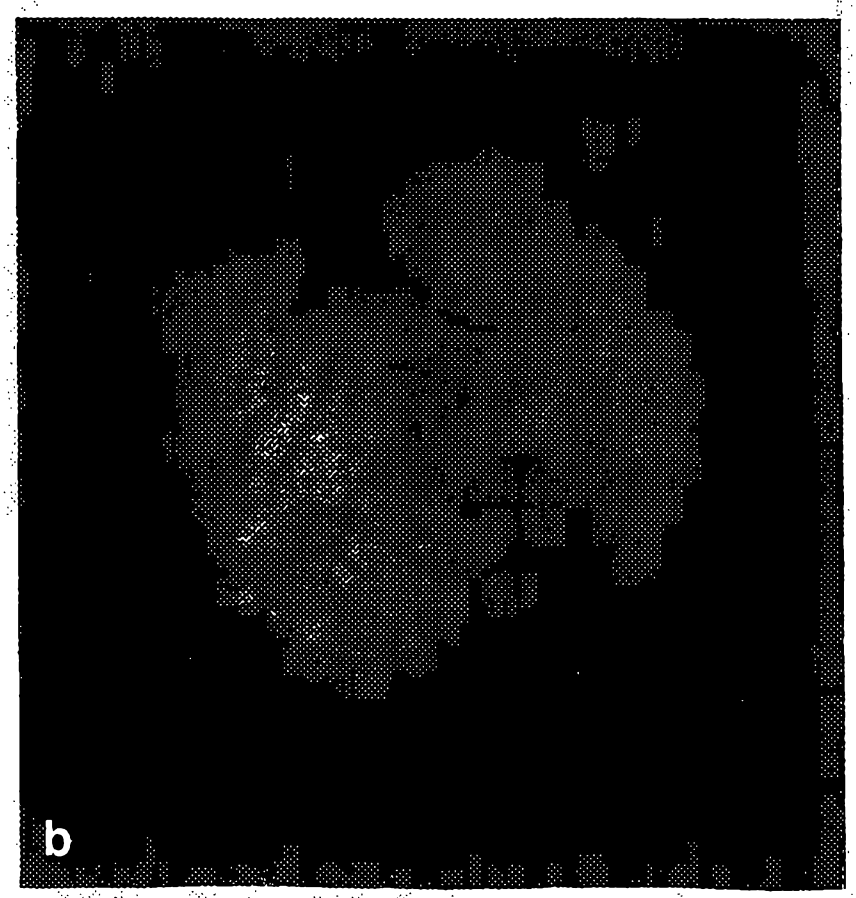


FIG.25(b)