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a Single Eucaryote (*S. Cerevisiae*) for Human Breast Cancer

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Genetic Instability and Checkpoint Genes: Insights From a Single Eucaryote (*S. Cerevisiae*) for Human Breast Cancer

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We study genes involved in cell cycle controls called checkpoints that block cell division after DNA damage. Checkpoint genes are relevant to breast cancer for each of the two aspects of this proposal we will continue to pursue. First, we proposed to isolate human checkpoint genes- now a putative *MEC1* homolog is already present in the *ATM* gene, recently described from human cells. *ATM* has a very high association with breast cancer, accounting for about 10% of all breast cancer cases. Our continuing goal is to identify other human homologs of yeast checkpoint genes, thinking they too may be relevant to breast cancer. Second, cancer progression is generally a multistep process in which mutations accumulate, allowing the cancer cell to grow under conditions where normal cells do not. Genomic instability may occur due to defects in checkpoints. We are studying in yeast cells how checkpoint mutations lead to genomic instability, including chromosome loss, recombination and point mutations. Thus far we find that all checkpoint mutants tested appear to have higher levels of loss and recombination, though our assay systems are still being optimized. We have seen no significant changes in point mutation frequency in checkpoint mutants.

**Checkpoint Genes/Genomic Instability/Cell Cycle/DNA Damage
Gene Amplification/*S. Cerevisiae***

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1. Abstract: Genomic Instability and Checkpoint Genes: Insights....

We study genes involved in cell cycle controls called checkpoints that block cell division after DNA damage. Checkpoint genes are relevant to breast cancer for each of the two aspects of this proposal we will continue to pursue. First, cancer progression is generally a multistep process in which mutations accumulate, allowing the cancer cell to grow under conditions where normal cells do not. Genomic instability may occur due to defects in checkpoints. We are studying in yeast cells how checkpoint mutations lead to genomic instability, including chromosome loss, recombination and point mutations. Thus far we find that all checkpoint mutants tested appear to have higher levels of loss and recombination, though our assay systems are still being optimized. We have seen no significant changes in point mutation frequency in checkpoint mutants. We have identified an instance of genome instability (we call "meta-stable 7") because we have identified in a system based on chromosome 7.

Our second goal was to isolate human checkpoint genes- now a putative *MEC1* homolog is already present in the ATM gene, recently described from human cells. ATM has a very high association with breast cancer, accounting for about 10% of all breast cancer cases. Our continuing goal is to identify other human homologs of yeast checkpoint genes, thinking they too may be relevant to breast cancer.

2. Introduction

Checkpoint genes delay cell cycle progression after DNA damage. Checkpoints insure genomic stability and may act as "tumor suppressors" to minimize the consequences of DNA damage. Two human genes of importance in cancer, p53 and recently ATM (Savitsky et al., 1995), are both checkpoint genes. ATM, for ataxi telangiectasia mutant, was recently identified by positional cloning, and has a high association with breast cancer (Swift et al., 1991). ATM proves to be related to our *MEC1* gene by both functional and protein sequence criteria (Figure 1). Both encode putative lipid or protein kinases (Hunter, 1995).

Our goals in this proposal are two. First, we are analyzing the relationship between checkpoint gene functions and four types of genomic instability in *Saccharomyces cerevisiae*, a simple eucaryotic cell. Second, we will isolate human checkpoint genes by complementation. We anticipate these studies will further our basic knowledge of checkpoint gene function and enhance our understanding of mechanisms of genomic instability in tumor cells. Our goals are:

Genomic Instability

1) Develop an assay of chromosome loss and mitotic recombination in the presence of checkpoint mutant genes. This assay is based on use of YAC artificial chromosomes, a stable but nonessential chromosome to the cell.

2). Develop an assay of point mutations in presence of checkpoint mutant genes.

3). Develop an assay of translocations between a YAC artificial chromosome and bona fide chromosome.

4) Develop an assay of gene amplification.

5) Analyze an instance of genomic instability

Human Checkpoint Genes

6) Isolate human checkpoint genes by complementation of yeast mutants.

3 . **Experimental Methods and Preliminary Results:**

Our progress through the first year includes the following, addressing each research objective stated above.

Part1- Genomic instability

1). *Chromosome loss and mitotic recombination assays:* We modified the YAC artificial chromosome (Sears et al., 1992) as described in our proposal. This required extensive molecular cloning to insert appropriate selectable markers in the ends of the YAC (see proposal for details). We then transferred these modified YAC into checkpoint mutant strains by karyogamy crosses (mating that allows transfer of only a chromosome into a recipient nucleus).

We found that all our checkpoint mutant strains showed elevated chromosome loss and recombination, especially *mecl* mutants. This is confirmed by report of mutation in *esr1*, which proves to be identical to *mecl* (Kato and Ogawa, 1994).

Our YAC-based chromosome loss system had unexpected complications; the YAC chromosomes we made in one yeast strain in which they are stable, yet when transferred to another yeast strain to test our checkpoint mutants they proved very unstable - even in a checkpoint normal cell! The second yeast strain thus proves to confer a high degree of instability on the YAC's.

We are taking two experimental approaches to resolve this issue. We are crossing our checkpoint mutations into a yeast strain in which the YAC's are stable (when those cells have intact checkpoint genes), and we

are using a chromosome loss assay of chromosome VII we had used previously in study of *rad9* mutants (Weinert and Hartwell, 1990).

Our preliminary results indicate that *mecl* mutants do show a tremendous instability of YACs, far greater than in our other strains. This is consistent with MEC1 having a key role in DNA replication compared to other checkpoint genes, a conclusion we and others have come to from other lines of research (Paulovich and Hartwell, 1995).

2). *Point mutation assays:*

We have analyzed point mutations by two systems; conversion of CAN1 to *can1* (selected by resistance to the drug canavanine) and conversion of SUP4 to mutant SUP4 (selected by loss of ochre suppression; Pierce et al., 1987). Both assays indicate that our checkpoint mutants do not confer any significant "mutator" phenotype as assayed by point mutation rates, either spontaneous or in the presence of DNA damaging agents.

3). *Translocation assays:*

We attempted to develop an assay of translocations by cloning a yeast gene into the human ALU sequence, to be placed back into the YAC chromosome and assayed for mobility. We completed initial cloning of URA3 yeast gene into an ALU sequence and will pursue that strategy in the coming year.

4). *Gene amplification assays:*

We were going to generate a ALU::HIS3 clone to bring attempts to look at gene amplification. We had difficulty with the translocation assay, and have therefore delayed development of a gene amplification assay.

5). *New Project- The analysis of the meta-stable chromosome VII.*

We have identified an instance of chromosome instability in *rad9* mutants we think will be very informative for genomic instability. A chromosome 7 disome (Figure 2, strain described in Weinert and Hartwell, 1990) allows us to select for loss of a marker at the end of the chromosome. This system was designed to identify chromosome loss and mitotic recombination events.

We identified serendipitously an event that shows remarkable instability, and has some hallmarks indicating a dicentric breakage-fusion-bridge cycle (McClintock, 1984). The initial events were identified on selective plates as lethal sectoring colonies (see Figure 2). Initial strains were haploids with 2 copies of chromosome. One copy had a dominant selectable gene CAN1- we used this marker to select for chromosome loss or recombination (identified by cells that become resistant to canavanine indicating that they have lost the CAN1 gene). When plated on agar medium containing canavanine, the initial cell with an intact CAN1 gene dies, and cells that lose CAN1 survive. These cells usually have undergone

mitotic recombination, loss, or point mutation events. Most can-resistant cells have undergone loss, some mitotic recombination, and very few point mutations.

What struck us in particular was evidence of an unusual event: some colonies were showing lethal sectoring ("nibbled" or "sectored" colonies). The sectored colonies occurred at about 10x higher rate in *rad9*⁻ than *RAD9*⁺ cells, and sectoring was inducible by irradiation (not shown), indicating these cells are experiencing some event related to DNA damage checkpoint controls.

The sectored colonies prove to have cells which are genetically unstable. This was identified by propagating the cells further on unselected medium. We surmise the following: consider an initial cell that has 2 chromosome 7s, a T (for top) and B (for bottom) homolog (Figure 2, top). Before selection, *CAN1* on the B homolog is lost. This gives rise to a cell with intact T and a B chromosome with some rearrangement (what is lost is denoted by dotted line in Figure 2). When propagated, this cell undergoes further events where either the T or B chromosome is lost (frequency of secondary events in this experiment was: 18% lose T, 11% lose B and 71% retain the T+altered B structure).

The site of instability on B was identified initially by genetics. T and B chromosomes are marked with alleles of *LYS2*, *CYH*, *TRP1*, *LEU1*, *ADE6* and *ADE3* (from the left to right ends, respectively). The + and - mark which homolog has wildtype (+) or mutant (-) allele. The unstable sectored colonies genetically are heterozygous for some markers and homozygous for others. The dotted line indicates regions that are homozygous.

When propagated further, cells with the T+altered B chromosomes continued to generate chromosome loss events that had lost one or other of the two homologs (an example is shown in Figure 2; bottom left).

Molecular Analysis of meta-stable 7

From cells undergoing the instability, we wished to determine their molecular structure. In strains undergoing the instability, we identified a chromosome that is physically larger than the initial chromosome 7 (Figure 3). We identified the larger chromosome using pulsefield gel electrophoresis that separates chromosomes by size on agarose gel matrix (Schwartz and Cantor, 1984). From the chromosomes present in 6 strains shown in Figure 3 (left panel), the starred bands represent extra bands corresponding to the meta-stable 7. That these extra bands are from chromosome 7 was demonstrated by hybridization of radio-labeled molecular probes from chromosome 7 (probe C, right panel). This probe identifies both the original 7 and modified 7's.

Molecular analysis then verified that DNA genetically absent from the chromosome (see Figure 2) was also not present on the meta-stable 7

chromosomes in Figure 3. For example, the starred chromosome in lane 4 has sequences C but not A (middle panel shows hybridization to probe A). We have shown that the strain has sequences from region B as well (not shown). The shorter modified chromosome 7s also have C (and B) but not A sequences.

It thus appears that the meta-stable 7 chromosome has physically lost DNA, depicted as the truncated chromosome in top of Figure 3. Yet, the truncated chromosome usually has the mobility of a larger chromosome by pulsefield gel electrophoresis (starred chromosome in lane 4). (The larger chromosome is of variable size in different isolates and is always extremely unstable. Most of the genetically unstable clones contain the larger chromosome seen in lane 4, though we have seen examples of smaller chromosomes (lanes 2 and 5) as well; not shown. The data in Figure 3 represent the structures seen but not the frequency at which they occur. >90% of extra chromosomes have the size shown in lane 4).

We are now focusing our attention on the structure of the larger chromosome (lane 4, starred). We have identified molecularly the breakpoints of the unstable chromosome to a region of 10-50kb kb on chromosome 7 (Figure 4). This was accomplished using yeast DNA cloned into a bank of lambda phages (Riles et al, 1993). Most unstable 7 chromosomes have similar though not identical breakpoints (judging from the intensity of the hybridization signals). Suffice it to say the breakpoints appear heterogeneous but cluster in this one region of the chromosome. This site appears, therefore, to be some sort of stabilization site and/or selectively broken (fragile?) site.

The DNA sequence of this region of chromosome 7 has recently been determined (Delius, personal communication: see references) allowing us to generate molecular probes to further test the nature of the rearrangement. We needed a cytogenetic tests of chromosome structure. Such a test is now possible, in collaboration with Ulli Weier at UC Berkeley. He has a method to inspect by immunofluorescence probes hybridized to intact chromosomes stretched out on glass slides (Weier et al., 1995). His method allow us to test the physical structure of the unstable chromosomes.

We have sent him isolated unstable and stable chromosome 7 from pulsefield gels. He prepares "stretched chromosomes" and studies their structures using fluorescently labeling DNA probes (FISH). To prepare "stretched chromosomes" he applies the isolated chromosomes to a glass slide, and upon dehydration the DNA molecule stretches across the slide, remaining intact. This "stretched chromosomes" are the subject to FISH hybridization using probes to specific regions of chromosome 7 (obtained from the yeast lambda clones; Riles et al., 1993).

We have just received our first data on structure of the stretched chromosomes. The unstable chromosomes structures show three

unexpected (and not yet explicable!) features. First, they do contain two same ends (with sequence "C" as in Figure 3). They appear to be products of a head to head fusion of chromosomes. Second, they do not appear to contain two centromeres- they are not dicentrics? This is inexplicable and we are eager to retest the structures with additional probes linked to the centromere. Third, some DNA circles were found as well- which we are told is very unusual in these sorts of preparations. This result is also currently inexplicable.

Model.

Our initial idea is shown in Figure 5. We hypothesized that an initial rare event lead to a chromosome break. The checkpoint defective cells continue through the cell cycle, rereplicate the broken chromosome, generating two truncated sisters (with sequence B near break points). These can then fuse, recombine with other homolog (not shown) be propagated as is or be lost. If the two fuse, this structure undergoes breakage-fusion-bridge cycle and rearrangements as shown in Figure 5 (McClintock, 1984).

Our genetic data are entirely consistent with this model (rare initial event inducible by radiation; high frequency secondary chromosome loss events, especially in a checkpoint mutant cell). The structures we have just obtained are consistent in one sense; the unstable chromosome has 2 "C" regions- it is in some sense a head to head fusion of the original chromosome. We have not yet seen, however, evidence for 2 centromeres, nor can we explain the circle chromosomes.

After we have obtained more data from the Berkeley group, we will design a model and try to test it.

Part 2- Isolation of Human checkpoint genes.

Our efforts at complementation of strains we have constructed have not yet been successful. Remarkably, the idea that checkpoint genes are conserved in humans and yeast has been verified - the ATM gene from humans is very similar functionally and by protein sequence to that of our *MEC1* gene (Figure 1). Others who have the ATM gene are attempting complementation of *mecl* mutants or test of dominant negative activity of *MEC1* in human cells (Steve Friend, Seattle, Wa; Mike Kastan, Baltimore, Md).

We have doubts about whether complementation will be successful, though we will continue with strains generated. We have tested homologs from fission yeast (*rad1+*, a homolog of our *RAD17* gene in budding yeast; provided by T. Carr; see Griffiths et al., 1995) and have not yet observed complementation nor dominant negative effects. ATM and *MEC1* bare

similar levels of homology (20% identity and 45% similarity) as do *rad1*⁺ and *RAD17* (Lydall and Weinert, 1995).

We will test other fission yeast genes, and continue to attempt complementation of our yeast mutants with human libraries as previously proposed. We are also linked to Phil Hieter's group and the continuing update of possible EST matches he provides us monthly. EST sequences are partial cDNA human sequences deposited in the data base frequently. Any putative match will be followed up by collaboration with those who identified the putative matching EST sequence.

4. Conclusions

First, analysis of genomic instability in checkpoint mutant cells is very much a priority. Our initial tests of *mecl* suggest it leads to high rates of mitotic recombination, and we will pursue this observation as discussed above. The point mutation frequencies do not seem to be elevated in our mutants. We will try to generate translocation and gene amplification assays.

We have an instance of genomic instability, called meta-stable 7 described above, that may provide us the opportunity to study genomic instability leading to a dicentric chromosome, as we postulate, and breakage-fusion-bridge cycle events. After molecular analysis of the putative dicentric, we will insert a gene whose amplification we can select (e.g. *HIS3*) and test if amplification can be detected at this locus.

Second we are still uncertain as to whether human checkpoint genes can be identified by complementation of yeast mutants. Such an effort has not yet been successful for *mecl* (Plon et al., 1993) and the level of homology between ATM and *MEC1* suggests why complementation may not work well. We are now scanning EST data bases for sequences that may be candidates, and will pursue those as the occasion arises.

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Table I Chromosome Loss and Recombination of YAC chromosomes in checkpoint mutants.

<u>Strain</u>	<u>Loss Frequency</u>	<u>Recombination Frequency</u>
Experiment 1		
MEC+	0.7 +/- 0.9 x 10 ⁻²	0.3 +/- 0.1 x 10 ⁻²
mec1		49 +/- 28 x 10 ⁻²
mec2 (rad53)	23.4 +/- 17.2 x 10 ⁻²	10.2 +/- 13.2 x 10 ⁻²
Experiment 2		
MEC+	0.13 +/- 0.17 x 10 ⁻²	1.1 +/- 2.1 x 10 ⁻²
rad9	0.71 +/- 0.76 x 10 ⁻²	2.8 +/- 3.2 x 10 ⁻²

Table 2 Point Mutation Frequency in Checkpoint mutant strains.

<u>Strain</u>	<u>Frequency $can1^R$ from $CAN1^S$</u>
MEC+	3.9 x 10 ⁻⁵
mec1	1.5 x 10 ⁻⁵
mec2 (rad53)	3.8 x 10 ⁻⁵
mec3	1.8 x 10 ⁻⁵
rad9	4.4 x 10 ⁻⁶
rad17	3.8 x 10 ⁻⁶
rad24	4.2 x 10 ⁻⁵

Figure Legends

Figure 1. MEC1 in family of PI 3 Kinases

An alignment of genes with PI3 kinase domains is shown. The PI3 kinase domain consists of sequences represented in the heavy boxes to the right end of all the genes. Other boxes consist of sequences of homology between genes as well. DNA-PK is also a PI 3 Kinase (see Hunter, 1995 for more details).

Figure 2. Phenomena: Meta-stable 7 chromosome in yeast.

This figure shows the sequence of events on how we identified this phenomena, the nature of secondary instability, and putative structure of the unstable chromosome.

The first plate (top left) contains canvanine and selects for the event initially. The other two plates are complete media plates. Genotypes of individual cells were determined by replica-plating onto defined media.

See Text for details.

Figure 3. An extra chromosome associated with meta-stable 7.

The top 3 drawings show the initial chromosome (normal 7), a truncated version showing (defined by genetic and molecular tests) and a dicentric (that may constitute the larger chromosome seen in lane 4).

The bottom panels show chromosomes isolated from unstable cells. In the left panel, all chromosomes are visualized by EtBr staining. Lane 1 is a control strain, and the other 5 lanes are from genetically unstable colonies. The middle panel is the same DNA probed with a sequence distal to the breakpoint (HKK2 DNA); the right panel is the same chromosomal DNA probed with a sequence to the right of the centromere (ADE3 DNA).

Figure 4. Physical Map of the region around the breakpoint.

The upper line shows restriction sites and fragments. The bottom line orients these sequences on chromosome 7.

Figure 5 Breakage-fusion-bridge cycle. This model depicts a hypothetical course of events to explain our meta-stable 7 phenomena. See text for details.

Figure 1.

MEC1 in family of PI3 kinases

modified from Savitsky et al. Science 268:1749

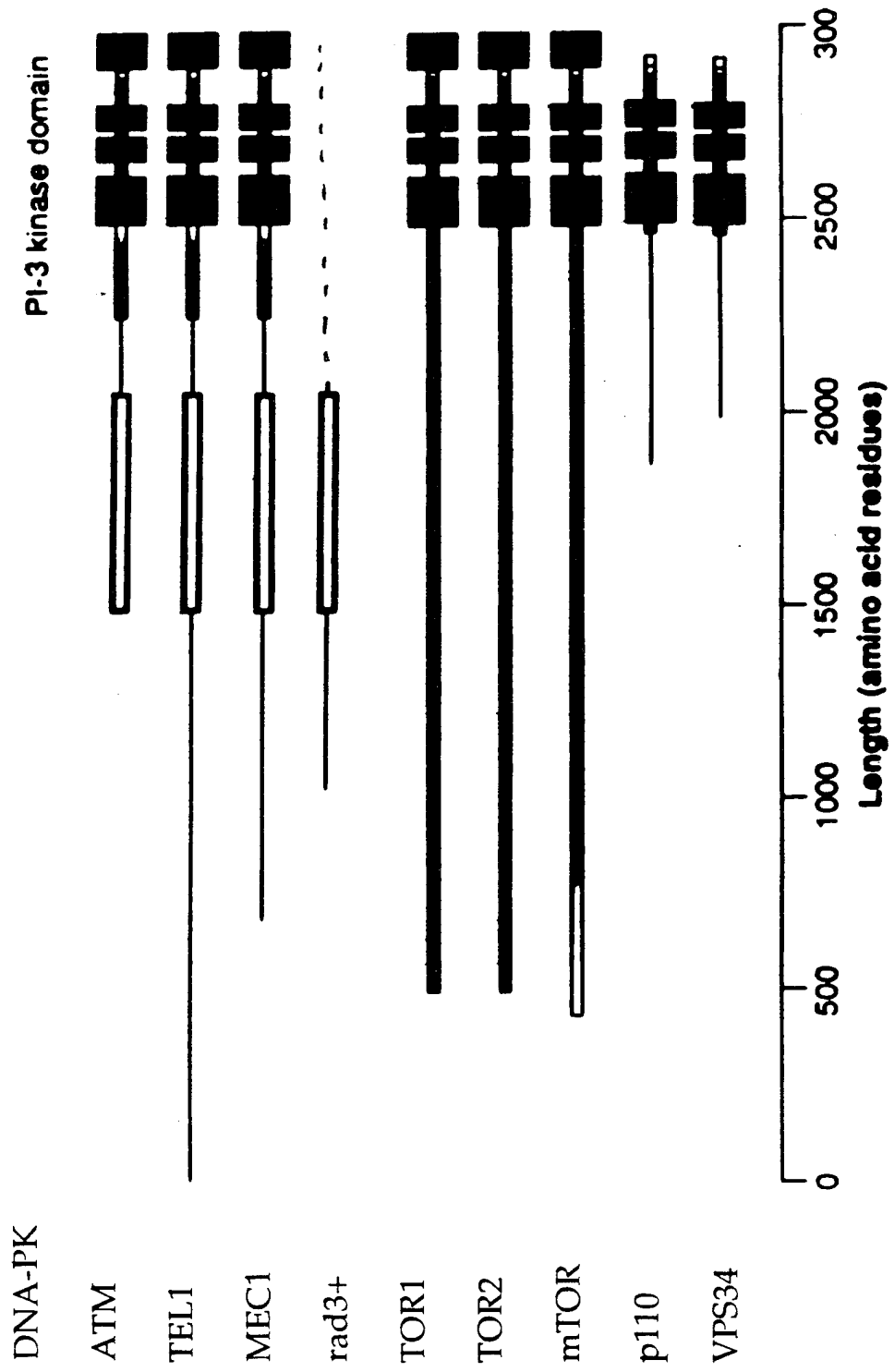


Figure 2

Phenomena: Meta-stable VII

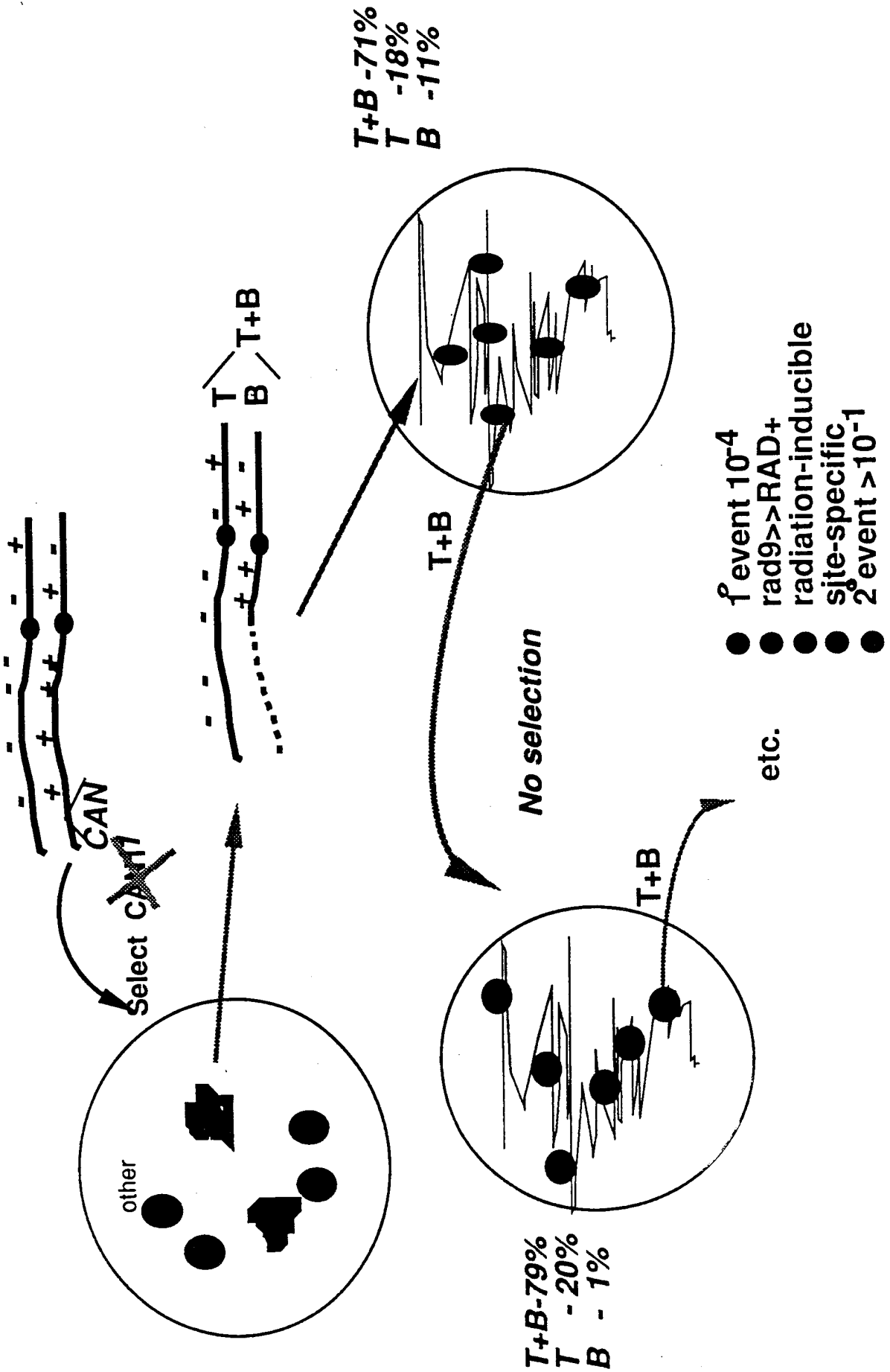


Figure 3

An extra chromosome associated with instability

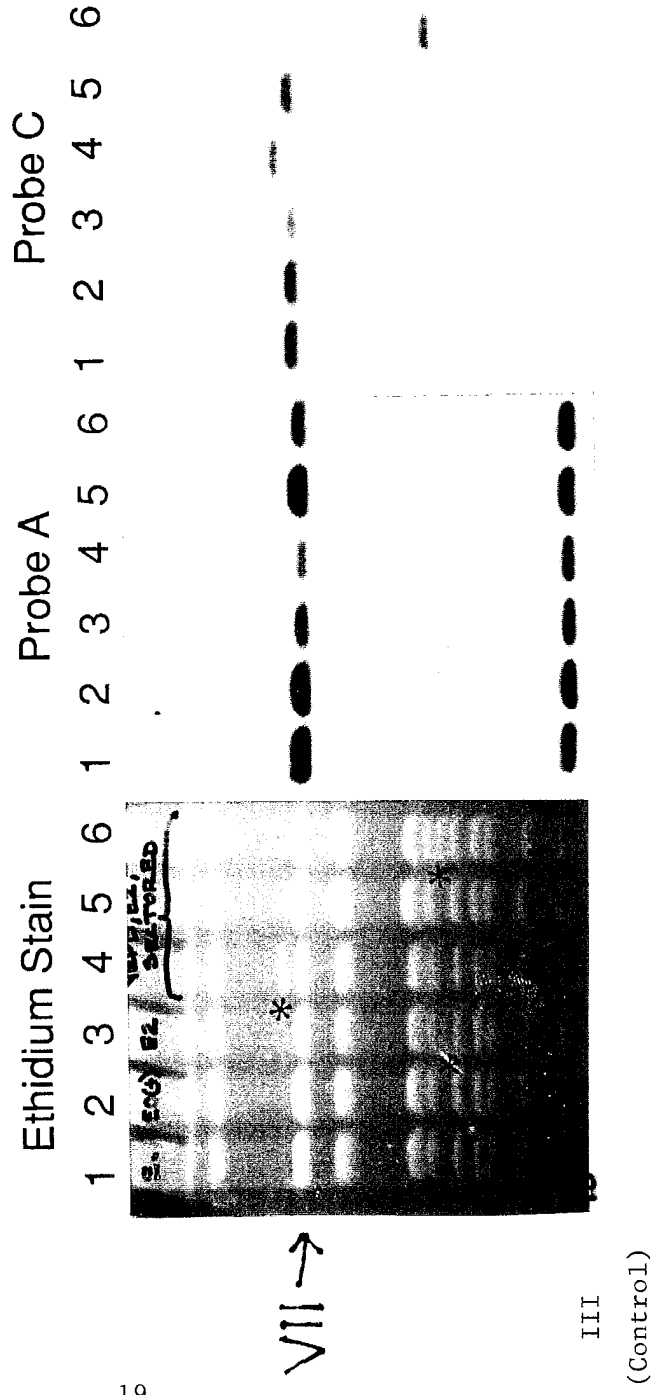
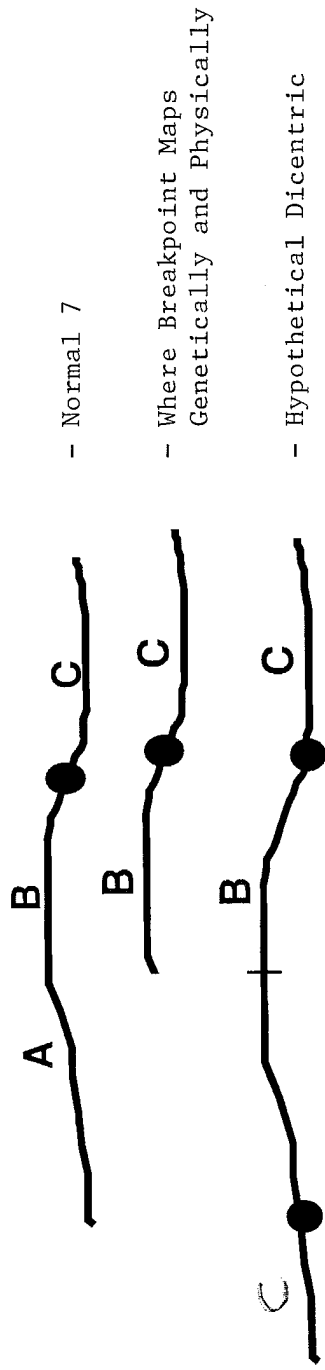


Figure 4

Chromosome: VII Arm: L Diagram 1 of 2

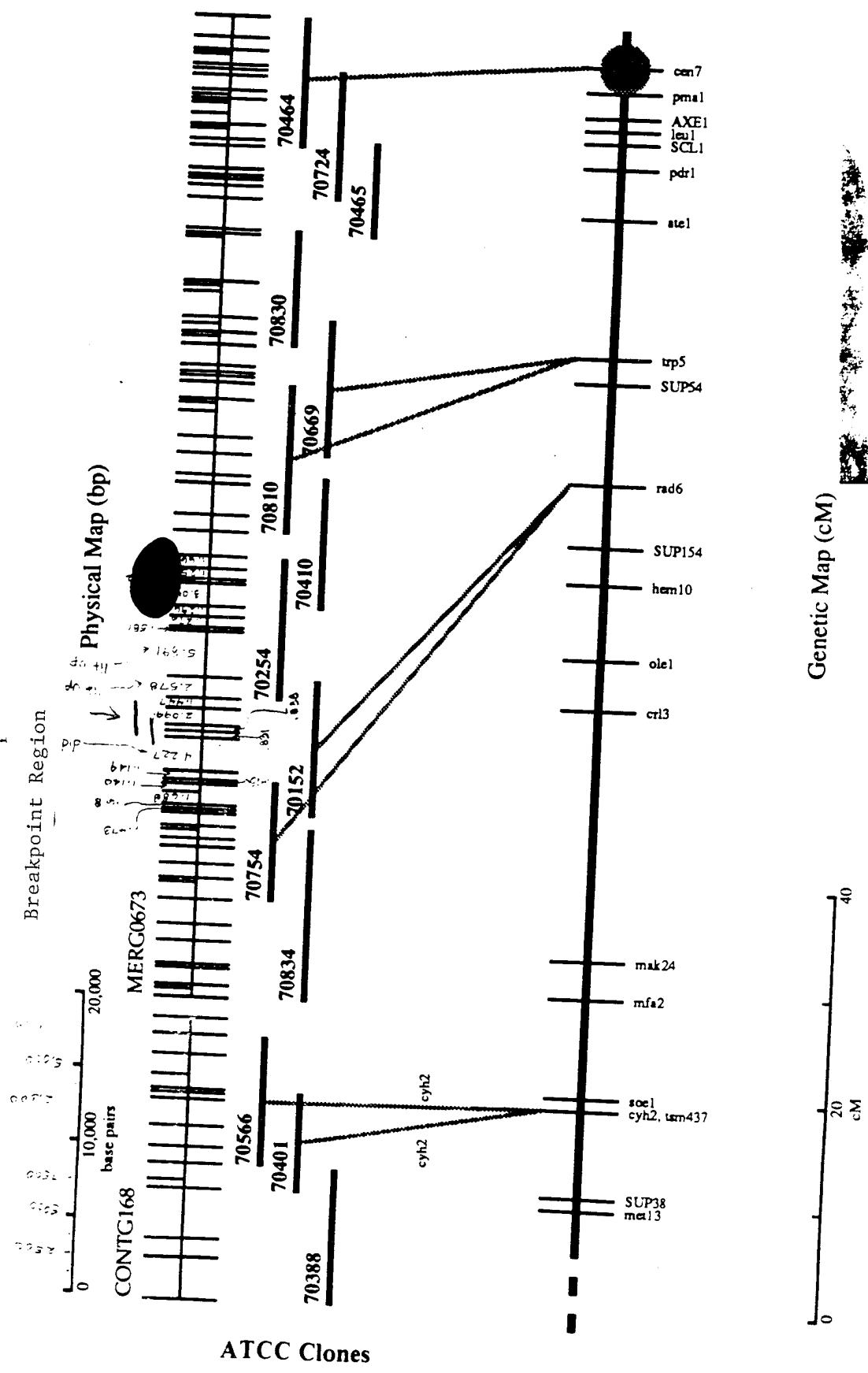


Figure 5

Breakage Fusion Bridge Cycle

