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Our research interest is focused on the identification of genes from chromosome 17. The isolation of genes transcribed from chromosome 17 will provide candidates for the proposed sporadic breast cancer genes and genes for other human disorders.The ability to isolate genes in a chromosome specific manner provides simultaneous identification of the expressed sequence and a chromosomal location. Our approach identifies expressed sequences by reciprocal probing of arrayed cDNA libraries and a chromosome specific cosmid library. To date from the cDNAs isolated from human chromosome 17 we have identified two very important genes. One gene, which encode a coactosin like protein (*CLP*) and maps to 17p11.2 has been demonstrated by us and our collaborators to be involved in the Smith-Magenis Syndrome, a neuro-muscular disorder. A second gene which encodes a putative transcription factor has been demonstrated by my laboratory to be a key gene in the mammalian circadian rhythm pathway. This gene which we have named *RIGUI* could initiate molecular studies into hypotheses that the responsiveness of patients to chemotherapy display circadian patterns.

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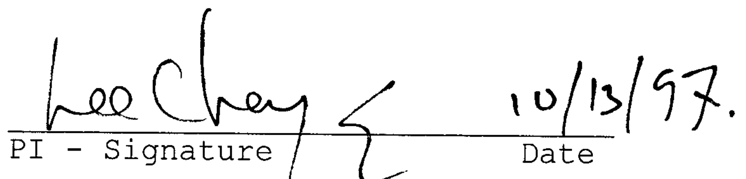

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Introduction

The role of genetics in cancer is now well established with the identification of several genes where the presence of mutation has been associated with cancer formation (1). It has been shown that mutations of tumor suppressor genes, as negative regulators of cell division, contribute to oncogenesis by interference with mechanisms restraining cell multiplication. Thus genes encoding proteins involved in cellular functions including signal transduction, transcription and phosphorylation/dephosphorylation cell cycle pathways are prime candidates for tumor suppressor genes. In familial forms of cancer, a combination of germ-line and somatic mutations on each allele results in chromosome loss or deletion, meiotic recombination, or gene conversion. Similar events uncovering recessive somatic mutations also occur in sporadic forms of cancer. The loss of genetic material inherited from one parent can be detected by loss of heterozygosity (LOH) analysis using genetic markers. LOH studies on tumors and linkage analysis in inherited forms of cancers have resulted in identification of several tumor suppressor genes (2). The gene for hereditary breast cancer, namely BRCA 1 is also associated with hereditary cancer of the ovary (2,3). The gene for BRCA 1 was mapped to chromosome 17q21, a region that is also associated with allele losses (loss of heterozygosity, (LOH)) in sporadic breast and ovarian cancer (4,5,6,7,8,9). After intensive investigation by many research groups, the gene BRCA 1 was identified via positional cloning methods (10). Many mutations in BRCA 1 gene were found in patients with hereditary breast and ovarian cancer (11,12). Surprisingly, these studies also show that mutations in BRCA1 are rare in sporadic breast and ovarian cancers that are thought to be due to susceptibility to the disease at this locus (13,14,15). Together, the LOH studies and the lack of mutation in BRCA 1 have led to the proposal that there is another gene within this region of 17q12-q22 that is associated with sporadic breast and ovarian cancer in women (16). Many studies have demonstrated that LOH in other regions of human chromosome 17 is associated with breast cancer (17,18). These studies indicate that a region telomeric to the P53 gene at 17p12-17p13.3 (about 3 cM) is believed to harbor a separate tumor suppressor gene associated with breast cancer. In the meantime, another study also shows that regions 17q24-25 are associated with another tumor suppressor gene (8,16). The challenge to identify these potential sporadic breast cancer genes on the chromosome 17 is expected to be great. The strategies for cloning a disease-related gene included either functional or positional cloning approaches (19). With the effort of the Human Genome Initiative in cDNA and expressed sequence tag (EST) mapping, a candidate gene approach to finding human disease genes has been predicted to be the future trend. Our research interest is focused on the identification of genes from chromosome 17 based on the reciprocal probing approach (20). The isolation of genes transcribed from chromosome 17 will provide candidates for the proposed sporadic breast cancer genes and genes for other human disorders. To date from the cDNAs isolated from human chromosome 17 we have identified two very important genes. One gene, which encode a coactosin like protein (*CLP*) and maps to 17p11.2 has been demonstrated by us and our collaborators to be involved in the Smith-Magenis Syndrome, a neuro-muscular disorder (21). A second gene which encodes a putative transcription factor has been demonstrated by us to be a key gene in the mammalian circadian rhythm pathway (22). This gene maps to 17p12 which we have named *RIGUI* could initiate molecular studies into the hypothesis that patients responsiveness to chemotherapy display circadian patterns.The next phase of this proposal will be focused on our continued effort to find candidate tumor suppressor genes associated with sporadic breast cancer on human chromosome 17 by combining candidate, functional and positional cloning strategies.

Results

We have reported a method for the isolation of chromosome specific cDNAs using high density arrayed cDNA and chromosome specific cosmid libraries (20). The ability to isolate genes in a chromosome specific manner provides simultaneous identification of the expressed sequence and a chromosomal location. This technology identifies expressed sequences by reciprocal probing of arrayed cDNA libraries and a chromosome specific cosmid library.

The isolated chromosome specific cDNA clones were sequenced through one pass sequencing from the 5' and 3'ends. The corresponding cosmids were used for *fluorescent in situ hybridization* (FISH) mapping to localize their chromosomal position. The sequence information was used to generate sequence tag site (STS) primers for polymerase chain reaction (PCR) mapping on chromosome 17 somatic hybrid cell-lines to further confirm the cDNA and the corresponding cosmid map position.

During our last report we described our goal of arraying 40,000 clones from a placental and an ovarian cDNA libraries. Our analysis of the human ovarian library indicates that the level of gene diversity is not high and to reduce redundancy of clones and to increase the level of gene diversity we have elected work with a human heart cDNA library. In line with this we carried out the arraying of a 20,000 clone human heart cDNA library and our search through this library has yielded many interesting genes. A total of 105 unique cDNAs of chromosome 17 have been identified and were characterized in much detail. This phase of the project is on target with our statement of work for the initial 36 months.

As mentioned in our 1996 report we have identified 72 cDNAs localized in the regions of 17p12-p13, 17q12-22 and 17q24-25, three regions targeted for study based on LOH evidences. The isolation of full-length cDNAs for selected genes that may encode for regulatory proteins is the target goal for the remaining year of this proposal. In the past year we have successfully completed 6 full-length cDNAs. These cDNAs were chosen on the basis that their partial cDNA sequences were found to encode to putative transcription factors, signal transduction molecules and other regulatory functions. One cDNA which we thought could be a transcription factor gene has turned out to be gene that may regulate the pacemaker function in the human and all mammalian circadian rhythms (22).

Identification of Mammalian Circadian Rhythm Gene

Biological rhythms are a fundamental characteristc to living systems. In reponse to daily envirnmental cues, the physiology and behavior of all living organism from bacteria to humans are controlled by circadian rhythms driven by endogenous oscillators. Although the behavioral and physiological properties of the mammalian circadian rhythms are well documented, the molecular and genetic mechanism are unclear. In *Drosophila*, two genes, *period* and *timeless*, are essential components of the circadian clock. A heterodimer of Per and Tim protein is thought to regulate the circadian process by creating a negative feedback loop controlling per and tim expression. However, in the decade since per was first found from *Drosophila* melanogaster, no mammalian *per* homolog had yet to be reported.

Using reciprocal probing between cDNA and cosmid libraries, we have identified a human chromosome 17 specific transcripts that encodes a bHLH-PAS protein domain with significant sequence similarity to Drosophila melanogaster Period. Overall protein homology (identical amino acid and conserved + neutral substitution) is 44%. We have named this gene RIGUI (after an ancient Chinese sundial).

Per and *tim* in Drosophila, circadian oscillator genes, are expressed in a periodic manner

during the 24 h day/night cycle. To examine whether expression of *RIGUI* behaves in a similar way, we studied its expression in mice. A murine brain cDNA library was screened with the human *RIGUI* 4.7 cDNA as probe, and a mouse homolog termed *m-rigui* was identified. The *m-rigui* encodes a protein of 1291 amino acids which has 92% amino acid identity with the human RIGUI. RNase protection assay using mouse retinae RNA collected every 4 hours during a 12 h light/ 12 h dark cycle showed that *m-rigui* mRNA level increased during the light phase and decreased during the dark phase.

In situ hybridization was carried out to determine the expression pattern of *m-rigui* in the specific regions of the brain. Expression in the suprachasmatic nucleus (SCN), the master regulator of circadian rhythms in mammalian systems, was the highest at ZT6 (whereby Zeitgeber time ZT0 is when lights were turned on and ZT12 is when lights were turned off) and the lowest at ZT18. We further observed the circadian pattern of expression in Purkinje neurons of the cerebellum and *pars tuberalis*, but expression patterns are shifted in phase from that of SCN. Expression analysis by *in situ* hybridization also revealed that circadian expression in the SCN is sustained in constant darkness, and a shift in the light/dark cycle evokes a propertional shift of *m-rigui* expression in the SCN. These are properties expected of a mammalian circadian genes. To date as far as we know, this is the first mammalian gene that fullfills these circadian rhythm properties. Taken together, the sequence homology and circadian patterns of expression suggest that *RIGUI* is a mammalian ortholog of *Drosophila period* gene, raising the possibility that a regulator of circadian clocks in mammals have been identified. (This work was recently published in the Journal *Cell* and has been accepted for presentation as a late breaking news in the American Society of Human Genetics meeting in Washington, D.C. October 27-November 1, 1997)

A gene involved in the Smith-Magenis Syndrome

Our present studies also identified a gene that encodes for a coactosin like protein (*CLP*) and maps to 17p11.2. Based on mapping studies we have been able to linked its involvment in the Smith-Magenis syndrome (SMS). Smith-Magenis syndrome is a contigous gene deletion syndrome with a frequency of 1/25,000 live births. The clinical findings of SMS include mental retardation, neurobehavioral abnormalities, sleep disturbances, short stature, minor craniofacial and skeletal anomalies, congenital heart defects and renal anomalies. This wide spectrum of phenotypic variation could have arise from a variation in the size of the DNA deletion. Our study shows that the *CLP* gene is duplicated in a repeat domain containing a gene cluster flanking the SMS deletion region. We were able to deomnstrate that the *CLP* gene flanks this repeat domain and that the duplication of this repeat leads to a recombination of the repeat domain resulting in the loss the of chromosomal region missing in SMS patients. (A paper describing this work is currently in press in Nature Genetics).

A disease gene for Spinocerebellar Ataxia type 6

Another area we have proposed to embark on in our 1996 report is involved the screening of genes for microsatellite (dinucleotide and trinucleotide) repeat sequences. Over the past seven years, 10 human diseases have been discovered that are associated with trinucleotide repeat expansion (23). The trinucleotide repeat expansion mechanism has provided a solution to the clinical termed anticipation. Anticipation is used in heritable diseases to describe the increasing incidence of a disease in a family from generation to generation as well as the earlier onset of the disease in individuals from generation to generation. Simple trinucleotide repeats are now proven to be the molecular origin for anticipation in ten of these heritable diseases. They include spinobulbar muscular atrophy (SBMA), fragile X syndrome (FX), Myotonic dystrophy (MD), Huntington's chorea disease (HD), Spinocerebellar ataxia type 1,2,3,6,and 7 (SCA), and Dentatorubral-pallidolusian atrophy (DRPLA). The exception to this rule was the gene for

Friedreich ataxia which is autosomal recessive without genetic anticipation. A novel trinucleotide repeat expansion of GAA within an intron sequence was discovered to cause Friedreich ataxia. This discovery suggests other variations of microsatellite sequences are involved in other human diseases. A mechanism based on microsatellite instability has been demonstrated for colon cancers due to mutations in DNA mismatch repair genes (25). Our goal is to identify genes with these microsatellite features and to type them on breast cell line DNA to determine the presence of aberrant alleles. The aberrant alleles can be identified from normal length polymorphism by comparing the alleles from disease state against non-disease state. We have only recently initiated this project for breast cancer. Our success for other human diseases was recently reported. We were able to identify a gene encoding for the $\alpha 1A$ calcium channel with a CAG repeat which displayed small expansion in about 10% of the patients diagnosed with Spinocerebellar ataxia. We have designated this gene to be Spinocerebellar ataxia type 6 (SCA6). (This work was recently published in Nature Genetics, vol 15, 62-70, 1997).

Conclusions

The proposed goals for the past year focused on detailed characterization of genes identified from human chromosome 17 have begun to yield important discoveries. Three very important genes, two of which are linked to human disorders and one which may be central to the human circadian pathway were discovered in my laboratory.This gene which we have named *RIGUI* could initiate molecular studies into hypothesis that the responsiveness of patients to chemotherapy display circadian patterns. This is a very important hypothesis that needs to be tested and the identifcation of the *RIGUI* gene allows for a precise marker for circadian rhythms.

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