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TITLE: The Roles of Chromosome Breaks and Telomere Dynamics in the Genomic Instability Associated with Human Breast Cancer

PRINCIPAL INVESTIGATOR: John H. Wilson, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030-3498

REPORT DATE: September 1998

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Fort Detrick, Maryland 21702-5012

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13. ABSTRACT <i>(Maximum 200 words)</i> Human tumors acquire a marked genomic instability as part of their progression from normal to malignant cells. This instability is likely due in part to the progressive shortening and transient loss of telomeres from chromosome ends. Loss of telomeres allows chromosomes to fuse end-to-end, triggering chromosome fusion-bridge-breakage cycles that lead to genome rearrangements, loss of heterozygosity, and gene amplification. The initial steps in chromosome fusion-bridge-breakage cycles are being studied by introducing site-specific double-strand breaks adjacent to interstitial telomere sequences in a marked region of specially engineered test chromosome. A modified gene for the Green Fluorescent Protein (GFP) is being constructed to permit detection of chromatid fusion, the first step in fusion-bridge-breakage cycles. By varying the length of the telomere sequence, we can measure the length of telomere sequence that is required to protect chromosome ends and prevent genomic instability in breast cancer cells. A test chromosome is being constructed using the APRT locus in CHO cells, which will permit a measure of the effects of telomere sequences on the frequencies of chromatid fusion and chromosome loss. After these initial studies we will transfer the engineered hamster chromosome by microcell fusion into human breast cancer cells at different tumor stages.			
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FOREWORD

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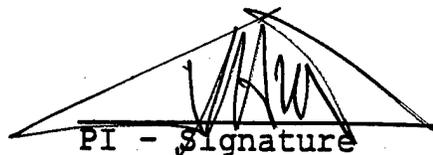
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INTRODUCTION

Virtually all tumor cells acquire genomic instability as they accumulate mutations in their progression from a normal state to a malignant phenotype. Instability of tumor genomes is due in large part to alterations in the regulation of the cell cycle, loss of cell-cycle checkpoints, and mutations in genes responsible for repair of genome damage. Recently it has become clear, however, that telomere loss may also contribute substantially to genomic instability by permitting chromatids or chromosomes to fuse, thereby initiating chromosomal fusion-bridge-breakage cycles, which play havoc with the genome. Such cells would normally die, but some survive by expressing telomerase, which stabilizes the genome, and allows the cells to progress to a fully malignant state. The possible role of telomere dynamics in promoting genomic instability and cancer has suggested a potential therapeutic approach to cancer treatment that involves blocking the function of telomerase. Our studies focus on the basic science that underlies the speculated role of telomerase, as well as the efficacy of the suggested treatment.

Malignancies arise through the combined effects of multiple mutations at a rate much higher than anticipated from the measured rates of mutation in normal cells (1). Thus, the acquisition of a mutator phenotype appears to be an essential step in the progression of normal cells to the malignant state (2). This mutator phenotype results in part from the loss of proper cell cycle regulation and dysfunction of cell cycle checkpoints (3). Recently, it has become clear that another potential cause of genomic instability is the loss of telomeres that normally cap the ends of chromosomes (4). In the absence of functional telomeres, chromosome ends can fuse to form dicentric chromosomes (5,6), which trigger fusion-bridge-breakage cycles (7,8) and can cause chromosomal rearrangements, gene amplification, and loss of heterozygosity (9,10)

Normal human chromosomes are capped by telomeres consisting of tandem TTAGGG repeats that may occupy several kilobases at the chromosome termini (11). Human telomeres appear to have an unusual nucleosome organization (12), cofractionate with the nuclear matrix (13), and have been shown to bind special proteins (14,15). In addition to their role as a chromosome cap, telomeres solve the end-replication problem by serving as substrates for telomerase, which adds TTAGGG repeats using an internal RNA template (16). Telomerase appears to be active in the germline, but significantly less so in somatic cells (17,18). As a result, telomeres in somatic cells grow shorter with each cell division (5,19,20).

Telomeres of primary human tumors, like those in normal somatic cells, also seem to lose telomere sequences (4,19,21). When telomeres become critically short or absent, chromosomes can fuse end-to-end to form dicentric chromosomes (5,6) that lead to chromosome rearrangements, gene amplification, and loss of heterozygosity. Although the resulting mutator phenotype may allow a rapid evolution of the tumor phenotype, it ultimately may also interfere with cell proliferation (4). At some stage tumor cells appear to activate telomerase, which restores their telomeres and partially stabilizes their genomes (22), allowing the genetically rearranged cells to grow unchecked. Since telomerase activity is typically high in tumor cells (17,23), inhibitors of telomerase could serve as cytotoxic agents in cancer therapy (20,22).

To date, all measurements of telomere length have been made on populations of cells and often on sets of chromosomes (4,19). These measurements are unsatisfying because the loss of a telomere from a single chromosome end may be sufficient to initiate genomic instability. We have developed a novel method for assessing the effect of the loss of an individual telomere on a defined chromosome. We have targeted 800 bp of telomeric sequence, which is known to be capable of seeding a new telomere (15,35), with an I-SceI recognition site at the seeding end, into the second intron of the APRT gene in CHO cells. We have shown that this interstitial telomeric sequence does not interfere with expression of the APRT gene, that it is stable by Southern blotting, and that it does not stimulate homologous recombination.

Interstitial telomere sequence does, however, stimulate illegitimate recombination, leading to deletions in the surrounding DNA (Kilburn, Shea, Sargent, Wilson, submitted). In tetraploid cell lines, in which the normally single-copy APRT chromosome is rendered nonessential, this interstitial telomeric sequence causes no increase in the rate of chromosome loss. We are now in a position to measure the effects of I-SceI expression, which cleaves off the end of the chromosome leaving a defined length of telomeric sequence at the new end of the chromosome.

A primary advantage of using the APRT gene in CHO cells as a site for introducing and testing various telomeric sequences is our previous experience with that locus. Both chromosomal (24,37) and targeted homologous recombination (25,26,27) are well characterized at APRT. Illegitimate recombination has also been characterized at this locus (28,29,38). We have adapted the FLP/FRT site-specific recombination system for use at APRT so that we can make modifications of the locus rapidly (39). We have experience with the rare-cutting endonucleases, PI-SceI (40) and I-SceI (37). We can introduce site-specific damage into this locus using triplex technology (30,31). And we have used this locus to investigate genomic instability associated with random integration (29).

The fundamental hypothesis guiding this study is that the loss of telomeres triggers genomic instability, leading to a loss of heterozygosity, gene amplification, and chromosome rearrangements.

The scope of this research is indicated by our technical objective, as outlined below:

Objective 1. Measure frequencies of chromosome loss after introduction of a site-specific double-strand break by I-SceI expression.

Objective 2. Use microcell fusion to transfer the test chromosome to a breast cancer cell line and repeat measurements of effects of different telomere lengths and telomerase expression.

Objective 3. Construct a color-based system for detecting chromatid fusion and gene amplification.

Objective 4. Characterize the color-based system in CHO cells for its ability to detect chromatid fusion and gene amplification.

Objective 5. Transfer the color-based detection system into breast cancer cells at different stages in the progression to malignancy and measure frequencies of chromatid fusion and gene amplification.

During this initial grant period, we have measured the ability of induced double-strand breaks to be healed by capture of telomere-bearing plasmid, and we have determined the frequency of chromosome loss after introduction of a site-specific double-strand break by I-SceI expression. Based on those results we have reevaluated the design of the appropriate test chromosome and begun its construction. In addition, we have begun to construct a color-based system for detecting chromatid fusion.

BODY

Can a telomere sequence join to a double strand break and heal the broken chromosome by seeding a new telomere?

The phenomenon of telomere assisted chromosome fragmentation (TACF) is well documented in various mammalian cells (15,35). Typically, a linearized plasmid, containing an internal selectable marker and a segment of telomere sequence at one end, is introduced into cells and positive selection for the marker is applied. Among the colonies that survive selection, a remarkably high proportion (20-70%) carry the plasmid and a newly seeded telomere at the end of a truncated chromosome. The mechanistic basis for the phenomenon of TACF is less clear. Do plasmids integrate randomly and subsequently stimulate a break due to instability of interstitial telomere sequence (35)? Are sites of random integration generally so unstable (independent of telomere sequence) as to frequently expose telomere sequence for seeding (29)? Do plasmids join to the centric end of a spontaneous double-strand break and then seed a new telomere? Do plasmids randomly integrate via the nontelomeric end only, leaving the telomeric end free to seed a new telomere (29)?

Our previous results suggest that the first mechanism---integrate then break---is unlikely because telomere sequence integrated at the APRT locus in CHO cells is only slightly destabilizing, far below the level needed to support breakage in 20-70% of cells as is characteristic of TACF (Kilburn, Shea, Sargent, Wilson, submitted). Since the modified APRT locus did not arise by random integration and is stable, we cannot shed light on the second mechanism in this experimental paradigm. To differentiate the second and third mechanisms we wanted to induce defined double-strand breaks at the selectable APRT locus in CHO cells to determine whether such breaks could capture a transfected plasmid and seed a new telomere.

In work that was initiated before this grant began, we constructed a targeted CHO cell line that carries the recognition site for the rare cutting endonuclease, I-SceI, in the second intron of the APRT gene and characterized the effects of cutting (38). By cotransfecting an I-SceI expression vector and a selectable plasmid bearing telomere sequence, we can select for integration and telomere seeding at a defined chromosomal break. Before we could apply this strategy, however, it was necessary to create tetraploid versions of this cell line so that any essential DNA in the largely haploid region surrounding the APRT locus would be rendered nonessential. Tetraploid cells were created by fusing the I-SceI-containing, APRT+ cell line with a cell line that is deleted for the APRT gene.

In experiments that overlap the beginning of this grant, diploid and tetraploid cell lines containing the I-SceI site at the APRT locus were transfected individually or cotransfected with the I-SceI expression vector and the linearized plasmid pSXNeoTTAGGG, which contains telomere sequence fused to a neomycin expression cassette. Transfected cells were selected for Neo+, for APRT-, and for Neo+APRT- cells. The data are presented in Table 1.

Table 1. Transfection of diploid and tetraploid cell lines with a plasmid expressing I-SceI, or a telomere-containing plasmid expressing Neo+, or both plasmids.

Transfected DNA	Neo+		APRT-		Neo+APRT-	
	Diploid	Tetraploid	Diploid	Tetraploid	Diploid	Tetraploid
pCMVI-SceI	NA	NA	3×10^{-4}	7×10^{-4}	NA	NA
pSXNeo	6×10^{-3}	2×10^{-3}	5×10^{-6}	8×10^{-5}	$< 2 \times 10^{-6}$	9×10^{-5}
pCMVI-SceI + pSXNeo	5×10^{-3}	1×10^{-3}	5×10^{-4}	4×10^{-4}	3×10^{-4}	1×10^{-2}

The data for Neo⁺ alone indicate that I-SceI cleavage does not significantly alter the baseline frequency of random integration in either diploid or tetraploid cells; however, it does indicate that the tetraploid cell line gives 3- to 5-fold fewer random integrants than the diploid cell line. The APRT⁻ data shows that in the diploid cells, I-SceI cleavage stimulates formation of APRT⁻ colonies about 100-fold, in agreement with our published data (38). Although the stimulated values are about the same in diploid and tetraploid cells, in tetraploid cells it represents only about a 5- to 10-fold stimulation over the frequency in the absence of I-SceI expression. The 10-fold higher background of APRT⁻ cells in tetraploid cells likely represents the frequency of loss of the APRT⁻ chromosome. (More about that below.) Most importantly, as shown in bold, tetraploid cells give a significantly higher frequency of Neo⁺APRT⁻ colonies when both plasmids are present than do diploid cells transfected with both plasmids or tetraploid cells transfected with pSXNeoTTAGGG alone.

These data in Table 1 suggest that a plasmid carrying a telomere sequence can join to an induced double-strand break and seed a new telomere. To examine that possibility further, we carried out a molecular analysis of 15 Neo⁺APRT⁻ colonies that arose in the above experiments. In principle, Neo⁺APRT⁻ colonies could arise in three ways: 1) independent integration and mutation, 2) integration at APRT, and 3) end joining followed by seeding. To distinguish these possibilities, we cut genomic DNA from individual colonies with BclI, which cleaves outside the APRT locus. The Southern blot of this digest was then successively hybridized to the Neo coding segment, the 3' end of APRT, and the 5' end of APRT. If integration and mutation are independent events, the neo and APRT probes will hybridize to different fragments; 8/15 colonies gave this pattern. If the neo plasmid integrates at the APRT locus, both APRT probes and the neo probe will hybridize to the same fragment; 5/15 colonies gave this pattern. If the neo plasmid end joined to one half of APRT at the break and seeded a new telomere, the neo probe and one APRT probe will hybridize to the same fragment; 2/15 colonies gave this pattern.

The two colonies that show evidence for telomere seeding at the site of the induced double-strand break provide proof of principle that a seeded telomere at the APRT locus can heal a chromosome break, which was a fundamental assumption of our proposed research. They also provide support for the hypothesis that TACF may arise by attachment of telomere-containing plasmids to transient double-strand breaks in the chromosome.

The structures of these two colonies also provide valuable information about the orientation of the APRT gene on the CHO chromosome, which was unknown at the time we began these studies. In both colonies the Neo gene is linked to the 3' half of the APRT gene, while the 5' half of the APRT gene is absent. Thus, the APRT gene is oriented with its 5' end toward the telomere and the 3' end toward the centromere. A defined orientation provides specific predictions for the outcomes of the experiments described in the next section and, in addition, provides a basis for our redesign of the test chromosome as discussed later.

In our Statement of Work, we had anticipated that these studies, which constitute part of Objective 1, would be completed before the grant began. They were actually completed within two months of the start of the grant.

Can a double strand break adjacent to telomere sequence on the chromosome induce chromosome truncation and the seeding of a new telomere?

To determine whether a double strand break adjacent to the seeding end of an interstitial telomere sequence can promote chromosome truncation and the seeding of a new telomere, we first inserted an I-SceI recognition sequence adjacent to the seeding end of telomere sequences present in either orientation in APRT gene targeting vectors. These vectors were used to modify the endogenous APRT locus in CHO

cells using site-specific recombination (38). As previously described (38), this procedure creates a targeted locus that contains a duplication of the APRT gene, with the telomere sequence located in the second intron of the downstream APRT+ copy of the gene. Between the two copies of APRT sequence are located two other selectable markers---herpesvirus TK and bacterial GPT---that have proven useful in the past. We created both orientations of the telomere sequence initially because we did not know the orientation of the APRT gene on the chromosome. The results described above gave a clear prediction for which orientation (CCCTAA) would permit truncation and seeding; the other orientation (TTAGGG) serves as a control.

Tetraploid cell lines were created, as described above, for these two cell lines and for two other control cell lines: one that carried just the I-SceI site and one that carried just the telomere sequence in the CCCTAA orientation. Each of these cell lines was transfected with an I-SceI expression vector and then subjected to selection for APRT- cells and for APRT-TK- cells. The results of these experiments are shown in Table 2.

Table 2. Effects of I-SceI expression in cell lines carrying an I-SceI recognition site adjacent to the seeding end of an interstitial telomere sequence.

Insert	APRT-			APRT-TK-		
	- I-SceI	+ I-SceI	Stimulat.	- I-SceI	+ I-SceI	Stimulat.
I-SceI site	3×10^{-4}	19×10^{-4}	6	NA	NA	NA
CCCTAA	11×10^{-4}	8×10^{-4}	0.7	3×10^{-4}	2×10^{-4}	0.7
I-SceI/CCCTAA	7×10^{-4}	10×10^{-4}	1.4	1.5×10^{-4}	3.6×10^{-4}	2.4
TTAGGG/I-SceI	14×10^{-4}	21×10^{-4}	1.5	8×10^{-4}	14×10^{-4}	1.7

In these experiments we had anticipated that the I-SceI/CCCTAA cell line would show a substantial stimulation of APRT- and APRT-TK- colonies when transfected with the I-SceI expression vector. Although a slight stimulation was observed with both selections, the stimulation was not significantly different than that observed with the control, TTAGGG/I-SceI cell line. These data clearly identify the problem in these experiments; the background levels of APRT- and APRT-TK- cells obscure any possible effects of chromosome truncation and seeding. In the absence of I-SceI treatment all cell lines have levels of APRT- cells that range on either side of 10^{-3} . Since the APRT locus is stable, we presume that these high levels represent the frequency of chromosome loss in the tetraploid cells.

A high rate of chromosome loss interferes with our analysis as we had initially proposed to carry it out. Although we know we can truncate the APRT chromosome and seed a new telomere from the work described in the first section, we can not demonstrate it in the experimental paradigm we had proposed. In principle, we can avoid the problems of chromosome loss by anchoring the chromosome with a positively selectable marker, such as the GPT gene in our constructs. In our existing constructs, however, the GPT gene is positioned so that it is lost when the chromosome is truncated. Below we discuss how it can be repositioned to anchor the marker chromosome against chromosome loss.

In our Statement of Work, we had anticipated that these studies, which constitute part of Objective 1, would be completed before the grant began. They were completed well into the first year. Because the outcome of these experiments indicated that the test chromosome would have to be redesigned, we opted not to undertake Objective 2 (Transfer test chromosome into breast cancer cells by microcell fusion) until we had redesigned the test chromosome.

How can the test chromosome be redesigned to anchor it against chromosome loss?

To anchor the test chromosome against loss, we want to place a selectable marker on the other side of the telomere sequence, so that when the chromosome is truncated the selectable marker will be

retained. To do this we will reposition the I-SceI/CCCTAA telomere sequence between the GPT gene and the TK gene. In this position, GPT+ (HAT) selection will allow cells with truncated chromosomes to survive, whereas cells that have lost the chromosome, and thus have lost GPT, will die. This strategy will work not only for our CHO cells, but also for breast cancer cells, which we can readily select to be HPRT- (allowing GPT to be selectable in those cells). In addition, since the TK gene will remain in its current location relative to the telomere sequence, it will be lost in a chromosome truncation event. Thus, simultaneous selection against the TK gene (FIAU) and for the GPT gene (HAT) can be used to specifically enrich for chromosome truncations, which are likely to be stable only when a new telomere is seeded. Since both TK and GPT can be used in breast cancer cell lines, this redesigned chromosome will permit us to select for chromosome truncation and seeding in breast cancer cells and meet technical objective 2. Additionally the test chromosome retains the ability to be rapidly modified by site-specific reconstruction of the APRT gene, which is essential for meeting our objectives.

To accomplish the intended modification, we are modifying our targeting vector so that it can accept the I-SceI/CCCTAA segment from the second intron in APRT into the sequences upstream of the GPT gene. Moving the I-SceI/CCCTAA segment into this restriction site will place it between the TK and GPT genes, yielding a targeting vector with all the essential features mentioned above. Although this construction is straightforward, it will nevertheless take us a few months to make the necessary modifications and test them out in cells.

The redesign and construction of the test chromosome was not considered in our original Statement of Work, and will uniformly shift the original schedule.

Can an intron be inserted into the gene for the Green Fluorescent Protein (GFP) gene?

Once we have built a test chromosome in which we can reliably detect chromosome truncation and the seeding of a new telomere, we want to use it not only to determine the minimum length of telomere sequence that can function in the seeding process, but also to detect chromatid fusion, the first step in the fusion-bridge-breakage cycle. To do so, we proposed to insert an intron into the GFP gene, turn the 3' half around relative to the 5' half, and place it on the test chromosome where it will be retained after chromosome truncation. I-SceI cleavage of a chromosome, followed by replication (or cleavage of both chromatids in G2) will expose the cleaved ends for fusion with one another. Fusion will juxtapose two copies of the GFP gene so that the transcript from one 5' half will be oriented correctly for splicing with the 3' half of the other copy, allowing expression of GFP.

To insert an intron in GFP, we scanned the sequence of enhanced GFP (eGFP) to find adjacent elements of the exonic portions of splicing signals that could be united with inserted intronic splicing elements. Several such sequences were found. To construct a functional intron, we used the known splicing elements of adenovirus E2A gene, which are commonly used in an adjacent laboratory. Oligos were synthesized so that we could insert the essential splicing elements by recombinant PCR. In this process we also inserted useful restriction sites in the intron and outside the two halves of the gene so that we can manipulate the two halves independently, as needed. We are now in the process of testing the ability of the construct to express eGFP. Once we have verified the ability of the artificial intron to function appropriately, we will invert the ends and move the construct into our test chromosome.

In our Statement of Work we had planned to be finished with the construction of our color-based test system by the end of the first year. Those plans have been shifted downstream slightly by the necessity to redesign and construct the test chromosome. Once that process is completed, and we have demonstrated truncation and telomere seeding, we will insert the modified eGFP gene and test the color-based detection system for chromatid fusion.

CONCLUSIONS

In this grant period we have shown that I-SceI-induced double-strand breaks in a chromosome can capture a plasmid bearing a telomere sequence, which in turn can seed a new telomere (Table 1). These studies revealed the orientation of the APRT gene on the chromosome, which was not previously known. This is a critical piece of information for the proper construction of the test chromosome. Experiments with chromosome truncation adjacent to an interstitial telomere sequence indicated that chromosome loss is a significant problem to be addressed in the construction of an appropriate test chromosome (Table 2). We have redesigned the test chromosome to take into account the problem of chromosome loss and have begun its construction. The redesigned test chromosome will retain the ability for rapid chromosome modification using site-specific recombination to reconstruct the APRT gene. It will position the positively selectable GPT gene on the centric chromosome fragment so that the truncated chromosome can be anchored against chromosome loss. It will carry the negatively selectable herpesvirus TK gene on the telomeric chromosome fragment so that chromosome truncation can be selected for. Since both the GPT gene and TK gene can be used in breast cancer cells, the modified test chromosome will represent a powerful general system for measuring the ability of different length telomere sequences to seed new telomeres. In addition, we have begun construction of a color-based assay for chromatid fusion, which will complement the studies with interstitial telomere sequence. We anticipate that these studies will yield significant new information on telomere dynamics in breast cancer cells.

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