Henry Ford Hospital Medical Journal

Volume 40 | Number 3 Article 15

9-1992

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# Isolation of YAC Clones From the Pericentromeric Region of Chromosome 10 and Development of New Genetic Markers Linked to the Multiple Endocrine Neoplasia Type 2A Gene

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*Genetic linkage mapping and contig assembly using yeast artificial chromosome (YAC) technology form the basis of our strategy to clone and define the genomic structure of the pericentromeric region of chromosome 10 containing the multiple endocrine neoplasia type 2A gene. Thus far YAC walks have been initiated from five chromosome 10 pericentromeric loci including RBP3, D10S94, RET, D10Z1, and FNRB. Long range pulsed-field gel electrophoresis maps are constructed from the YACs isolated to define clone overlaps and to identify putative CpG islands. Bidirectional YAC walks are continued by rescreening the YAC library with sequence-tagged site assays developed from end-clones. Several new restriction fragment length polymorphisms and simple sequence repeat polymorphism markers have been identified from the YAC clones. In particular, two highly informative $(CA)_n$ dinucleotide repeat markers, sTCL-1 from proximal chromosome 10p (16 alleles, PIC = 0.68) and sJRH-1 from the RBP3 locus (18 alleles, PIC = 0.88), provide useful reagents for a polymerase chain reaction-based predictive genetic test that can be performed rapidly from small amounts of DNA. (Henry Ford Hosp Med J 1992;40:210-14)*

Efforts to identify the genetic defect(s) responsible for the multiple endocrine neoplasia type 2 (MEN 2) syndromes based on their known chromosomal map position rely on the interplay between linkage analysis and physical mapping. This "positional cloning" approach begins with genetic linkage analysis in large disease pedigrees to identify the smallest interval containing the disease locus defined by flanking markers. Subsequently, physical mapping strategies such as hybrid panel mapping and contig construction using large insert cloning systems (e.g., cosmids and yeast artificial chromosomes [YACs]) may be employed to further characterize the region of interest. In addition, detectable germline or somatic chromosomal rearrangements may be sought which could potentially provide important clues to the location of the defect(s).

Genetic linkage studies from several laboratories have assigned the multiple endocrine neoplasia type 2A disease gene (MEN2A) to the interval between the flanking markers RBP3 in 10q11.2 and D10S34 in 10p11.2 (1-7). Linkage of the disease locus to chromosome 10 pericentromeric markers has also been shown in families segregating the defect(s) responsible for the MEN 2 syndrome with the mucosal neuroma phenotype (MEN 2B) (8,9) and familial medullary thyroid carcinoma (9-11). Currently, at least five markers have been described which map within this interval and show no recombination with respect to the MEN2A locus, including D10S97 (12), D10S102 (7,13), D10S94 (14), the ret proto-oncogene (7,15), and D10Z1 (5,6, 16). Further progress using meiotic mapping is limited by the reduced recombination rate, especially in males, which has been reported (5,17) in this region and the availability of critical recombinants within the interval of interest.

We have employed YAC walking and physical mapping to further characterize the pericentromeric region of chromosome 10 containing MEN2A. The requirements for success using this approach are the availability of multiple markers within the region of interest, which can be used as entry points for bidirectional chromosomal walking, and efficient isolation of terminal sequences from YAC clones for library rescreening. In this report we summarize the results of the YAC mapping effort, including the strategies used for identification of YAC end-clones, detection of chimeric YAC clones, and screening for highly informative markers within the cloned DNA.

## Methods

### Development of sequence-tagged sites

Oligonucleotide primers were chosen from published or derived sequences from each entry point locus, and a specific polymerase chain reaction (PCR) assay defining the sequence-tagged site (STS) (18) was developed. The primer choice computer program named PRIMER (provided by M. Daly, S. Lincoln, and E. Landers, Whitehead Institute) was used to choose optimal primer pairs based on calculated melting temperatures and low intra- and inter-primer sequence homology. Genetic se-

Submitted for publication: November 15, 1991.
Accepted for publication: January 27, 1992.
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quence data bases (e.g., GenBank) were searched with the primer sequences to test for homology to known human sequences or repetitive elements. Thermal cycling reaction conditions were optimized for each PCR assay. Then, the specificity of each primer pair was tested by PCR amplification from a panel of somatic cell hybrid DNAs (obtained primarily from the NIGMS mutant cell repository, Camden, NJ) and was shown to amplify a product of expected size only from DNA templates containing chromosome 10.

The PCR amplification was performed from 50 ng of total genomic human DNA in a reaction volume of 5 μL including a final concentration of 200 μM for each dNTP, 1 μM for each primer, 50 mM KCl, 10 mM Tris-HCl pH 8.4, 1.5 mM $MgCl_2$ and 0.5 units of AmpliTaq (Perkin-Elmer Cetus, Norwalk, CT). The reaction was carried out for 30 cycles of 94 °C for 1 minute, 50 to 65 °C for 2 minutes, and 72 °C for 2 minutes in a Perkin-Elmer Thermal Cycler.

### PCR-based YAC library screening

The human genomic YAC library at the Center for Genetics in Medicine (Washington University, St. Louis, MO) was screened using a previously described strategy based on PCR amplification from DNA pools (19). First, PCR amplification is performed from 36 DNA pools representing the entire library. Each pool consists of DNA from the 1,920 YAC clones which are contained on five single membranes (384 clones each). In a second round of screening, PCR amplification is performed from each positive pool along with the pooled DNAs from the clones from each of five single membranes. In the final step of library screening, the radiolabeled PCR product is hybridized to the positive single membranes to identify the desired YAC clones.

### Pulsed-field restriction mapping of YAC clones

High molecular weight genomic yeast/YAC DNA was prepared (20) and digested with rare-cutting restriction enzymes according to conditions specified by the manufacturer. The DNA fragments were separated in a 1% agarose gel in 0.5X TBE by pulsed-field gel electrophoresis, depurinated in 0.1 N HCl and then transferred to nylon filters (Zeta-bind, AMF/Cuno, Meriden, CT) in 1.5 M NaCl and 0.25 N NaOH. Left and right terminal fragments were identified by hybridization with probes specific for the right and left vector arms of pYAC4 (21). In addition, double digest and partial digestion experiments followed by hybridization with radiolabeled YAC-derived DNA probes were used to order the internal fragments.

### Construction of subclone libraries and screening for new restriction fragment length polymorphism probes and dinucleotide repeats

Subclone libraries were prepared by partial digestion of yeast/YAC DNA with MboI or Sau3A to generate fragments 13 to 22 kb in size, followed by ligation into the BamHI site of λEMBL3A or λDASH II (Stratagene, La Jolla, CA). Libraries of approximately five yeast genome-equivalents in size were plated and nylon filter plaque lifts were prepared. Human sequence-containing clones were identified by hybridization with radiolabeled total human DNA (22). Human sequence-containing clones were tested for the detection of restriction fragment length polymorphisms (RFLPs) by Southern hybridization to screening panels containing genomic DNAs from six unrelated individuals restricted with BamHI, BglII, EcoRI, HincII, HindIII, MspI, PstI, TaqI, EcoRV, HinfI, PvuII, and RsaI (23). Lambda subclones containing $(CA)_n$ repeat elements (24) were identified by hybridization with poly(dC-dA)$_n$ × (dG-dT)$_n$ (Pharmacia, Piscataway, NJ) labeled by nick-translation. The $(CA)_n$-positive lambda clones were then further digested to fragments 200 to 500 bp in size and subcloned into M13 or pBluescript (Stratagene, La Jolla, CA) for sequencing and development of primers for PCR amplification of the CA repeat elements.

**Table 1**
**YAC Clones Identified by Screening of a Human Genomic Library with Chromosome 10 Pericentromeric STS Markers**

| Clone | Locus | Size (kb) |
|---|---|---|
| yIR-1* | RBP3 | 200 |
| yIR-2 | RBP3 | 750 |
| yIR-3 | RBP3 | 425 |
| yIR-4 | RBP3 | 240 |
| yZ-1 | D10Z1 | 135 |
| yZ-2† | D10Z1 | 175 |
| yZ-3 | D10Z1 | 60 |
| yZ-4 | D10Z1 | 80,430 |
| yZ-5 | D10Z1 | 115 |
| yFN-1 | FNRB | Unknown |
| yFN-2 | FNRB | 170 |
| yFN-3 | FNRB | 185 |
| yFN-4 | FNRB | 100 |
| yFN-5 | FNRB | 300 |
| yFN-6 | FNRB | 195, 375 |
| yFN-7 | FNRB | 260 |
| yFN-8† | FNRB | 350 |
| yFN-9 | FNRB | 300 |
| yFN10* | FNRB | 280 |
| yFN-11 | FNRB | 300 |

*Clones known to be derived entirely from chromosome 10.
†Clones known to be chimeric.

## Results

### Identification of YAC clones from chromosome 10 pericentromeric loci

YAC screening has been initiated from five chromosome 10 pericentromeric markers tightly linked to MEN2A, including RBP3, D10S94, RET, D10Z1, and FNRB. Currently, a total of four clones have been identified for the RBP3 locus, five clones for the chromosome 10-specific alphoid repeat marker D10Z1, 11 clones for the FNRB locus, and six clones for the D10S94 locus (Table 1) (YACs isolated from the D10S94 and RET loci are in the preliminary stages of characterization and will not be discussed in detail).

The PCR assay used to screen for YAC clones from the RBP3 locus was developed from the cDNA sequence of the interstitial retinol-binding protein gene (IRBP) (25). Two of the YAC

**Table 2**
**New Genetic Markers Detecting RFLPs From the Pericentromeric Region of Chromosome 10**

| Locus | Probe | Enzyme | Alleles | Heterozygosity | PIC* | Reference |
|---|---|---|---|---|---|---|
| FNRB | pTCL-10 | BglII | 3 | 0.55 | 0.46 | (28) |
| FNRB | LTCL-2 | MspI | 2 | 0.43 | 0.34 | (28) |
| RBP3 | JH-1 | BglII | 2 | 0.41 | 0.32 | (9,26) |
| RBP3 | JH-2 | TaqI | 2 | 0.48 | 0.37 | (9) |
| RBP3 | JH-3 | HindIII | 2 | 0.46 | 0.36 | (9) |
| RBP3 | JH-4 | TaqI | 3 | 0.34 | 0.28 | (9,26) |

**Dinucleotide Repeat Polymorphisms From the Pericentromeric Region of Chromosome 10**

| SSRP† | Dinucleotide Repeat | Number of Alleles | PIC* | Reference |
|---|---|---|---|---|
| sTCL-1 | $(CA)_n$ | 16 | 0.68 | (36) |
| sJRH-1 | $(CA)_n$ | 18 | 0.88 | (36) |

*PIC denotes polymorphism information content.
†SSRP denotes simple sequence repeat polymorphism.

clones identified from the RBP3 locus have been characterized in some detail. The YAC clone yIR-1 is a 200 kb continuous segment of chromosome 10 containing the IRBP gene (26). Four new RFLP markers (JH-1, JH-2, JH-3, and JH-4) have been identified from this clone which greatly increase the informativeness of this locus when haplotyped with the previously described RFLPs at RBP3 (Table 2). In addition, a highly informative dinucleotide repeat element, sJRH-1 (Table 2), has been identified from the 750 kb clone yIR-2 and shown to be tightly linked to the MEN 2A disease locus. The 750 kb clone is known to contain the smaller 200 kb clone.

Two separate PCR assays were developed from different parts of the 3.6 kb FNRB cDNA sequence (27). The first set of primers was chosen from the middle of the cDNA sequence and the second set of primers was chosen about 300 bp after the signal sequence but before the cysteine-rich repeat region. A total of 11 clones were identified by screening of the YAC library with these two assays (Table 1). An approximately 280 kb YAC, yFN-10, is derived entirely from chromosome 10 and contains a putative CpG island identified by the cluster of rare-cutting restriction sites in close proximity to the FNRB gene (Figure) (28). An additional clone, yFN-8, has been characterized and found to be a chimeric clone containing a portion of chromosome 10 and a portion of chromosome 4 (28). Two new RFLP probes (pTCL-10, LTCL-2), as well as a highly informative dinucleotide repeat polymorphism (sTCL-1), have been identified from the FNRB YAC clones (Table 2) which enhance the informativeness of this locus for linkage mapping and predictive genetic testing.

Finally, a PCR assay specific for chromosome 10 centromere alpha satellite sequences was developed and used to screen the YAC library (29), and five clones were identified (Table 1).

### Rapid detection of chimeric YAC clones using IRS-PCR

In order to quickly confirm the chromosomal origin of YAC clones and to detect probable chimeric clones (derived from two different chromosomes), we have employed interspersed repetitive sequence polymerase chain reaction (IRS-PCR) (30-32). This technique involves the generation of the specific set of DNA fragments amplified from each YAC clone using primers directed at interspersed repetitive sequences, such as the Alu-517 primer (30) or the PDJ34 primer (33), and hybridization of the products generated to a "slot blot" containing PCR products generated with the same primer from a panel of somatic cell hybrid lines.

First, the Alu primer is used to amplify the specific population of fragments or "fingerprint" of Alu-PCR products from each member of a panel of somatic cell hybrid lines representing virtually all 24 human chromosomes. The majority of these hybrids contain only one human chromosome or a few human chromosomes. The PCR products generated from each hybrid line are then spotted onto a nylon filter (slot blot). Second, the Alu primer is used to amplify the specific DNA fragments from the YAC clone, and the resulting products are then radiolabeled by the random hexamer priming method (34) and used as a hybridization probe against the slot blot of hybrid line PCR products. This method is very sensitive because of the amplified nature of the products, requiring exposure times on the order of hours. A strong hybridization signal is usually obtained from each line containing a chromosome of origin for the YAC clone, although a faint background signal is sometimes seen for other lines. In addition to confirming derivation of at least a portion of the YAC clone from the appropriate chromosome, this method provides a rapid way to identify probable chimeric clones based on strong hybridization signals representing two different chromosomes. This initial characterization can be performed rapidly in a single PCR reaction from yeast mini-prep DNA or potentially even from a crude lysate of yeast cells.

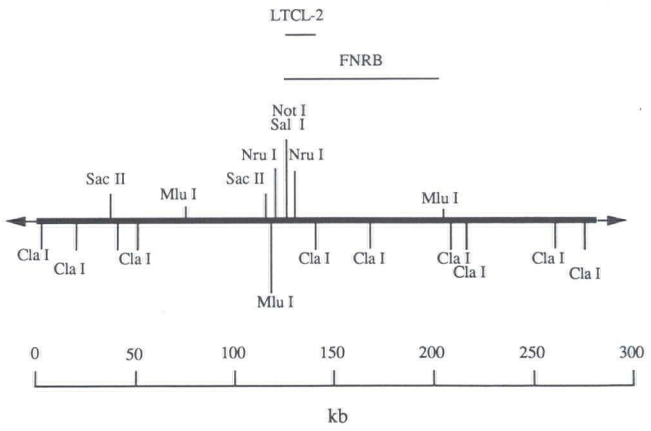
### Strategies used for identification of terminal sequences from YAC clones

YAC end-clones may be identified by standard subcloning techniques followed by hybridization with probes specific for either the right or left vector arm of pYAC4. This strategy is vir-

tually always successful, but is relatively labor intensive and therefore is not well-suited to the simultaneous characterization of multiple clones of interest. Lambda subclone libraries constructed from genomic yeast/YAC DNA are screened with sequences derived from pBR322 which hybridize specifically to either the left or right vector arm. Primers have been synthesized for the convenient generation of these probes by PCR amplification from 1 ng or less of pBR322 DNA. The products are radiolabeled during the thermal cycling reactions. The chromosomal origin of the lambda end-clones may then be determined by hybridization to Southern blots containing DNAs from a somatic cell hybrid panel, or by genetic linkage analysis if the clone identifies an RFLP.

A variety of PCR-based strategies for the rapid isolation of YAC end-clone sequences have been described. Specific amplification of the junction of the vector arm and the terminal portion of the human insert using a vector arm-derived primer and a primer directed to Alu repetitive elements is termed Alu-vector PCR (33). This method depends on the presence of an Alu repeat element in the human insert in the proper orientation and within a distance which may be amplified by PCR. An additional rapid method for isolating the terminal (end-clone) sequences from YAC clones is provided by the recently described "vectorette" technique (35). This strategy involves digestion of the YAC clone with relatively frequently-cutting restriction enzymes (chosen not to cleave the vector between the cloning site and the sequences for available vector arm primers), ligating a specially designed linker with a noncomplementary central region termed a "vectorette" onto the population of restriction fragments, and then specifically amplifying the terminal sequences using a vector-derived and a linker-derived primer. Because the vectorette has an internal region of noncomplementarity, only those fragments undergoing an initial round of extension from the vector arm primer will have the needed sequences present to extend from the vectorette primer. This fact provides specific amplification of only the end-clone sequences from the complex background. The products generated by Alu-vector PCR or the vectorette method may then be sequenced by standard strategies for direct sequencing of PCR products (or after cloning into a plasmid vector).

Currently, 20 (91%) of the 22 ends from the 11 YAC clones which have been most extensively characterized have been isolated using one or more of the above methods. Nine (41%) of the ends were isolated by lambda subcloning and hybridization with probes specific for the right or left YAC vector arms. Mapping information for these clones was obtained either by hybridization to somatic cell hybrid blots or by linkage analysis. Seven (32%) additional ends identified by lambda subcloning and plaque hybridization were further subcloned into a plasmid vector for sequencing of 150 to 250 bp of the human insert near the vector-insert junction. New STS assays are developed from this sequence information for mapping by PCR amplification from a panel of somatic cell hybrid DNAs. Finally, specific PCR products from four (18%) of the ends have been identified by either Alu-vector or alphoid-vector amplification of the vector-insert junction. Five of the ends were isolated by both subcloning and PCR-based methods.




*Figure—Rare-cutting restriction map of yFN-10 YAC clone with the map positions of the RFLP probe LTCL-2 and the FNRB gene indicated (28).*

## Conclusion

Our group has approached characterization of the pericentromeric region of chromosome 10 containing the MEN 2 gene(s) using combined genetic linkage mapping and physical mapping strategies. YAC walks have been initiated from five loci linked to the MEN2A locus, including RBP3, D10S94, RET, D10Z1, and FNRB. New highly informative markers, including two dinucleotide repeat polymorphisms, have been developed from the YAC clones identified from the RBP3 and FNRB loci. These markers have increased the informativeness of the flanking markers and should provide a higher likelihood of identifying critical recombinants in the disease gene region as well as a refined genetic map. In addition, the highly informative dinucleotide repeat polymorphisms may be applied to a rapid PCR-based predictive genetic test for the MEN 2 syndromes.

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