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Applications of Strand-Specific *in situ* Hybridization

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Abstract

This is the final report of a three-year, Laboratory-Directed Research and Development (LDRD) project at the Los Alamos National Laboratory (LANL). Fluorescence *in situ* hybridization (FISH) is used to determine the location of specific DNA sequences on chromosomes. It is an effective tool in genomic mapping and is finding increasing use in medical diagnosis. A "strand-specific" version of FISH has been developed in the Life Sciences Division of LANL. The new procedure, named CO-FISH, reveals not only location but also the 5'-to-3' direction of a target sequence, such as the sense strand of a gene. This project was designed to investigate applications of the new technique. Strand-specific FISH was found to be useful and informative for genomic mapping of repetitive DNA sequences. The method provide a valuable new tool for investigating the mechanisms of aneuploidy inducing agents and the cytogenetic phenomena called lateral asymmetry. Finally, using strand-specific FISH, we were able to detect certain types of chromosome aberrations (isochromosomes, inversions and Robertsonian translocations) that can be difficult to observe with standard techniques.

Background and Research Objectives

A conspicuous limitation of the standard FISH procedure is its inability to determine the 5'-to-3' direction of single-stranded DNA target sequences with respect to chromosomal reference points such as centromeres and telomeres. We have removed this limitation by making *in situ* hybridizations strand-specific [1,2].

The process begins with cultivation of cells for a single round of DNA replication in a medium containing the nucleotide analogue bromodeoxyuridine (BrdU). During the course of semiconservative DNA synthesis, BrdU partially replaces thymidine in the newly replicated DNA strands. Every replicated chromosome is now composed of sister chromatids that are singly substituted with BrdU in opposite strands of the DNA molecule.

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Cell cycle progression is blocked in mitosis with Colcemid, a microtubule inhibitor. Cells are harvested and fixed, and microscope slides are prepared by standard cytological procedures. After staining with the fluorescent DNA-binding dye Hoechst 33258, cells are exposed to long-wave ultraviolet light. UV exposure induces nicks in the BrdU-substituted DNA strand, but not to any appreciable extent in the opposite strand.

Nicked DNA serves as a substrate for exonuclease III (Exo III), an enzyme that excises nucleotides from one strand of double-stranded DNA starting at the sites of nicks. Following UV exposure, Exo III digestion removes nucleotides from the BrdU-substituted DNA strand while leaving the unsubstituted strand largely intact. It is important to note that complementary single-stranded regions are created on sister chromatids.

Single-stranded nucleic acid probes can now be hybridized to the chromosomes without thermal denaturation. For a single-copy sequence, such as the sense strand of a gene, a hybridization signal is found on one chromatid only; base pairing cannot occur with the complementary target sequence on the sister chromatid. When a probe is hybridized to a multiple-copy target sequence, a fluorescent signal on only one chromatid indicates the repeats have a head-to-tail arrangement on the chromosome. In contrast, if the repeats exist in a mixed orientation, a signal will appear across both chromatids. This information is not obtainable through standard FISH methodology. The technique has been named CO-FISH (Chromosomal Orientation FISH).

This project was designed to investigate potential uses of the new technique. CO-FISH was examined for application in genomic mapping, as a tool for cytogenetic research capable of providing unique information, and as a means for detecting hard-to-observe chromosome aberrations. Five publications and a "Research Highlights" article in the Life Sciences Division Annual Report have resulted from this work, and one additional manuscript has been accepted for publication.

Importance to LANL's Science and Technology Base and National R&D Needs

This project provides new tools for scientific research. These same tools eventually may find application in medical diagnosis. As such, this project strengthens LANL's position in biotechnology.

Scientific Approach and Accomplishments

Genomic Mapping of Repetitive DNA Sequences. FISH is commonly used in mapping to determine chromosomal locations of cloned genomic fragments. In addition to location, the directionality of these cloned fragments with respect to chromosomal landmarks like telomeres

and centromeres can also be determined using CO-FISH . This information should facilitate genomic mapping.

Approximately 20 repetitive DNA sequences were probed by CO-FISH [1-8] and mapping the sequences known as sat I (3) and sat III to their corresponding chromosomal locations has been completed.

Mechanisms of Aneuploidy-Inducing Agents. Aneuploidy refers to a gain or loss of one or more whole chromosomes in a cell. The resulting gain or loss of genetic information may alter the cellular phenotype. Aneuploidy is frequently observed in tumor cells, and is thought to be one factor contributing to uncontrolled cellular proliferation that is a characteristic of malignancy. While aneuploidy occurs spontaneously at a low rate, it can be induced at much higher frequencies by certain chemical agents.

In mitosis the two identical chromatids of a mitotic chromosome normally separate from one another and are pulled by microtubules into the newly forming daughter cells. This process ensures that upon cell division both daughter cells receive an equal complement of genetic material. The most commonly accepted mechanism proposed to explain aneuploidy is called "non-disjunction." It postulates that the two sister chromatids fail to separate and both are pulled into one daughter cell. The result would be an extra chromosome in one daughter cell and a chromosomal deficit in the other. Another possible mechanism of chromosome loss centers around the phenomenon of chromosome lagging, which refers to the occasional observation of slow movement of one chromatid during the separation process. This chromatid may not be incorporated within the nucleus of the daughter cell and will eventually be lost from the cell.

The centromeres of the sister chromatids of a mitotic cell can be labeled separately using CO-FISH and followed individually. This provides a means of distinguishing between the two competing explanations for aneuploidy induction. If non-disjunction is correct, the two chromatids should be observed to travel together during the separation process. In contrast, a lagging chromatid would appear as an isolated entity. Our investigation of aneuploidy-inducing agents is continuing.

Investigation of Lateral Asymmetry. When mouse cells are grown in BrdU for one replication cycle and stained with the fluorescent dyes Hoechst 33258 or DAPI, an unequal brightness between sister chromatids is observed in the centromeric regions of mitotic chromosomes [9,10]. Similar observations were made with human cells, but fewer chromosomes were affected [11-15]. Reports of this phenomenon, which was given the name "lateral asymmetry," were first published over 20 years ago along with a plausible mechanistic explanation. It was proposed that the chromosomal segments exhibiting asymmetrical fluorescence were thymidine-rich in one strand of the DNA double helix. One round of DNA

synthesis in BrdU produced chromosomes where one chromatid contained more of the nucleotide analog than the other. Lateral asymmetry, it was reasoned, resulted from fluorescence quenching by BrdU that was greater in the more heavily substituted chromatid. In support of this hypothesis were observations that one strand of mouse satellite DNA contained approximately twice the thymidine content as the other [16], and that fluorescence was indeed quenched in solutions of BrdU-substituted DNA [17]. However, the technology available at the time could not determine which of the chromatids (i.e., the bright one or the dim one) contained the greater amount of BrdU. Thus the proposed mechanism lacked one crucial piece of supporting evidence.

CO-FISH has allowed us to reexamine the phenomenon of lateral asymmetry [6]. In this procedure, the DNA of singly BrdU-substituted chromosomes is made single-stranded by nicking the DNA with UV light at the sites of BrdU incorporation and enzymatically digesting the substituted strand with exonuclease III. The treatment yields chromosomes where sister chromatids contain single complementary DNA strands. A single-stranded DNA probe was prepared having a sequence chosen from the thymidine-rich strand of the mouse major satellite. This probe hybridizes only to the single-stranded chromatid containing its complementary sequence. Thus the probe identifies the BrdU-rich chromatid (i.e., before the enzymatic treatment). Comparing the hybridization pattern to photographs of the same cell taken previously after Hoechst staining provided a test of the proposed mechanism.

Our observations demonstrated a coincidence between the mouse major satellite sequence and chromosomal regions displaying lateral asymmetry. They also unambiguously identified BrdU-rich chromatid segments as the ones that fluoresce less brightly. This new data supports the mechanism originally proposed, i.e. lateral asymmetry is caused by differential fluorescence quenching related to the quantity of incorporated BrdU. Other observations suggest that differential loss of DNA from the heavily substituted chromatid also contributes to asymmetrical brightness.

Detection of Isochromosomes, Inversions and Robertsonian Translocations.

Chromosome aberrations are found in nearly all types of cancer that have been adequately studied [18]. Aberrations also are a common feature associated with birth defects [19]. In many cases chromosome aberrations are thought, or have been demonstrated, to play a causal role in the disease process. The association between certain disease states and particular chromosome aberrations is highly specific. Therefore, the appearance in a patient of one of these aberrations is considered to be diagnostic for the associated disease. As a result, considerable effort has been devoted to improving chromosome aberration detection methods to provide better tools for clinical diagnosis and scientific investigation of disease. Three types of disease-associated aberrations, isochromosomes, inversions, and Robertsonian translocations,

are commonly identified with chromosome banding, a method that is laborious and requires considerable technical skill. We reasoned that CO-FISH might provide a less cumbersome means for detecting these aberrations.

CO-FISH was applied to two cell lines, one carrying an isochromosome formed by duplication of the long arm of chromosome 13 and the other by duplication of the short arm of chromosome 18 [7]. These aberrations, initially detected by chromosome banding, were observed in cells from patients with congenital abnormalities. Using centromeric probes, we were easily able to confirm the presence of these aberrations from the "side-by-side" hybridization signal resulting from the duplication of centromeric repeats in an inverted order.

Robertsonian translocations are created by fusion within the centromeric regions of two "V-shaped" chromosomes to form a single "X-shaped" chromosome. The expected CO-FISH hybridization pattern of a centromeric probe is very similar to that of isochromosomes. This expectation was confirmed by successful observation of Robertsonian translocations in human and mouse cells.

Chromosomal inversions form when a chromosome is broken in two places followed by reinsertion of the chromosomal segment back into the chromosome in an inverted order. It was reasoned that inversions might be detected by CO-FISH using two probes, one hybridizing to a sequence within the inverted region and the other hybridizing outside to serve as a reference. This approach was successfully applied to detecting inversions in chromosomes 1, 8, and 10 [8].

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