Grant Title: Chromosome Region-Specific libraries for Human Genome Analysis
Grant No.: DE-FG02-91ER11139.A000
P.I.: Fa-Ten Kao, Ph.D.

DOE/ER/61139--1

DE92 001741

PROGRESS REPORT (March 1, 1991 to August 31, 1991)

We have made significant progress since the start of the current grant in March 1, 1991. The following summarizes our progress to date and outlines our proposed research for the coming year.

(1) To further develop microdissection and PCR-assisted microcloning by the linker-adaptor method.

Previously, we have successfully constructed a very large microdissection library of human chromosome 21 using the linker-adaptor method (ref. Appendix Fig. 1 and Publications 1,2). We have now further improved the efficiency and reliability of this technology and have used it to construct additional libraries, including the libraries from 12cen-12q14 and 21pter-21q11.2. Characterization of these libraries have proved that they are all of excellent quality. Furthermore, trypsin-banded and Giemsa-stained human chromosome preparations fixed in 100% methanol were used for microdissection and no damage was found in the dissected chromosome sequences amplified and cloned into pUC19. Only 20 dissected chromosome fragments were needed from each region for PCR amplification. Both MboI and Sau3A linker adaptors were used and proved successful in yielding 30,000-40,000 microclones which is about several genome equivalents of the dissected region. Characterization of the 12cen-q14 library showed that about 40% of the microclones contained repetitive sequences, an indication of a good quality library. Over 90% of the microclones were identified to derive from chromosome 12 and regionally mapped to the dissected region. Thus, we feel confident that we can apply this technology to any human chromosomal regions of interest to construct large region-specific libraries with high quality for human genome analysis.

In addition, we have continued to improve conditions for eliminating DNA contamination during microdissection and PCR microcloning procedures. Exposure of dissecting needles and micropipets to UV prior to use has proved highly effective. Careful handling of reagents in all steps is also crucial.

In separate library construction experiments, we have compared the insert sizes in the resulting microclones using either MboI or Sau3A cleavage of the dissected sequences. In general, Sau3A yielded microclones with smaller inserts (200-250 bp), whereas MboI yielded larger inserts (400-450 bp). This difference may be due to the cleavage of the methylated G at the 6th position (G^m ATC) by Sau3A, but not by MboI, thus yielding larger inserts by MboI.

For demonstrating the usefulness of microclones in genomic expansion, we tested microclones containing small inserts (a few hundred bp) by screening human YAC libraries. Microclones from both chromosome 21 and 12 libraries were used as labeled probes in screening of the Washington University YAC library and identified several corresponding YAC clones with inserts ranging from 150 to 450 kb (ref. Appendix Fig. 2 and Publication 3). Thus, a large collection of YAC clones from a dissected region can be quickly assembled and used for contig

MASTER

construction of the region.

Due to the short insert size, it is relatively easy to determine the sequence in the microclones for use in STS production. We have developed a convenient procedure for sequencing double-stranded, supercoiled plasmid DNA directly from pUC19 microclones, thus eliminating the need for subcloning of the inserts into M13 for single-strand sequencing (ref. Appendix Fig. 3). Using the two specific primers flanking the insert in microclones, sequencing can be carried in both directions to generate a total readable sequence over 800 nucleotides in a single sequencing reaction. Thus, if necessary, microclones can be quickly and conveniently sequenced and converted to STS as genomic landmarks for the dissected region.

(2) To develop the PCR microcloning by the terminal deoxynucleotidyl transferase (TDT) method.

The development of PCR microcloning by the TDT method is particularly appealing because this method can make microcloning extremely easy and can be carried out in test tubes instead of microdrops under the microscope. Thus after microdissection, the chromosome fragments can be transferred to Eppendorf tubes for all the subsequent manipulation, including proteinase K digestion, phenol/chloroform extraction, BglI/SstI cleavage, oligo(dG)-tailing by TDT, PCR by oligo(dC) primer, etc. No microscopic manipulation with microdrops is needed. Recently, we have compared the efficacy of PCR amplification of the dissected sequences tailed with either oligo(dG) or oligo(dT) and amplified by oligo(dC) or oligo(dA) primers respectively. The results showed that both procedures were equally effective and yielded excellent PCR products with very little background. Thus, we will apply this method to the microdissected fragments from human chromosome 2, region 2qter-q37, to construct a library for detailed characterization and evaluation of the quality of the library.

(3) To apply the developed microtechnology to human chromosomes 2 and 5 to construct region-specific libraries for use by LLNL and LANL for their physical mapping and contig construction.

As a valuable resource for the human genome program, we will apply the developed microdissection and PCR microcloning to construct region-specific libraries of various human chromosomes for physical mapping, STS production and contig construction. In particular, the large numbers of microclones from specific chromosomal regions should well represent the dissected region and samples of microclones from each region can be used to screen YAC libraries to isolate corresponding clones with very large inserts. We have already demonstrated the feasibility of expanding the genome coverage by a thousand-fold by one round of screening of the YAC library. If we dissect a 10 mb region, it should be relatively simple to use microclones from the library to isolate enough YAC clones to saturate the entire region with contigs.

We have already started on chromosome 2. We microdissected 20 fragments from the terminal portion of the short arm of chromosome 2, region 2pter-p23. After dissection, the fragments were treated with proteinase K and phenol, digested with MboI, ligated to MboI linker-adaptor, amplified with PCR. The PCR products showed nice yield with no background in the control drop. This is the first requirement for the production of a good quality library. We are now proceeding to perform transformation and to construct the library and to analyze samples of the microclones in the library for the following:

- (a) To determine the percentage of repetitive and unique sequence microclones in the library.
- (b) To determine the average insert size in the microclones.
- (c) To isolate unique sequence microclones as labeled probes to hybridize to DNAs from human and our panel of cell hybrids to determine their human origin and chromosome 2 specific.

The collection of unique sequence microclones will be made available to Dr. Anthony Carrano of LLNL for use in the screening of cosmid or YAC libraries for isolating clones with large inserts for contig construction of this dissected region.

(4) Plan for the coming year

For the coming year, we will continue construction of the microdissection library for chromosome 2, region 2pter-p24, and to characterize samples of microclones from the library as outlined above. Unique sequence microclones from this region will be used by LLNL for physical mapping purposes.

For the coming year, we will also dissect the terminal portion of the long arm of chromosome 2, region 2qter-q37. We will use the TDT method for constructing a microdissection library. We will then carefully characterize microclones from this library in order to critically evaluate the quality and characteristics associated with this method. Particularly, we will compare this library with the linker-adaptor library to determine the usefulness of each method. If the TDT library is as valuable as the linker-adaptor library, it has the advantage of easy construction because no nanoliter volume manipulation under the microscope is necessary.

During the coming year, we will start to dissect the terminal region of the short arm of chromosome 5, region 5pter-p15. Either the linker-adaptor or the TDT method will be used. The resulting library will be characterized and evaluated and if promising, unique sequence microclones will be isolated and made available for Dr. Robert Moyzis of LANL for their use in physical mapping and contigs construction of this chromosome.

In addition, we will continue to explore the usefulness of region-specific microclones in isolating large numbers of cDNA clones from a defined chromosome region. Initially, we will use unique sequence microclones from chromosome 21 to establish an efficient protocol for screening cDNA libraries. As soon the pilot studies prove promising, we will use this method to isolate as many cDNA clones as possible from the dissected regions on chromosome 2 and 5 for the various genomic studies using expressed gene sequences.

In this connection, we have used a human liver λ gt11 cDNA library for screening (ref. Appendix Fig. 4 and Publications 1,4). Filters containing 5×10^5 phage plaques each were prepared. Either one or two filters were usually used in each screening. We used mixed probes in various numbers in order to determine the most effective number to use. The insert in each unique sequence microclone was individually amplified by PCR and purified by GeneClean. Various numbers of purified inserts were mixed to prepared labeled probes. We found that a mixture of 20 probes was optimal for yielding a hybridizing signal strong enough to identify positive plaques. It has the additional advantage in that generally only 0 or 1 cDNA clone will be present in a group of 20 probes tested, thus eliminating the effort to identify individual cDNA

clone if more than one are present in each screening. From the first 200 unique sequence microclones from the chromosome 21 library, we identified at least 5 different cDNA clones. These cDNA clones have been purified by secondary and tertiary screenings, and the corresponding microclones have also been identified from the mixed 20 microclones used in each screening.

In a second series of screening, we used the normalized cDNA library from Dr. Sherman Weissman's lab (Patanjali et al. PNAS 88, 1943-1947, 1991). This is a human thymus λ gt10 cDNA library normalized for more equal representation of the various poly(A)⁺ RNA species in the library. After screening with the same 200 unique sequence microclones, we have identified not only the 5 cDNA clones isolated previously from the liver cDNA library, but also identified 2 additional cDNA clones, for a total of 7 different cDNA clones. This pilot study showed that it is highly efficient to use mixed probes for screening cDNA libraries to isolate as many cDNA clones as possible from a defined region of the chromosome. We estimated that on the average, about 20 different cDNA species could exist in a chromosomal region of 10 mb. Thus, it would be feasible to screen about 400 unique sequence microclones to isolate 20 cDNA clones from the dissected 10 mb region. This procedure will isolate all the cDNA clones present in a particular cDNA library used for screening. The only limitation is that we cannot isolate those cDNA species that are not present in the library. However, we can use several cDNA libraries constructed from different tissues and different developmental stages for a more complete coverage of the expressed sequences in the dissected region. This pilot study is continuing by adding other normalized cDNA libraries such as the brain.

The cDNA clones isolated from a particular chromosome region can be sequenced, even partially, and used to search the GenBank for homologies with existing gene sequences or domains and motifs to define possible functions, or to identify new genes. We will undertake some of these studies using the cDNA clones isolated so far to complete this pilot study designed for exploitation of the usefulness of microclones in isolating chromosome region-specific expressed gene sequences.

We have acquired a Zeiss Axiovert 10 microscope through the current grant budget. This microscope is equipped to accommodate the motor-driven, electronically controlled Zeiss micromanipulator already available in our lab. Using this micromanipulator, microdissection can be carried out mechanically and more accurately. The dissecting glass needle is mounted to the manipulator and can be moved in all three directions at a maximum speed of 1 mm per sec. With the addition of this equipment, we can perform microdissection at a higher efficiency and increased precision.

(5) Summary

We have made important progress since the beginning of the current grant year. We have further developed the microdissection and PCR-assisted microcloning techniques using the linker-adaptor method. We have critically evaluated the microdissection libraries constructed by this microtechnology and proved that they are of high quality. We further demonstrated that these microdissection clones are useful in identifying corresponding YAC clones for a thousand-fold expansion of the genomic coverage and for contig construction. We are also improving the technique of cloning the dissected fragments in test tube by the TDT method. We are applying both of these PCR cloning technique to human chromosomes 2 and 5 to construct region-specific

libraries for physical mapping purposes of LLNL and LANL. Finally, we are exploring efficient procedures to use unique sequence microclones to isolate cDNA clones from defined chromosomal regions as valuable resources for identifying expressed gene sequences in the human genome. We believe that we are making important progress under the auspices of this DOE human genome program grant and we will continue to make significant contributions in the coming year.

Publications:

- (1) Kao, F.T. and Yu, J.W (1991) Chromosome microdissection and cloning in human genome and genetic disease analysis. Proc. Natl Acad. Sci. USA 88, 1844-1848.
- (2) Kao, F.T. and Yu, J.W. (1991) Chromosome region-specific libraries for human genome analysis. Abstract presented in the DOE Human Genome Workshop for Grantees and Contractors, Feb. 17-19, 1991, Santa Fe, NM.
- (3) Mendez, M.J., Yu, J., Kao, F.T., Klapholz, S., Brownstein, B.H. and Gemmill, R.M. (1991) Rapid screening of a YAC library by pulsed field gel Southern blot analysis of pooled YAC clones. Abstract presented in the Cold Spring Harbor Meeting on "Genome Mapping and Sequencing", May 8-12, 1991.
- (4) Yu, J., Hartz, J., Xu, Y. and Kao, F. (1991) Chromosome microdissection and PCR microcloning in human genetic disease analysis. Abstract presented in the 8th International Congress of Human Genetics, Oct. 6-11, 1991, Washington, D.C.

Appendix Figures

1. A diagram showing microdissection and PCR microcloning procedure.
2. Screening of YAC library using pools of microclones.
3. Direct sequencing from the double-stranded plasmid DNA of microclone 21F-13.
4. Screening of cDNA library using pools of unique sequence microclones.

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

MICRODISSECTION

Screening of the human YAC library from Washington University using microclones. The library contains about 56,000 YAC clones (5x human genome equivalent) with a mean insert size of 250 kb. Four unique sequence microclones from chromosome 21 library were pooled as mixed probes. The gel shown on the left contained 50 lanes with 384 YAC clones per lane separated by PFGE. DNA was transferred to filter for Southern hybridization. After screening 2/3 of the library, five positive YAC clones were identified with inserts ranged 50-360 kb. Three of the positive YAC clones were shown in the autoradiogram on the right. (In collaboration with Drs. Robert Gemmill and D. Patterson)

BamHI 1 11 21 31 41
 GGATC TACTCTACAA CACTTTGGCA TCCTCCGAAA GAGGTTGCTC TTTCAGTTCT
 51 61 71 81 91 101
 GTCCAAAGAC TTCACAGACA TGGATAAAAA TTCGCCAGCC AGAGGCCTGG TGTGCTGCAG
 111 121 131 141 BamHI
 GGA ACTGTTT CGGTCTGCAA CGGGAACACG ATATTGC GATCC

Nucleotide sequence of microclone 21F-13 isolated from a
 microdissection library of human chromosome 21. The insert
 contains 147 bp cloned into the BamHI site of plasmid pUC19.
 Sequencing was done using double-stranded, supercoiled plasmid
 DNA.

cDNA library screening using PCR amplified inserts pooled from 10 unique sequence microclones to prepare labeled probes. 100,000 phage plaques from a human liver cDNA library were screened for each group of pooled microclones. In the primary screening (shown in A and B), each filter contained 50,000 phage plaques. Positive cDNA clone 21E-C1 was isolated and plaque purified by secondary screening (shown in C containing 400 phage plaques) and by tertiary screening (shown in D containing 14 plaques). This cDNA clone, 21E-C1, corresponds to microclone 21E-181, has an insert of 1.5 kb, and was regionally mapped to 21q21.3 using hybrid mapping panel.

END

**DATE
FILMED**

11/13/91