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Technical Progress Report
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An Improved Method for Producing Radiation Hybrids Applied to Human Chromosome 19

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Molecular and Cytogenetic Characterization of the Radiation Hybrids

At the initiation of the grant we had just produced radiation hybrids from a monochromosomal microcell hybrid containing human chromosome 19 as its only human component (PK87-19). Radiation hybrids were produced using doses of radiation ranging from 1000-8000 rads. Lethally irradiated cells were then fused to hamster recipients (CHTG49) and selected for growth in histidinol. Approximately 240 clones were isolated and 75 clones were expanded for the isolation of DNA.

Southern Analysis and PCR

We have spent a considerable amount of time characterizing these hybrids to determine what region of the chromosome they contain. This has been accomplished by analysis using Southern blots or PCR. We prefer to detect as many markers as possible using PCR. This is a more rapid method for screening a large number of hybrids which uses 1% of the DNA required for a Southern hybridization. We have analyzed the hybrids using PCR for specific sequences as well as Alu-PCR using the primers described by Nelson et al (1989). The hybrids were also analyzed by Alu-PCR by Kathy Yokobata at Lawrence Livermore Laboratory using the A1S primer (Cotter et al., 1991). Due to the low number of long interspersed repeats on chromosome 19 we did not analyze the hybrids with primers for this type of repeat. DNA sequences for markers and genes located on chromosome 19 were obtained from the Genbank or EMBL database as well as searches of the current literature. The DNA sequences were analyzed using the Oligo primer program and appropriate primer sequences were chosen. Oligonucleotide primers were synthesized in the Clinical Molecular Biology Lab on an Applied Biosystems Synthesizer and used to amplify DNA from the hybrids. Primer only, UV135 DNA, CHTG49 DNA, human placental DNA and PK87-9 DNA were included as controls on each PCR run. PCR products were fractionated on NuSieve agarose gels. Depending on the sequence amplified, there may or may not be a hamster band present. In most cases the hamster band could be distinguished from the human specific band based on a difference in size. This band then served as an internal control for the PCR amplification. In several instances the hamster band co-migrated with the human band. In those cases, we tried to choose a third primer further upstream or downstream in order to generate different size fragments in human DNA compared to hamster DNA. An example of the Alu PCR is shown in Figure 1. A representative example of PCR using specific marker primers is shown in Figure 2.

We used Southern blot analysis to detect DNA markers for which no sequence information was available. These probes were purchased from ATCC or obtained courtesy of other investigators. 5-10ug of hybrid DNA was digested with Bam, Hind III or EcoRI and fractionated on 0.8% agarose gels. The DNA was transferred to Zetaprobe membrane and hybridized with probes labelled with 32^{P} dCTP using the random primer method. Hamster DNA, human DNA and PK87-19 DNA were run as controls. Bands present in PK87-19 and human DNA but absent in hamster DNA were scored in the hybrids. The

markers tested and their location on the chromosome is shown in Figure 3. The current status of our characterization is shown in Table 2. This table lists the hybrids and markers tested to date. From the data it appears that we have generated overlapping hybrids extending out from the short arm of the chromosome. From the marker analysis it appears that the retroviral vector has been inserted into the short arm of chromosome 19.

In Situ Hybridization Studies

In situ hybridization is the best technique to visualize the chromosome fragment in the radiation hybrid cell and to determine the number of human fragments contained in each cell line. We chose nonisotopic in situ hybridization because we wanted to avoid the hazards and administrative problems associated with using radioisotopes. Initially we started using a colorimetric method of in situ hybridization because of the lack of a fluorescent microscope.

Briefly, with the nonfluorescent, nonisotopic method, the slide was treated post-hybridization with streptavidin-alkaline phosphatase conjugate (SA-AP). This step was followed by incubation with the chromogen nitroblue tetrazolium (NBT) and the enzyme substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in subdued light for signal detection.

We have also employed a similar system using streptavidin-horseradish peroxidase (Detek-I-hrp Signal Generating System, Enzo Diagnostics, Inc., N.Y., N.Y.) enhanced with DAB and silver (Amersham Corporation, Arlington Heights, Ill.) using the protocol supplied by the manufacturer.

These methods work well for whole chromosome hybrids but are not as sensitive as fluorescent in situ hybridization (FISH) for the detection of chromosome fragments. Since the Cytogenetics Laboratory was able to purchase a fluorescent scope with funds from the department in 1991, we have started using fluorescent in situ hybridization for the routine analysis of the radiation hybrids.

For fluorescent in situ hybridization, standard procedures were employed using total human genomic DNA labeled with biotin. The first day of the procedure includes pretreatment of the slides in RNAse and 2XSSC, dehydration in a cold graded ethanol series, denaturation of slides in a formamide solution at 70-71°C, the application of heat-denatured probes, and overnight hybridization in a humid 37°C chamber. The second day, after coverslips are removed, slides are post-washed in formamide solutions at either 37°C. After the slides are washed in phosphate buffered detergent, probe detection and amplification are carried out using a series of blocking reagents, FITC-labeled avidin, and anti-avidin antibodies, which are applied in subdued light. The final counter-staining steps include application of propidium iodide, antifade and coverslips.

Slides are viewed at either 40X, 60X or 100X magnification and photographed with a Zeiss epifluorescence photomicroscope using an FITC exciter filter set and Ektachrome ASA 200 color film. Direct positive print film (Polaroid 669, Polaroid) was used to make prints.

Summary of Fluorescent In Situ Hybridization Results

We are currently hybridizing biotin-labelled total human genomic DNA to metaphase chromosome spreads prepared from the hybrid cell lines. Table 2 lists the hybrids for which we have prepared metaphase chromosome spreads via conventional cytogenetic

harvesting techniques and those hybrids which have been tested by FISH. Figure 4 shows two examples of the results with FISH on the radiation hybrids.

We found that each hybrid cell line had an optimal time for harvest, however, by splitting the cells 1:4, 18 hours before harvest resulted in an adequate number of cells in metaphases to produce slides for FISH. Of the 9 hybrid cell lines analyzed to date by FISH, all have had only a single chromosome fragment. Some of the cell lines had 2 fragments in a few spreads and will need to be rechecked. This is a higher fraction then we expected. Additional fragments can be randomly carried along and we expected to see cell lines with more than 1 piece. This has been our experience with radiation hybrids for chromosome 9. Chromosome 19 is a much smaller chromosome and therefore the total number of breaks on the chromosome would be less. Also we have only analyzed 9 hybrids and over the next year we hope to analyze the rest of the hybrids which will provide a better estimate of the fraction of hybrids that contain 1 fragment vs. more than 1 fragment.

Other details of cell culture, harvest and slide processing will be reported elsewhere (Mark, Santoro and Jackson, Manuscript to be submitted to Somatic and Cell Genetics).

Introduction of Additional Markers into Human Chromosome 19

One of the original goals described in the proposal was the introduction of additional markers into chromosome 19 at different sites on the chromosome. Towards that goal we have been sequencing the region of SP-1 between the hisD gene and the 3 prime LTR of the vector in order to be able to more easily construct additional retroviral vectors.

In collaboration with Dr. Jude Samulski we have introduced a neomycin resistance marker into the long arm of human chromosome 19. It had been observed that adeno-associated virus-2 (AAV-2) a defective parvovirus exhibits site specific integration into human chromosome 19. Dr. Samulski has localized the viral insertion site to the region of 19q13.4-qter using in situ hybridization ( ). The gene encoding neomycin resistance was inserted into the AAV genome and this construct used to infect the hybrid cell line PK87-19. Several clones have now been isolated. Further analysis will be done to confirm the presence of the neo^r marker in 19q.

References

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