Unique Behavior of Abnormal Chromosomal Distribution Exhibited by a Human Ch.21 YAC

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<Abstract>

A unique clone was found in the genomic library specific for human chromosome 21 that was constructed by us previously. The YAC (yeast artificial chromosome) clone exhibited unusual chromosomal distribution as described below. It showed several bands that were considered as artificial chromosomes by means of karyotype analysis with CHEF. Human specific short-repetitive-sequence, *Alu* hybridized to the bands. It was doubtful whether different clones are contaminated, since a single yeast cell usually does not contain several artificial chromosomes. Thus single colonies were isolated from the original clone and CHEF analysis was performed for them through six generations. As the result, it was shown that four different karyotypes including the original clone itself are generated from the original one. A clone expressing such a unique chromosomal behavior has not ever been reported before. The original clone is considered to be lacking in the function of regulatory factors for normal chromosomal distribution. It might be possible that novel factors would be identified from this unique clone. We report in this article the analytical results of abnormal behavior in chromosomal distribution of this clone.

Key words : Homologous recombination, Chromosome, *Saccharomyces cerevisiae*

INTRODUCTION

Homologous recombination of DNA is essential for maintaining the eukaryotic cell life. Studies using a budding yeast provide the most recent knowledge of the mechanism on DNA recombination event as follows. Meiosis involves the formation of homolog pairs of DNA, and then the chromosomes are connected by a specific structure: the synaptonemal complex. The interaction between homologs includes recombination at the DNA level, which is initially introduced by double-stranded break (DSB) of DNA. An interesting fact is that the meiotic recombination event called as crossover is strictly regulated to occur on more than one place of a single chromosome (obligate crossover). And also the event occurs on only few places of a single chromosome[1] (crossover interference). Defects in the genes related to meiotic recombination are lethal for most of the organisms. For human, defects in these essential genes cause critical disorder in chromosome number and its structure, but in most cases embryos fall in death with several exception such as Down syndrome (trisomy or partial deletion of chromosome 21), Turner syndrome (trisomy of chromosome X), Klinefelter syndrome (monosomy of chromosome Y) and so o n[2]. In a somatic chromosome division, homologous recombination contributes to repair DNA damage of DSB during the cell division cycle between late S-phase and G2-phase[3]. Non-homologous end-joining (NHEJ) is another rescue system from DNA damage caused on between G1-phase and early S-phase. These facts suggest that homologous recombination is essential for both

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meiosis and mitosis. One of the hallmarks of tumor cells is their highly rearranged karyotypes with respect to both chromosome number and the structural integrity of each homologous pair. And there are also several human diseases caused by chromosomal rearrangements. These chromosomal rearrangements are consequence of the loss of fidelity in repairing DSBs. These dangerous lesions are corrected by two primary pathways of NHEJ and homologous recombinational repair (HRR). Ataxia telangiectasia, Nijmegen breakage syndrome, Bloom syndrome, Werner syndrome, and Fanconi anemia are the chromosome instability disorders that are defective in the genes involved in the DSB repair pathways[4]. Recent studies employing mouse models have shown that the absence of either pathway leads to genomic instability, including potentially oncogenic translocations[5]. Disruption of the genes such as RAD51, MRE11, RAD50 and NBS1 that are related to homologous recombination are lethal in mammalian[4].

For pursuing the causative factors on some human diseases, yeast is an excellent tool for analyzing the gene and protein function, resulting in primary understanding of the biological regulation systems that are conserved in human. Comparative merits on yeast genetics are as follows, many analytical techniques are accumulated, cell generation time is 2hrs which is adequate for experiments, even the function of essential genes can be studied with the use of a diploid strain, meiosis can be easily introduced in yeast cells and most meiotic factors are conserved in human, more than 70 primary phenotypes are convenient to identify pleiotropic phenotypes associated with primary or suppressor mutations[6], and several useful Databases are constructed by the efforts of international projects and are available for anyone. Those databases are genomic sequence database[7], proteome database[8], micro-array database[9], two-hybrid database[10], and gene deletion database[11]. The availability of the full *Saccharomyces cerevisiae* genome offered a perfect opportunity for revising the number of homologues to human disease-related proteins. It was identified that 285 yeast proteins were similar to 155 human disease-related proteins[12]. We have been aiming to obtain useful mutants to identify novel factors associated with homologous recombination event. In this report, as a candidate for our targeting mutants, we show the behavior of a unique clone exibiting unstable chromosomal distribution. The clone was identified from a human chromosome 21 equivalent library[13] which was constructed from a line of human-rodent somatic hybrid cells with *Alu* selection by conventional hybridization techniques[14].

MATERIALS AND METHODS

Yeast strains, plasmids, and chemicals.

Saccharomyces cerevisiae strain AB1380 (Mat-a, ade2-1, can1-100, lys2-1, trp1, ura3, his5[psi+]), pYAC55, and the HY-1 YAC clone (125-kb DNA fragment from EB virus transformed human peripheral lymphocytes[15]. Chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA except ones indicated below.

YAC selection.

Single colony that possesses YAC was isolated on the double selection medium (Ura$^-$ and Trp$^-$), SD agar plate. The composition of SD medium is 0.17% yeast nitrogen base without amino acids, 0.5% ammonium persulfate, 0.056% amino acid mix without uracil and tryptophan[16], 2% glucose and 2% agar (pH5.8).

Contour-clamped homogenous electric field (CHEF) gel electrophoresis.

CHEF gel electrophoresis was performed as described elsewhere[17], using a CHEF-DR III system (Nippon Bio-Rad Laboratories, Fukuoka, Japan). The 1% agarose gels in 0.5×TBE (45mM Tris-borate, 45mM boric acid, 1mM pH8.0EDTA) were prepared by pouring 100 ml agarose (Agarose NA, Pharmacia AB, Uppsala, Sweden) into a 12 ×12㎠ frame. Electrophoresis was carried out in 0.5×TBE as running buffer at a constant temperature at 14℃, which was maintained by recirculation of the buffer through a heat

exchanger, at a constant voltage of 170V for 20 hrs. The stepped switching interval was 60sec for the first 12hr and 90sec for the next 8hrs.

Preparation of agarose embedded yeast DNA.

A single colony on YPD (1% yeast extract, 2% peptone, 2% glucose, pH5.8) agar plate was inoculated into 2 ml YPD broth. After grown for 20hrs to stationary growth phase, the cells were collected by centrifugation at 5,000×g, 5min, at 25℃. The supernatant was decanted and the cells were re-suspended in 1ml SE (75mM NaCl, 25mM pH7.4EDTA). Cells were collected again by centrifugation at 5,000×g, 5min, at 25℃. 50μl of SE and 150μl of agarose suspension buffer (ASB) which was equilibrated to 55℃ in a water bath were added. ASB was freshly prepared in each experiment. 200μl of ASB was mixed with following four solutions, 500μl of 3.5% low melt agarose (BRL, Gaithersburg, MD, USA) which was equilibrated to 55℃ in a water bath after autoclaved in SE for 1min at 121℃, 20μl of 1M DTT (filter-sterilized after dissolved in water), 10μl of 1mg/ml Zymolyase (Kirin 100T, Kirin Brewery Co. Ltd, Tokyo, Japan) suspended in sterile water, and 470μl of SE. After immediately combining the cell mixture, transfer the mixture plug mold and solidify for 8min at -20℃. Each solidified agarose plug was pushed into a well of 20 well micro plate and 80μl of 1M DTT (filter-sterilized after dissolved in water), 40μl of 1mg/ml Zymolyase (Kirin 100T, Kirin Brewery Co. Ltd, Tokyo, Japan) suspended in sterile water, 1880μl of SE were added into each well. After incubated for 1hr at 37℃, each agarose plug was washed with ES (0.5M pH9.5EDTA, 1% sodium sarcosinate) twice for 10min. 1ml ES and 50μl of proteinase K (Merk, Darmstadt, FRG) solution (10mg/ml in 50% glycerol) was added to each well and incubated at 55℃ for 20 hrs. After proteinase K treatment, each agarose plugs were washed with 1×TE (20mM Tris-HCl, 1mM EDTA, pH7.4) twice for 20min and stored at 4℃ until use.

Southern blot analysis.

CHEF gels were treated with 0.25N HCl for 15 min, then with a solution of 0.4N NaOH and 0.6M NaCl for 15min, and finally with a solution of 1.5M NaCl and 0.5M Tris-HCl (pH 7.5), before transfer to nylon membranes (Pall, Glen Cove, NY, USA) as described elsewhere[18]. Hybridization was carried out in a solution of 1M NaCl, 10% dextran sulfate and 1.0% sodium dodecyl sulfate with 100μg/ml yeast tRNA as carrier and approximately 10^6 cpm/ml of ^{32}P-labeled probe. The probes used were a 280-bp *Bam*HI fragment of BLUR-8 (*Alu* probe)[19], 2670-bp *Pvu*II/*Bam*HI digest of pBR322 (left arm probe) and 1692-bp *Bam*HI/*Pvu*II digest of pBR322 (right arm probe), radiolabeled with [^{32}P]dCTP with an oligopriming labeling kit[20] (Pharmacia-LKB). Membranes were washed twice with 2×SSC at room temperature for 10min, then once with 2×SSC, 1% SDS at 65℃ once for 30 min, and finally once with 0.1×SSC at room temperature once for 30min. Autoradiography was performed at -80℃ for a day. Filter was served for re-hybridization after labeled probe was completely washed off with the treatment of boiling for 30min in 0.1×SSC.

RESULTS AND DISCUSSION

The YAC (yeast artificial chromosome) cloning system allows the cloning of large fragment of exogenous DNA that is several hundred kilobases in length[15]. The schematic view of the cloning system is shown in Fig.1. The vector incorporates all necessary functions into a single plasmid that can replicate in *Eschericia coli*. This plasmid is called as a "yeast artificial chromosome" (YAC) vector. Cleavage at *Bam*HI sites adjacent to the TEL sequences produces termini that heal into functional telomeres *in vivo*. And ARS (ARS1) and a centromere (CEN4) enable the inserted DNA fragment to replicate as a normal yeast chromosome. During the characterization of YAC clones from the genomic library of human chromosome 21, we found a unique clone denoted as EG12C6 whose artificial chromosome changes in size at every event of single colony isolation on

SD medium. This was a novel mutant not ever reported before. In order to examine whether it was just the result of different yeast contamination or there was a regulatory pattern in the unstable chromosome distribution on each single-colony isolation stress, we repeated single colony isolation and the karyotype analysis by CHEF electrophoresis through six generation. A hundred single-isolated colonies were analyzed in each generation, and we confirmed that there are four types of clones including the original clone generated from EG12C6 (named as Original, Type 1, Type 2 and Type 3).

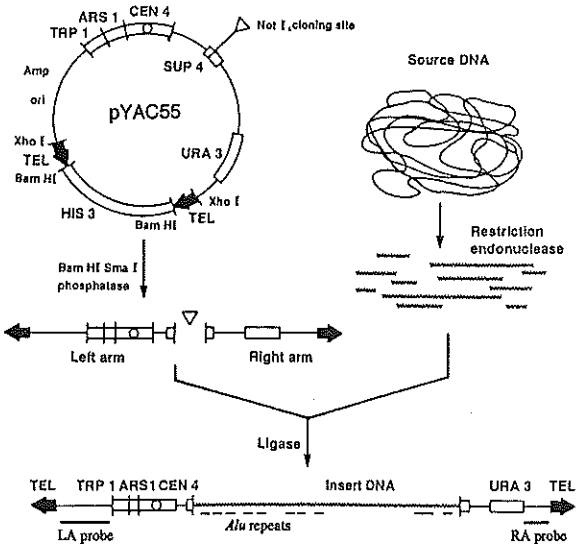

Fig. 1. Yeast artificial chromosome (YAC) cloning system. Since the EG12C6 YAC clone was selected with *Alu* probe from the pool of genomic library that was constructed with a mixed Chinese hamster and human chromosome 21 genomic DNA[13], *Alu* should exist in the cloned insert fragment even if the genomic nucleotide sequencing has not yet be performed. Thus, the position of *Alu* sequence is drawn on the inserted fragment at random. "LA" and "RA" indicate the probe position on the left arm and right arm of the vector, respectively.

Fig.2. shows the result of CHEF gel analysis and southern hybridization for the four types of clones obtained from the EG12C6 YAC clone. The HY-1 marker strain has an artificial chromosome of 125-kb size derived from human genome and three probes (*Alu*, LA and RA) hybridized to the 125-kb band, suggesting that this clone was a stable and a typical YAC clone. Original showed two extra bands of 200-kb and 280-kb on the stained gel as shown in Fig. 2, A. The extra 280-kb band overlapped with the yeast host chromosome VI (280 kb). The reason why the extra band was exogenous is that the eye-inspected intensity of the 280-kb band was obviously stronger than the other host's chromosomes and the extra band was slightly smaller than the yeast host chromosome VI, showing slightly smear broadness in the 280-kb band, and also that three probes (*Alu*, LA and RA) hybridized to the extra band, indicating a normal YAC. *Alu* probe hybridized to both of the two extra bands, but the eye-inspected intensity of the autoradiogram was obviously different, showing stronger signal on the 280-kb band. This suggests the human DNA might be integrated into the yeast chromosome VI, and then two chromosomes, both a YAC and an abnormal yeast chromosome VI containing human DNA might exist within one cell, resulting in stronger signal on Southern blot probed with *Alu*. Abnormality of this clone was that a faint signal was shown at the position of 125-kb band on the Southern blot probed with *Alu*, but no trace of signal was seen even after overexposure (data not shown) at that 125-kb position on the Southern blot probed with LA and RA (Fig.2, B, C and D). The reason that causes these phenomena is unsolved. However, a possible explanation could be that there was a different clone generated in very little ratio and the clone possesses the 125-kb human DNA lacking vector arms, probably also lacking the vector telomeres. In this speculation, the 125-kb chromosome must have functional telomeres acquired by integration from some chromosomes of the yeast host. It is unclear whether two types of cells that contain the 200-kb YAC and the 280-kb YAC have grown as mixed in liquid culture, or one cell contains both of the YACs. Single colony isolation of Original generates all four types of clones including Original itself as shown on Fig.3, A. The ratio of occurrence is 14% for Original, 49% for Type 1, 12% for Type 2 and 25% for Type 3.

Type 1 showed two extra bands, the 200-kb vivid and the faint 125-kb band. The faint band might indicate that the extra smaller

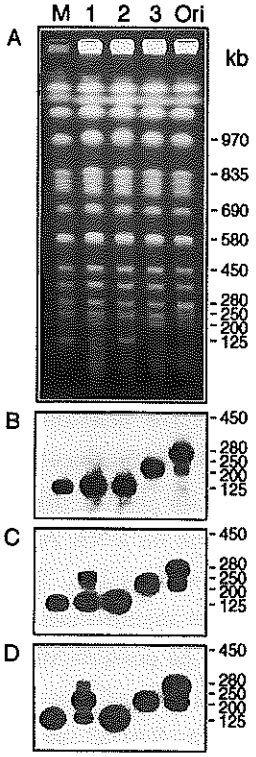

Fig. 2. CHEF gel analysis of the EG12C6 YAC clone. A: a gel stained with ethidium bromide. B, C and D: Southern hybridization with *Alu* probe, LA probe and RA probe, respectively. Lane M (marker) is the HY-1 YAC clone. Lane Ori is the original EG12C6 YAC clone. Lane 1, 2 and 3 are Type 1, Type 2 and Type 3 of the EG12C6 YAC clone, respectively.

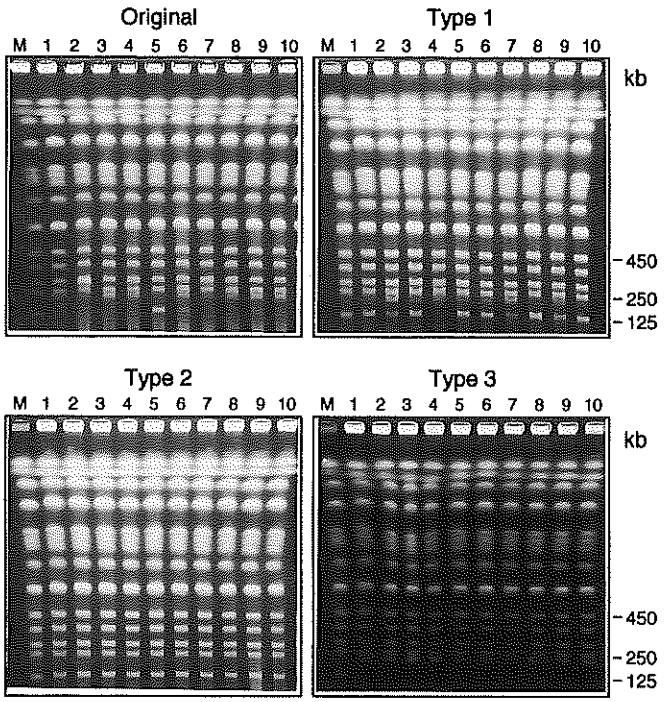

Fig.3. CHEF gel analyses of the each ten single-colonies that were isolated from the original clone (shown as Original), Type 1, Type 2 and Type 3 of the EG12C6 YAC clone. Gels were stained with ethidium bromide. Lane M (marker) is the HY-1 YAC clone. Lane 1-10 are the single-isolated clones from a parental clone on SD agar plate.

chromosome was easily lost during cell division in liquid culture. Or on the contrary, it is also possible that Type 2 was generated from Type 1 during liquid cultivation. *Alu* probe hybridized to the smaller 125-kb band, but did not hybridize to the 200-kb band, indicating that the 200-kb band did not contain human DNA, or at least did not contain *Alu* repeat. Possibly some host chromosomes might be broken down into the 200-kb size. Or it is also possible that 200-kb DNA was replicated from a normal host chromosome and acquired replication function by some process, absorbing the essential fragments of centromere and telomere. LA probe hybridized both to the smaller 125-kb band and the host chromosome I (250kb). This indicates some portion of vector left arm was integrated into the host chromosome I. RA probe hybridized to the three bands, that is, the 125-kb, the 200-kb and the 280-kb bands. The 280-kb band might be the host chromosome VI (280 kb) or the same extra band as seen in Original. Taking these facts into consideration, the 125-kb band seemed to be normal YAC possessing human DNA, the left arm and the right vector arm. Also the vector arms that might harbor the short sequence of human DNA lacking *Alu*, might integrate into yeast host chromosomes. The extra 200-kb band did not contain human DNA containing *Alu* and the vector left arm. It is uncertain that this 200-kb chromosome contained an integrated host telomere on one termini of the chromosome, since LA probe did not contain telomere sequence of the vector. Single colony isolation of Type 1 generates Type 1, Type 2 and Type 3 in the ratio of 72%, 14% and 14%, respectively. The example of CHEF analysis on

single-isolated colonies from Type 1 is shown on Fig. 3, B.

Type 2 and 3 showed typical behavior as same as a common stable YAC. *Alu*, LA and RA probes hybridized to only the extra band shown on the stained gel and did not hybridize to any yeast host chromosome. These two types of clones were stable even after the single colony isolation on SD agar plate as shown on Fig. 3, C and D. These results indicate that the artificial chromosomes of Type 2 and Type 3 replicated normally.

These four clones are generated from a single YAC clone. The question is the reasons why chromosomal distribution of Original and Type 1 was unstable and why those of Type 2 and Type 3 were stable. The events of homologous DNA recombination or DNA deletion are caused by cooperative action of many factors related to cell division. For example, factors for DNA replication and DNA repair, cyclins, condensins, cohisins, adherins, separins, securins, and so on. The known functions of these factors must be only a part of the whole event process. Original and Type 1 are lacking the function of normal chromosome distribution at the somatic cell division. Interestingly, the defect is recovered in Type 2 and Type 3. We speculate that a recombination hot spot existing in the inserted human DNA is deleted in Type 2 and Type 3, resulting in stable chromosomal condition. In this speculation, it is possible that the recombination hot spot of the inserted human DNA was associated with the specific DNA sequence of the yeast host genome. The gene containing the recombination hot spot could be identified by means of mRNA subtraction between stable cells and unstable cells, or by means of the complementation of the unstable cells. There are several recombination hot spots reported. One of the hot spots is LCRs[21] (low-copy repeats). LCRs are usually of 10-400kb in size and exhibit 95-97% similarity in human genome. LCRs constitute about 5% of the human genome. LCRs seem to play a major role in both primate karyotype evolution and human tumorigenesis. *Alu* repeat clusters also mediate chromosomal rearrangements[22]. Other repetitive sequences like SINEs (short interspersed elements) and LINEs (long interspersed elements) might be playing important role. The other hot spot is highly GC rich region[23]. Another hot spot is the MHC (major histocompatibility complex) gene locus that has a single-nucleotide polymorphism[24]. Minute analysis on this YAC clone may leads to find a novel hot spot for DNA recombination.

It is reported that the deletion of RAD52 enhances the chromosomal translocations in budding yeast[25]. RAD52 is the RECA like protein and plays an essential role on DNA replication, DNA repair, and DNA recombination. The RAD52 gene is highly conserved in mammalian genome including human. It might be possible that the function of RAD52 or the function of some other proteins works together with RAD52 such as MRE11, NBS1, etc is blocked in Original and Type 3 clones for some reason. And the block is possibly unlocked in Type 2 and 3 clones.

One of the targets for the study on homologous recombination is to get the clue for tumorigenesis. Most human cancer cells show signs of genome instability, ranging from elevated mutation rates to gross chromosomal rearrangements and alterations in chromosome number. Little is known about the molecular mechanisms that generate this instability or how it is suppressed in normal cells. Intensive studies on genome instability using the yeast *Saccharomyces cerevisiae* have implicated more than fifty genes in the suppression of genome instability, including genes that function in S-phase checkpoints, recombination pathways, and telomere maintenance. Human homologs of several of these genes have well-established roles as tumor suppressors[26]. This suggests that the mechanisms preserving genome stability in yeast are the same mechanisms that go awry in cancer. In this point, further study on the EG12C6 YAC clone may have a significant role.

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原 著

ヒト21番染色体YACクローンの示すユニークな染色体分配挙動

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＜要 旨＞

我々が先に構築したヒト21番染色体特異的なゲノムライブラリーの中から、ユニークなクローンが発見された。このYAC（酵母人工染色体）クローンは通常見られない次のような染色体挙動を示した。このクローンはCHEFによる核型分析では人工染色体と思われる数本のバンドを示し、これにヒト特有の短鎖反復配列である*Alu*がハイブリダイズした。一般にはひとつの酵母細胞に複数の人工染色体が存在することは無いので、クローンのコンタミネーションが疑われた。そこでオリジナルクローンから単一コロニーを6世代に渡って分離し、その各世代でCHEF解析を実施した。その結果、オリジナルクローンから、それ自身も含めると4種のクローンが派生することが分かった。このような挙動を示すクローンはこれまで報告されていない。このオリジナルクローンでは、染色体分配を正常に制御する因子が機能しないために異常分配を引き起こしているものと考えられる。このクローンを用いて染色体分配に関わる新たな因子の同定が期待される。本稿では、このクローンの異常な染色体分配挙動の解析結果を報告する。

キーワード：相同的組み換え　染色体　出芽酵母