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AUTHORITY

USAMRMC ltr, 1 Jun 2001.

AD_____

Award Number: DAMD17-97-1-7165

TITLE: Chromatid Paints: A New Method for Detecting Tumor-Specific Chromosomal Inversions

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REPORT DATE: October 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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Patricia A. Hodson 10/5/05

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1999	Final (30 Sep 97 - 29 Sep 99)

4. TITLE AND SUBTITLE
Chromotid Paints: A New Method for Detecting Tumor-Specific Chromosomal Inversions

5. FUNDING NUMBERS
DAMD17-97-1-7165

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, October 1999). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The primary purpose of this project was to identify genes participating in the transformation of a normal cell into a cancerous one. This process frequently is associated with chromosomal changes. Therefore we chose an approach based on methods of molecular cytogenetics. The approach was intended to improve the technology itself and to utilize it in the search for cancer-related genes. Focusing on the relationship between DNA repair and tumor suppression., three discoveries were made. First, we found that the homologous recombination repair genes, xrcc2 and xrcc3, are required to maintain the stable transmission of genetic information from one cell cycle to the next. Thus these genes could be classified as tumor suppressor genes. Second, members of the Ku DNA double-strand break repair pathway are required to cap ends of mammalian chromosomes. When any of these genes is defective, chromosomes are subject to end-to-end fusions. Third, it was found that the BrCa2 gene is required for repair of DNA damage induced by x-rays and mitomycin C. BrCa2 also has a role in preserving chromosomal stability, thus explaining why it is a tumor suppressor. That BrCa2 mutation leads to a profound sensitivity to mitomycin C has potential significance to breast cancer therapy.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, IDEA Award	43
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Edward H. Rothman 11-5-99

PI - Signature Date

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Introduction:

A key goal of this project was to identify genes that are involved in cancer. All cancers have chromosomal aberrations. Specific aberrations are often associated with only one, or a small number, of tumor types. Thus karyotype analysis is useful in establishing a precise diagnosis. Other aberrations arise during the later stages of disease, are associated with more aggressive malignancy, and so are useful as prognostic indicators and for treatment planning. Oncogenes or tumor suppressor genes are frequently observed to be located at the sites of tumor-specific chromosome aberrations, and are known to suffer mutation as a result of chromosomal rearrangement. These observations provide a firm scientific foundation for the clinical use of karyotype analysis. Furthermore, finding new tumor-specific chromosome aberrations is often the initial step in discovering new cancer genes. As such, karyotype analysis is an important tool for advancing our knowledge of carcinogenesis. Despite their known association with cancer and birth defects, chromosomal inversions are perhaps the most difficult of all aberrations to detect on a routine basis. Therefore, it is doubtful that we know the full extent to which inversions are associated with cancer. It was the goal of this research project to develop a new tool for karyotype analysis that we call "chromatid painting". A chromatid paint is a complex mixture of labeled probes prepared using DNA cloned from a specific human chromosome. When hybridized to a metaphase chromosome spread, the probes anneal to multiple target sequences along the length of one chromatid of the chromosome. With fluorescence microscopy, the chromatid appears to be painted. A fluorescent DNA-binding dye counterstains the sister chromatid. The visual effect is that the two halves of the target chromosome fluoresce with different colors. With a chromatid paint, an inversion will appear as a switch in labeling from one chromatid to the other within the inverted region. Since the pattern is easily recognized, chromatid paints would permit rapid identification of inversions. In addition, breakpoints can be localized precisely, and smaller inversions can be detected than is now possible using chromosome banding. Any new aberrations revealed by this method will help to improve diagnostic and prognostic capabilities, and could be the beginning of an effort to identify genes at the breakpoints.

Body:

As stated in the Statement of Work, our first Technical Objective was to perfect the steps in preparing a chromatid paint according to the procedure described in the proposal. This procedure requires directional cloning of chromosomal DNA, PCR amplification, probe preparation, and fluorescence *in situ* hybridization (FISH) painting. The first Technical Objective was divided into tasks designed to test and optimize each step in the procedure. Accomplishments and progress are reported below.

Testing the adequacy of chromosomal DNA as a template for DNA polymerization. The cloning procedure requires copying DNA from fixed cells attached to a glass substrate. Any step in the process potentially could damage the DNA and render it unsuitable as a template. A test was devised for examining the adequacy of chromosomal DNA as a template for DNA polymerization. Chromosomal DNA was made single-stranded by the CO-FISH procedure (1). A short synthetic oligomer was then hybridized to a repetitive DNA sequence in the chromosomal DNA, and a primer extension reaction initiated. During DNA polymerization a fluorescently tagged nucleotide was incorporated into the newly synthesized DNA. This label made it possible to detect DNA polymerization by fluorescence microscopy. The procedure resembles a PRINS

(primed *in situ* synthesis) reaction (2), but unlike PRINS the polymerization was expected to be strand-specific as indicated by a signal on one chromatid for tandem repeats. Two primers were utilized, one to telomeric DNA $(TTAGGG)_n$ and the other to a $(AATGG)_n$ repeat. Results demonstrated that chromosomal DNA prepared in this manner is indeed a suitable template for DNA synthesis. Also confirmed was the 'one-sided' nature expected of fluorescent signals from head-to-tail repetitive sequences (for example, chromosome 1 centromere targeted with the 5-mer) and two-sided signals from repeats that are known to exist in a mixed orientation (chromosome 9 centromere). Using this testing procedure, we optimized our cell fixation protocol to maximize preservation of chromosomal DNA. Best results were achieved by initially fixing cells in methanol and adding acetic acid just before dropping cells onto coverslips. The procedure itself is a novel and useful addition to the techniques available to molecular cytogenetics.

Directional cloning from cells on a coverslip. Our goal was to directionally clone chromosomal DNA, *i.e.* copy and amplify DNA segments from a single-stranded chromatid, according to the procedure described in the proposal. Hamster/human hybrid cells containing one human #11 chromosome were fixed and dropped onto microscope coverslips. These cells had been synchronized by mitotic shake-off so that most were in the G_1 phase of the cell cycle. Chromosomal DNA was made single-stranded by the CO-FISH technique (1). Upstream (5') and downstream (3') oligomers were hybridized to the single-stranded chromatids. These oligomers contained flanking primer hybridization sites for later PCR amplification. DNA polymerization and ligation joined the up- and down-stream oligomers. The up-stream oligomer had an Alu binding site allowing specificity for human DNA. Single cells were isolated on fragments of the coverslip and placed into a PCR tube for amplification. Analysis of the product by gel electrophoresis and quantification by spectrophotometry indicated successful cloning and amplification.

DNA amplification from small amounts of template. The quantity of DNA available for PCR amplification is expected to be small, *i.e.* ~1pg. While successful PCR amplification starting from such small amounts of template has been reported previously, the procedure is not routine. In addition, PCR artifacts are potentially more troublesome in the preparation of chromatid paints, which need to be strand specific, as opposed to chromosome paints where strand specificity is not an issue. We therefore sought to optimize DNA amplification starting with 1 pg of a defined template. Analysis of the amplified product by gel electrophoresis allowed us to detect PCR artifacts. We learned that one of our PCR primers had a tendency to produce artifacts in blank (no template) reactions. A new primer was designed and tested with better results. Various PCR conditions (annealing temperature, number of cycles, buffer, etc.) were tested in order to optimize the reaction. We found that the type and quantity of DNA polymerase was important to achieving successful amplification of template without producing excessive PCR artifacts. Our best results were obtained by initializing the PCR amplification with a small amount of Pfu-turbo thermostabile DNA polymerase and a low primer concentration. After 15 cycles additional primer was added to the reaction along with a larger quantity of the DNA polymerase Ampli-Taq Gold. This second polymerase activates gradually with each successive cycle. This amplification strategy was chosen because excessive polymerase and high primer concentrations have been shown to produce PCR artifacts. We can now successfully perform DNA amplification starting with very small template quantities. However, mispriming remains a problem. Defined as unwanted priming of DNA synthesis, mispriming is caused by primers annealing to other than at

the desired location, or by the 3' end of a template strand hybridizing to an internal portion of another template strand. Mispriming is not a seroius problem with ordinary chromosome paints since the PCR product still contains amplified chromosomal DNA. On the other hand, mispriming destroys the strand specificity that is required for a chromatid paint. Mispriming during PCR amplification was the single most important factor in limiting our ability to produce a fully useable chromatid paint. The complex mixture of templates derived from directional cloning contains enough sequence homologies to cause significant mispriming no matter how carefully PCR reaction conditions are chosen.

Preparing and testing a chromatid paint. A complex probe (paint) was prepared by performing a single-stranded DNA amplification using a single primer. The probe was labeled by incorporating the fluorescently tagged nucleotide, Cy3-dCTP, during amplification. The probe was painted (hybridized) to both human cells and the chromosome 11 hybrid cells. Early results showed that in the hybrid cells the human chromosome was clearly painted, while the hamster chromosomes showed little fluorescence. The human cells showed a contrast in fluorescence intensity between different chromosomes, but all were labeled to some extent. Of particular concern, these early experiments did not show differential fluorescence intensity between sister chromatids as would be expected of a chromatid paint. We attributed this negative result to three possible factors, cloning of some hamster DNA, Alu hybridization during painting that was not completely blocked by our painting protocol, and mispriming during PCR amplification. We attempted to solve the first two potential problems by modifying our painting protocol to include blocking with human Cot 1 DNA, hamster Cot 1 DNA, and a synthetic oligomer having the same sequence as the Alu binding site in the up-stream oligomer. With these changes, hybridizations improved to the point where a pattern consistent with that expected of a chromatid paint was observed in some, but not all cells. While these results were encouraging, further modifications did not significantly improve the early results. The most likely problem is that mispriming links together cloned DNA segments into opposite orientations, thus destroying the strand specificity required of a chromatid paint. In order to minimize this problem, we devised new strategies as discussed below.

Microdissection approach to directional cloning. Low template quantity has been shown to be a source of PCR artifacts. We considered several strategies for increasing template quantity, and chose to pursue an approach based on chromosomal microdissection. Initial steps in the procedure, *i.e.* primer extension and ligation, are unchanged. However, in an additional step a centromeric probe is hybridized. This probe both identifies a specific chromosome and distinguishes between the two sister chromatids.

It is desirable (although not absolutely necessary) to microdissect and pool only chromatids that had not engaged in a sister chromatid exchange (SCE). For the CO-FISH procedure, normally cells are grown for one cell cycle in bromodeoxyuridine (BrdU). In these "first cycle cells" SCE can not be visualized. We therefore worked with cells grown in BrdU for two cycles in an attempt to distinguish between the singly and doubly substituted chromatids. Since it is desirable to simultaneously detect both the fluorescent probe and SCE, we counterstained with the fluorescent dye DAPI. It was observed that DAPI staining resulted in poor differentiation between sister chromatids. Another fluorescent dye, SYBR Green II, was tested. This dye shows enhanced fluorescence yield when bound to single-stranded DNA. The result was a very strong differentiation between sister chromatids. This procedure for simultaneously detecting fluorescent

probes and SCE has not been described before. In addition to serving our immediate needs, we have every reason to believe that it will be a useful technique in its own right.

Using microdissection several chromatids having the same DNA polarity were scraped from cells attached to a coverslip and placed into a PCR tube. This work was done in collaboration with Dr. Joel Bedford of Colorado State University, an expert in microdissection. We also attempted to adapt an in-house micromanipulation facility for this purpose, but had better results working with Dr. Bedford and his technical staff. Microdissected DNA was PCR amplified, labeled and hybridized to human cells. Results were about the same as achieved earlier. Again mispriming is the most likely problem.

Cytogenetic evidence that DNA repair genes as tumor suppressor genes. The most important goal of this project was to identify genes that participate in the development of tumors. On the basis of data linking DNA double-strand break (dsb) repair gene defects to cancer proneness, dsb repair genes had been suggested to act as tumor suppressor genes. However, the mechanism of action remained elusive. We took the opportunity to work with several experts in the field of DNA repair to pursue an investigation this problem. Molecular cytogenetic techniques, some developed in the PI's laboratory, were used to determine how chromosomal stability depends on dsb repair genes. Chromosomal stability refers to the stable inheritance of genetic information through the precise duplication and segregation of chromosomes as cells go through the cell division cycle. DNA dsb repair gene defects have the potential to disrupt the orderly transmission of genetic information through the genesis of chromosome aberrations.

Cells with defects in the xrcc2 and xrcc3 genes were the first to be investigated. These genes are required for DNA repair by homologous recombination. The work was performed by a Postdoctoral Research Associate, Dr. Xiao Cui, in Dr. Goodwin's laboratory. Colonies derived from single cells were subjected to karyotype analysis. This procedure allows us to pinpoint which aberrations were generated during the course of clonal expansion. Dr. Cui observed that deficiencies in either xrcc2 or xrcc3 resulted in a state of chromosomal instability characterized by the frequent occurrence of spontaneously arising chromosomal rearrangements. Chromosomal instability may be a driving force in carcinogenesis since it can lead to the kinds of chromosome aberrations that are observed in human tumors. These aberrations can cause amplification of oncogenes or deletion of tumor suppressor genes. The link between xrcc2 and xrcc3 genes deficiency and chromosomal instability identifies these genes as tumor suppressors and provides a mechanistic explanation for their action. This work was published in Mutation Research (3).

A second investigation was carried out by Ms. Susan Bailey, a graduate student at the University of New Mexico. Dr. Goodwin is Ms. Bailey's research advisor. For her thesis, Ms. Bailey has been investigating the role of DNA dsb repair genes in normal telomere maintenance and function. The investigation focussed on an essential function of telomeres, the ability to cap chromosome ends and thereby prevent illegitimate recombination. Mouse cell lines having defects in one of several genes (Ku70, Ku80, DNA-PKcs, p53, PARP, and ATM) were examined by FISH using a telomere probe. Defects in three of these genes (Ku70, Ku80 and DNA-PKcs) led to the appearance of highly unusual chromosome aberrations. These aberrations are characterized by telomere-to-telomere end joining called telomeric fusions. In contrast, repair-proficient cells never had telomeric fusions. These observations were the first to demonstrate this new role for DNA dsb repair genes. They also revealed a previously unsuspected form of chromosomal instability that has the potential to influence tumor development by creating a genetic imbalance.

This finding requires further study. A manuscript describing this work has been accepted for publication in the Proceedings of the National Academy of Sciences (4).

A third investigation was carried out in collaboration with Dr. Mark Brenneman, who was a Postdoctoral Research Associate at Los Alamos National Laboratory for two years before taking a similar position at the University of New Mexico. Dr. Brenneman is an expert on DNA repair by homologous recombination, and has a special interest in the BrCa2 tumor suppressor gene. He wanted to test the hypothesis that BrCa2 acted as a tumor suppressor because it had a role in DNA repair. Mouse cells having a mutation in the BrCa2 gene were examined by the clonogenic survival procedure and found to have a moderate sensitivity to ionizing radiation, and a pronounced sensitivity to the crosslinking agent mitomycin C. These experiments confirmed that BrCa2 is a DNA repair gene. Further experiments demonstrated that BrCa2 mutation resulted in spontaneous chromosomal instability, thus providing a mechanistic explanation for BrCa2's role as a tumor suppressor gene. A manuscript describing this work is in preparation (5). BrCa2 mutation is common in breast cancer. The sensitivity to mitomycin C suggests that tumors with BrCa2 mutation may be treatable with this drug or another crosslinking agent. We intend to pursue the implications of this finding in the future.

Key Research Accomplishments:

- Developed new cytogenetic procedures.
- Determined that xrcc2 and xrcc3 DNA repair genes are required to maintain chromosomal stability. These genes are likely to act as tumor suppressors.
- Discovered that DNA double-strand break repair genes are required to cap the ends of mammalian chromosomes thereby preventing illegitimate recombination.
- Determined that the BrCa2 gene is required for DNA crosslink repair. This finding suggests that drugs like mitomycin C may be useful for treating breast cancers having BrCa2 mutations.

Reportable Outcomes:

Manuscripts:

X. Cui, M. Brenneman, J. Meyne, M. Oshima, E. Goodwin and D. Chen, The XRCC2 and XRCC3 repair genes are required for chromosome stability in mammalian cells. Mutation Res. 434(2), 75-88 (1999).

Bailey, S.M., Meyne, J., Chen, D.J., Kurimasa, A., Li, G.C., Lehnert, B.E. and Goodwin, E.H. (1999) DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes. Proc Natl Acad Sci (accepted).

Brenneman, M.A., Cui, T.X., Donoho, G., Goodwin, E.H., Hasty, P. and Chen, D.J. BrCa2 mutation causes hypersensitivity to both gamma rays and mitomycin C, and induces chromosomal instability. (manuscript in preparation).

Meetings: Some parts of this work were presented at meetings of the Society of Toxicology and the International Congress of Radiation Research.

Degrees obtained that were supported by this award: Ms. Susan M. Bailey was partially supported by this grant. She is expected to complete her Ph.D. by August, 2000.

Funding applied for based on work supported by this award: A proposal has been submitted to NIH to continue investigation of the role of DNA repair genes in telomere biology.

Employment or research opportunities applied for and/or received on experiences/training supported by this award: Dr. Xiao Cui applied for and received a position at Sloan-Kettering Memorial Institute.

Conclusions:

Significant progress was been made towards achieving the goal of creating a chromatid paint. In the process, two new and useful cytogenetic techniques were devised. However, a fully utilizable chromatid paint was not realized. We attribute the sole remaining difficulty to mispriming during PCR amplification, and will continue working towards solving this problem. In other work, we investigated the relationship between DNA repair and tumor suppression. Three important discoveries were made. First, we found that the xrcc2 and xrcc3 genes, whose function is in DNA repair by homologous repair, are required to maintain the stable transmission of genetic information from one cell cycle to the next. Thus these genes could be classified as tumor suppressor genes. Second, members of the Ku pathway of DNA double-strand break repair genes are required to cap the ends of mammalian chromosomes. When any of these genes is defective, an unusual type of genomic instability results that is characterized by end-to-end fusions between chromosomes. Third, it was found that the BrCa2 gene is required for repair of DNA damage induced by x-rays and mitomycin C. BrCa2 also has a role in preserving chromosomal stability, thus explaining why it is a tumor suppressor. The discovery that BrCa2 mutation leads to a profound sensitivity to mitomycin C has potential significance to breast cancer therapy and will be pursued in the future.

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4. Bailey, S.M., Meyne, J., Chen, D.J., Kurimasa, A., Li, G.C., Lehnert, B.E. and Goodwin, E.H. (1999) DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes. Proc Natl Acad Sci (in press).

5. Brenneman, M.A., Cui, T.X., Donoho, G., Goodwin, E.H., Hasty, P. and Chen, D.J. BrCa2 mutation causes hypersensitivity to both gamma rays and mitomycin C, and induces chromosomal instability. (manuscript in preparation).

Personnel receiving pay from the research effort.

Edwin H. Goodwin, Ph.D.
Julianne Meyne, Ph.D.
Susan M. Bailey, M.S.
Xiao Cui, Ph.D.

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DNA Repair

Mutation Research 434 (1999) 75–88

Accelerated publication

The *XRCC2* and *XRCC3* repair genes are required for chromosome stability in mammalian cells

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Received 21 December 1998; received in revised form 4 March 1999; accepted 5 March 1999

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The *XRCC2* and *XRCC3* repair genes are required for chromosome stability in mammalian cells

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Received 21 December 1998; received in revised form 4 March 1999; accepted 5 March 1999

Abstract

The irs1 and irs1SF hamster cell lines are mutated for the *XRCC2* and *XRCC3* genes, respectively. Both show heightened sensitivity to ionizing radiation and particularly to the DNA cross-linking chemical mitomycin C (MMC). Frequencies of spontaneous chromosomal aberration have previously been reported to be higher in these two cell lines than in parental, wild-type cell lines. Microcell-mediated chromosome transfer was used to introduce complementing or non-complementing human chromosomes into each cell line. irs1 cells received human chromosome 7 (which contains the human *XRCC2* gene) or, as a control, human chromosome 4. irs1SF cells received human chromosome 14 (which contains the *XRCC3* gene) or human chromosome 7. For each set of hybrid cell lines, clones carrying the complementing human chromosome recovered MMC resistance to near-wild-type levels, while control clones carrying noncomplementing chromosomes remained sensitive to MMC. Fluorescence in situ hybridization with a human-specific probe revealed that the human chromosome in complemented clones remained intact in almost all cells even after extended passage. However, the human chromosome in noncomplemented clones frequently underwent chromosome rearrangements including breaks, deletions, and translocations. Chromosome aberrations accumulated slowly in the noncomplemented clones over subsequent passages, with some particular deletions and unbalanced translocations persistently transmitted throughout individual subclones. Our results indicate that the *XRCC2* and *XRCC3* genes, which are now considered members of the *RAD51* gene family, play essential roles in maintaining chromosome stability during cell division. This may reflect roles in DNA repair, possibly via homologous recombination.

Keywords: *XRCC2*; *XRCC3*; Chromosomal stability; Chromosome rearrangement; Human–hamster hybrid cells; DNA repair; Homologous recombination; Microcell-mediated chromosome transfer

1. Introduction

The X-ray repair cross complementing (XRCC) genes correct the phenotypes of certain mutated rodent cell lines for sensitivity to ionizing radiation and other DNA damaging agents. The human *XRCC2*

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0921-8777/99/$ - see front matter
PII: S0921-8777(99)00010-5

and *XRCC3* genes were originally identified by their ability to complement the irs1 and irs1SF mutant cell lines isolated from V79 and AA8 Chinese hamster cells respectively [1–5]. The irs1 and irs1SF cell lines have similar phenotypes of sensitivity to DNA-damaging agents. Both are moderately sensitive to X-ray or gamma radiation (~ 2-fold), to UV radiation (2- to 3-fold), and to ethylmethanesulfonate (EMS, 2- to 10-fold) [1,2]. However, both show extreme sensitivity to DNA cross-linking agents, such as cisplatin and nitrogen mustard [6] and particularly mitomycin C (MMC, 60- to 100-fold) [1,3,4]. In addition, both irs1 and irs1SF suffer increased rates of spontaneous and X-ray-induced chromatid and chromosome breaks [2,3,7].

The complementing human genes have been cloned recently [3,8,9]. Sequence analysis of the human *XRCC2* and *XRCC3* genes has revealed that both bear homology to the *RAD51* genes of yeast and mammals [8,9]. The *RAD51* protein plays a critical role in repair of DNA double-strand breaks by homologous recombination in the yeast *Saccharomyces cerevisiea* [10], and is well conserved in higher eukaryotes [11–13]. The realization that *XRCC2* and *XRCC3* belong to the *RAD51* family of genes has fueled speculation that they also function in a pathway for DNA repair by homologous recombination.

Chromosome instability in the irs1 and irs1SF cell lines has been investigated by following the fate over many cell generations of human marker chromosomes introduced via microcell-mediated chromosome transfer. This method offers advantages over an assessment using only the endogenous chromosomes. Because a human marker chromosome can be painted with a human-specific fluorescent probe, any alteration to it can be readily seen against the background of hamster chromosomes. Breakage, deletion and translocation events involving the marker chromosome can be scored unambiguously. By following alterations of a marker chromosome over the expansion of multiple clonal cell populations, and applying a statistical analysis, it becomes possible to characterize chromosome instability quantitatively in a mutant cell line. The *XRCC2* and *XRCC3* loci have previously been mapped to human chromosomes 7q36 and 14q32.3 [3–5]. Chromosome transfer was used to introduce one copy of human chromosome 7 or 14 into irs1 and irs1SF mutant cells, respectively. Noncomplementing control chromosomes (4 and 7) were also transferred into irs1 and irs1SF cells. The results demonstrate that the *XRCC2* and *XRCC3* genes are essential for chromosome stability.

2. Materials and methods

2.1. Cell culture and microcell-mediated chromosome transfer

Human–hamster hybrid cells were grown in alpha-Modified Eagle's Medium (Gibco) with 10% fetal bovine serum, 100 units/ml of penicillin (Gibco) and 100 μg/ml of streptomycin (Gibco), 0.5 μg/ml Fungizone (Gibco), and 400 μg/ml G418 (Gibco). Cultures were maintained at 37°C in a humidified incubator with 5% carbon dioxide. Mouse A9 hybrid cell lines carrying single human chromosomes with a neomycin resistance marker (*NEO*) have been established and previously described [14]. Chromosome transfer was carried out according to the method of Kurimasa et al. [15]. Briefly, A9-human hybrid cells were treated with colcemid (0.05 μg/ml) for 48 h to form microcells, which were then harvested by centrifugation and filtration, and fused with irs1 or irs1SF cells using polyethylene glycol. G418 at 800 μg/ml was used to select for the *NEO* marker on transferred human chromosomes in hybrid cells. Hybrid clones of G418-resistant irs1 or irs1SF cells were isolated and transferred into T25 flasks for expansion. Each primary clone was screened by fluorescence in situ hybridization (FISH) of metaphase chromosome spreads for the presence of a single human chromosome. For passage of primary hybrid clones, confluent cultures were trypsinized and one tenth to one fifth of the cells were reseeded to a fresh T25 flask. For subcloning of primary clones, 100 cells were plated per 10 cm culture dish. After 10 to 14 days, secondary clones arose and were transferred individually to T25 flasks for expansion.

2.2. MMC exposure

Wild-type cell lines or complemented clones (V79, and irs1 with chromosome 7; AA8, and irs1SF with

chromosome 14), were plated for colony formation assays at 200 cells/10 cm dish, with MMC (Sigma, St. Louis, MO) at 0, 50 and 100 nM. Mutant cell lines or noncomplemented clones (irs1, or irs1 with chromosome 4; irs1SF, or irs1SF with chromosome 7) were plated at 200 and at 2000 cells/10 cm dish, with MMC at 0, 10 and 50 nM; at least 3 dishes per treatment. After ten to fourteen days, cell colonies were stained with 0.2% crystal violet in 70% ethanol. Colonies of fifty or more cells were counted.

2.3. Chromosome slide preparation

For chromosome harvest, cells were treated with 0.05 μg/ml colcemid (Gibco) for 1.5 h. The cells were then trypsinized, centrifuged and resuspended in hypotonic saline (0.075 M KCl) at 37°C for 12 min. The cells were fixed in a 3:1 mix of methanol and acetic acid and stored at −20°C. Fixed cell suspensions were transferred to glass slides and allowed to air-dry. For routine observations, chromosome slides were Giemsa stained.

2.4. Fluorescence in situ hybridization painting

After two weeks aging in air, chromosome slides were denatured in 70% formamide in 2 × SSC at 70°C for 2 min. Four single-strand Alu-repeat oligomers were synthesized as probes for human chromosomes:

A. GGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGA;
B. TCGGCCTCCCAAAGTGCTGGGATTACAGGCGTGAGCCACC;
C. GGAGGCTGAGGCAGGAGAATCGCTTGAACCCGGGAGGCGG;
D. CCGCCTCCCGGGTTCAAGCGATTCTCCTGCCTCAGCCTCC.

The four oligos were mixed in a 1:1:1:1 ratio. 0.5 μg/reaction of mixed probe were labeled with Cy3-dCTP (Amersham Life Science) using a terminal transferase reaction (Terminal Transferase Kit, Boehringer Mannheim). A synthetic single strand telomere-repeat oligomer $(TTAGGG)_7$ was labeled by the same method with Oregon green-dUTP (Molecular Probes, Eugene, OR, USA). A hybridization mixture comprising 0.3 μg/ml human Alu probe DNA and 0.25 μg/ml telomere probe DNA in 50% formamide, 2 × SSC, was applied to the slides. After overnight hybridization at 37°C, the slides were washed in 2 × SSC at 42°C three times, 15 min each, and then for 5 min in 1% Triton-X 100, phosphate buffer (pH 8.0). Chromosomes were counterstained with DAPI in antifade solution.

2.5. Statistical analysis

To quantitatively express quantitatively chromosomal change, the Chromosome Instability Index (CII) was applied. CII is defined as the mean number of unique rearrangements per cell in a population expanded from a single cell. CII for control and mutant cells (or, in the present case, complemented and noncomplemented mutant cells) are comparable only between clonal populations expanded for the same number of cell doublings. Statistical analysis and determination of clonal instability was done as follows. From all control (complemented) clones, an average CII ± standard error was calculated. Individual control clones are judged to be stable if their CII falls within a 98% confidence interval from the mean. No more than one clone in a hundred would be expected to exceed the upper boundary of this interval. By pooling data from stable control clones, a representative control CII was calculated. Each repair-deficient clone was then evaluated to determine whether it had a CII greater than the representative control CII, and if so the clone was labeled unstable. In performing this evaluation, an adjustment was made to the representative CII to account for the different lengths of the human marker chromosomes in the control and mutant hybrid cell lines. A 2 × 2 matrix (stable/unstable vs. control/mutant) was constructed. A chi-squared test was applied to determine whether there was a statistically significant difference in the proportions of stable to unstable clones between the control and mutant cell lines. A mutation was judged to induce chromosome instability only when the chi-squared test indicates a significant difference. A *t* test was also applied to compare the average CII between complemented and noncomplemented cell lines.

3. Results

3.1. Correction of MMC sensitivity and cloning efficiency in XRCC2- and XRCC3-complemented human–hamster hybrid cell clones

After microcell-mediated chromosome transfer, four groups of human–hamster hybrids, each carrying a single human chromosome, were isolated and confirmed by G-banding (data not shown). Clones of irs1 cells carrying human chromosome 7 (which contains the *XRCC2* gene) or human chromosome 4 (as a control) were designated irs1-C7 and irs1-C4 respectively. Clones of irs1SF cells carrying human chromosome 14 (which contains the *XRCC3* gene) or human chromosome 7 (as a control) were designated irs1SF-C14 and irs1SF-C7 respectively.

To determine whether appropriate human chromosomes could correct the mutant phenotypes of irs1 and irs1SF cells, several independent hybrids of each group were tested for resistance to MMC. Testing for complementation could also have been done by measuring resistance to ionizing radiation. However, while irs1 and irs1SF are only moderately sensitive to radiation, they are very highly sensitive to MMC. Recovery of MMC resistance therefore is a more sensitive test of complementation.

MMC sensitivity was measured in four irs1-C7 clones and three irs1-C4 clones, and these were compared to the sensitivities of irs1, and to V79, the parental cell line from which the irs1 mutant was derived. The relative sensitivities of the four cell types to MMC are shown in Fig. 1. The irs1-C4 clones were no more resistant to MMC than irs1 itself. The irs1-C7 clones recovered resistance to levels that appear slightly higher than those of V79, but no statistical significance can be attached to the difference. The irs1 and irs1SF cell lines also show reduced cloning efficiency relative to the parental lines from which they were derived. Cloning efficiencies (C.E.) were therefore measured as an additional indicator of complementation. The C.E. for irs1-C7 was 0.79 ± 0.02 (average $\pm$ standard error of 4 clones); somewhat lower than for wild-type V79 (0.92 ± 0.02). Curiously, the C.E. for irs1-C4 was only 0.28 ± 0.04 (average of 3 clones); even lower than for irs1 (0.50 ± 0.01).

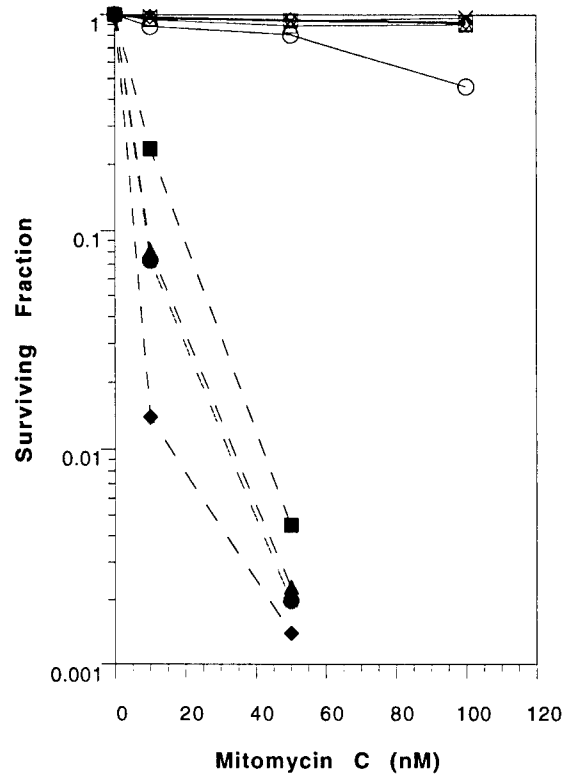

Fig. 1. MMC sensitivity of *XRCC2* complemented and noncomplemented cell clones. Four clones with human chromosome 7, irs1-C7#4 (□), irs1-C7#5 (◇), irs1-C7#7 (△) and irs1-C7#8 (×) showed resistance to MMC close to that of the wild type V79 (○), while three clones with human chromosome 4, irs1-C4#3 (●), irs1-C4#4 (▲), and irs1-C4#6 (◆) retained a sensitivity to MMC close to that of the mutant parent irs1 (■).

Similarly, MMC sensitivity and C.E. were measured in five irs1SF-C14 clones and four irs1SF-C7 clones, and these were compared to the sensitivity of irs1SF and of AA8, the parental cell line from which the irs1SF mutant was derived. Relative MMC sensitivities are shown in Fig. 2. The irs1SF-C7 clones were not consistently more resistant than irs1SF itself. The irs1SF-C14 clones substantially recovered MMC resistance, though not fully to the level of parental AA8 cells. The incomplete complementation seen here by chromosome transfer is similar to that reported previously for complementation by cDNA expression [3,9]. Incomplete complementation in this instance cannot be attributed to the absence of regulatory elements, since a complete chromosomal locus was transferred. The C.E. of irs1SF-C14 ($0.68 \pm$

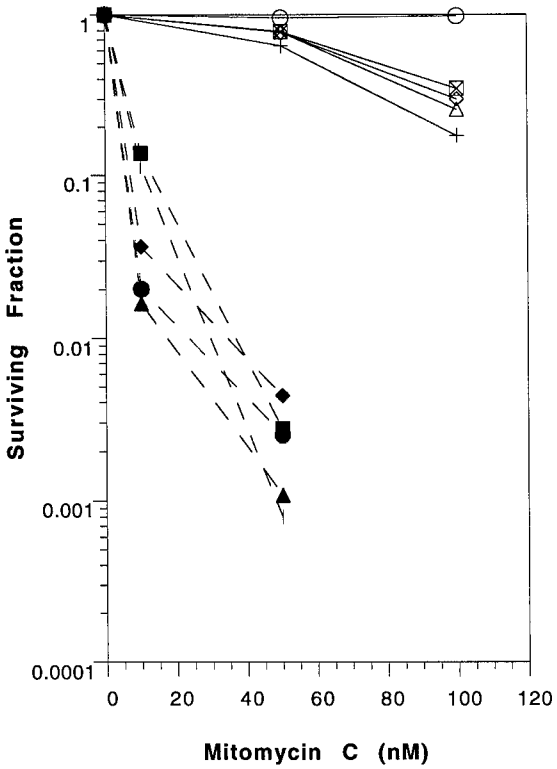

Fig. 2. MMC sensitivity of *XRCC3* complemented and noncomplemented cell clones. Five clones with human chromosome 14, irs1SF-C14#1 (□), irs1SF-C14#2 (◇), irs1SF-C14#3 (×), irs1SF-C14#6 (+), and irs1SF-C14#7 (△) showed increased resistance to MMC, though less than that of the wild type AA8 (○), while four clones with human chromosome 7, irs1SF-C7#1 (●), irs1SF-C7#2 (■), irs1SF-C7#3 (◆) and irs1SF-C7#4 (▲) retained a sensitivity to MMC close to that of the mutant parent irs1SF (|).

0.02; average of 5 clones) was nearly that of AA8 cells (0.74 ± 0.02). The C.E. of irs1SF-C7 was much lower (0.28 ± 0.02; average of 4 clones) and very similar to that of irs1SF (0.22 ± 0.01).

Thus human chromosomes 7 and 14, carrying the *XRCC2* and *XRCC3* repair genes respectively, are able to complement the MMC sensitivity of irs1 and irs1SF mutant cells (at least partially) and increase C.E. to near wild type levels.

3.2. Correction of chromosome stability in XRCC2- and XRCC3-complemented cells

The stability of the human marker chromosome was assessed in ten irs1-C7 clones, eight irs1-C4 clones, nine irs1SF-C14 clones and eight irs1SF-C7 clones. After chromosome transfer, individual hybrid colonies were expanded to a confluent T25 culture flask; estimated to represent at least twenty cell generations. FISH painting with a human Alu DNA probe was used to identify the single human chromosome against a background of hamster chromosomes (Fig. 3). Chromosome aberrations involving the human marker chromosome were scored in 100 metaphase cells for each clone. It should be noted that all hybrid cell lines in this study were maintained under G418 selection for the *NEO* gene on the human marker chromosomes. Chromosome rearrangements that result in loss of the *NEO* gene could not be transmitted under these conditions, and hence were not scored.

The results for irs1-C7 (*XRCC2*-complemented) and irs1-C4 (noncomplemented) clones are presented in Table 1. In irs1-C7 clones, only 13 cells with rearrangements of the human chromosome were found among the 1000 metaphase cells examined; a frequency of 1.3%. In contrast, irs1-C4 clones showed rearrangements of the human chromosome at a frequency of 39.5%, about thirty-fold higher. Several types of chromosome rearrangements were seen in irs1-C4 cells, including breaks, deletions (including terminal and interstitial deletions), balanced translocations (trans I), unbalanced translocations (trans II) and insertions (portions of the human chromosome inserted into hamster chromosomes). Chromosome 'gaps' cannot be reliably resolved by fluorescence microscopy, and so were not scored.

The average CII of irs1-C7 (complemented) clones was 0.013 ± 0.003. For irs1-C4 (noncomplemented) clones, mean CII was 0.325 ± 0.094, about twenty-one times higher when adjusted for different lengths of the human marker chromosome. By t test, the difference is significant to a level of $p < 0.005$. The CII for each of the irs1-C7 clones was then individually compared to the mean for the group (control mean). None of the ten clones exceeded the mean by more than 2.3 standard deviations (the upper boundary of a 98% confidence interval), and hence all were considered to be stable. CII for individual irs1-C4 clones were then compared to the control mean. All eight irs1-C4 clones exceeded the mean by more than 2.3 standard deviations, and so were judged unstable. A chi-square test was used to com-

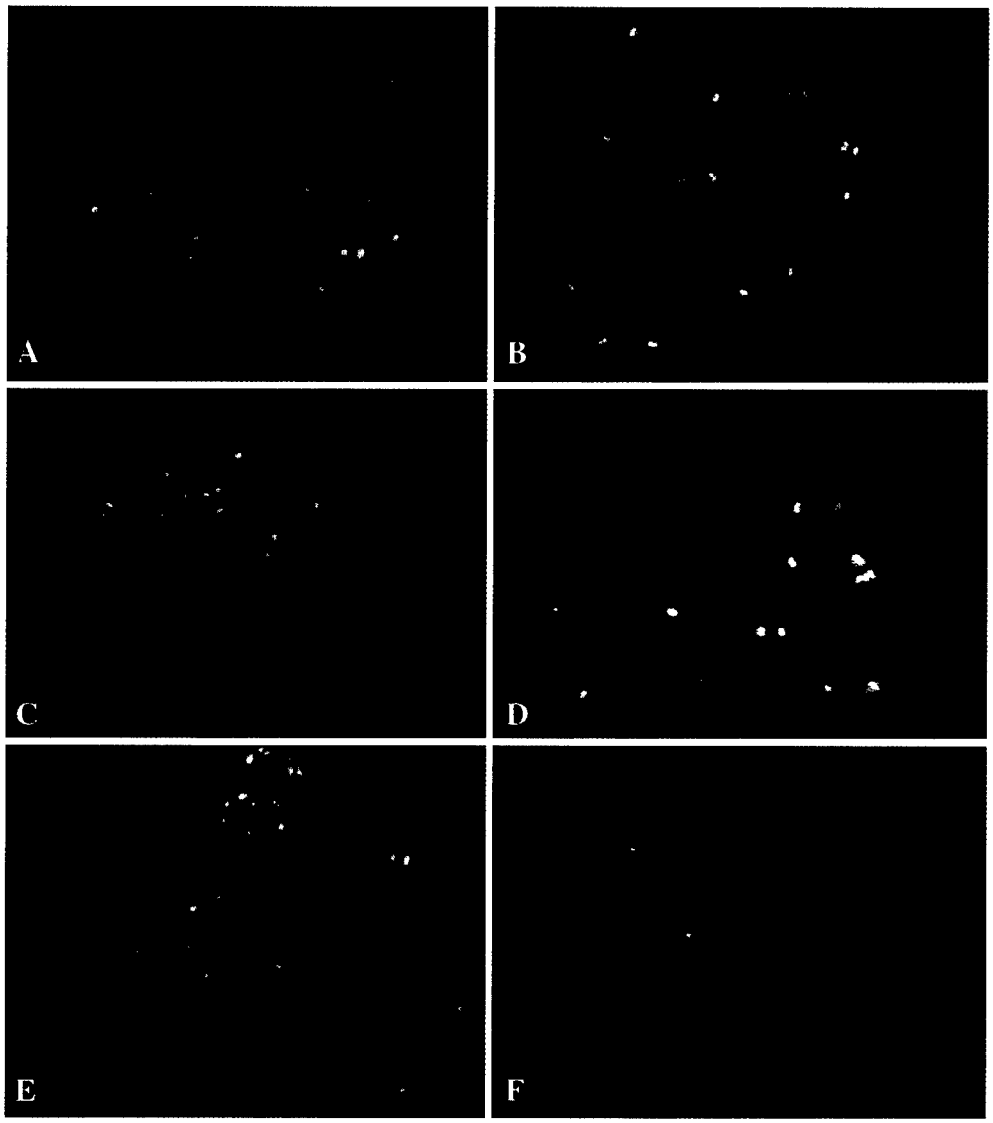

Fig. 3. Rearrangement of the human marker chromosome in human–hamster hybrid cells after FISH. The human chromosome has been hybridized with a human Alu probe (red). Hamster chromosomes appear blue. ITB (interstitial telomere band) are visualized using a mammalian telomeric probe $(TTAGGG)_7$ (green). A to F show different types of rearrangements that occurred in the human marker chromosome in noncomplemented *XRCC2* or *XRCC3* mutant cells: A. normal human chromosome 7 in an irs1SF-C7 cell; B. a fragment of human chromosome 7 inserted into a hamster chromosome in an irs1SF-C7 cell; C. a break in human chromosome 7 in an irs1SF-C7 cell; D. a balanced translocation with an ITB proximal to the junction site in an irs1SF-C7 cell; E. an unbalanced translocation in an irs1-C4 cell; F. an unbalanced translocation with an ITB proximal to the junction site in an irs1SF-C7 cell.

pare the irs1-C7 and irs1-C4 clones in aggregate. A significantly greater proportion of irs1-C4 clones were unstable than of irs1-C7 clones ($p < 0.001$).

A similar trend is apparent when irs1SF-C14 (*XRCC3*-complemented) clones and irs1SF-C7 (non-complemented) clones are compared (Table 2). Among 900 irs1SF-C14 metaphases, only 3 were found with rearrangements of the human chromosome, a frequency of 0.33%. For irs1SF-C7 clones, the frequency of metaphases with a rearranged hu-

Table 1
Chromosome rearrangements among irs1-C7 (*XRCC2*-complemented) and irs1-C4 (noncomplemented) primary clones

Primary clones	Metaphases scored	Metaphases with aberrations (%)[a]	Chromosome instability index[b]	Type of chromosomal aberrations[c]					
				Deletion[d]	Break[e]	TransI[f]	TransII[g]	Insertion	Others
irs1-C7									
#1	100	1	0.01	1	0	0	0	0	0
#2	100	2	0.02	2	0	0	0	0	0
#3	100	2	0.02	2	0	0	0	0	0
#4	100	0	0.00	0	0	0	0	0	0
#5	100	1	0.01	0	0	1	0	0	0
#6	100	1	0.01	0	0	0	1	0	0
#7	100	3	0.03	2	0	0	0	0	1
#8	100	1	0.01	1	0	0	0	0	0
#9	100	2	0.02	1	0	1	0	0	0
#10	100	0	0.00	0	0	0	0	0	0
irs1-C4									
#1	100	77	0.77	21	16	39	1	0	0
#2	100	53	0.53	22	0	24	0	0	7
#3	100	5	0.05	0	0	4	1	0	0
#4	100	24	0.22	3	1	4	13/15	0	1
#5	100	59	0.57	5	1	5/7	39	6	1
#6	100	12	0.12	4	0	7	0	1	0
#7	100	78	0.27	4	1	7/46	12/24	2	1
#8	100	8	0.07	4	0	1/2	2	0	0

[a]Only rearrangements of the human chromosome were scored.
[b]CII, the average number of unique rearrangements of the human chromosome within a clone.
[c]Unique rearrangements and total rearrangements scored. For example, '39' (irs1-C4#1 transI) means 39 unique rearrangements; '13/15' (irs1-C4#4 transII) means that 13 of total 15 transII are unique.
[d]Including terminal and interstitial deletions.
[e]Not including gaps.
[f]Balanced translocation.
[g]Unbalanced translocation.

man chromosome was 30.9%, about 93-fold higher. The average CII for irs1SF-C7 (noncomplemented) clones was 0.264 ± 0.064, about 50-fold higher than for irs1SF-C14 (complemented) clones (0.003 ± 0.002). By t test, the difference is significant to a level of $p < 0.001$. When the CII of individual irs1SF-C14 clones were compared to the mean for the group (control mean), all were found to differ by less than 2.3 standard deviations. However, all eight of the irs1SF-C7 clones differed by more than 2.3 standard deviations from the control mean and are hence unstable. By a chi-square test, the difference in proportions of unstable clones among the noncomplemented versus complemented clones is significant to a level of $p < 0.001$.

To further assess stability of the human marker chromosome in *XRCC3*-complemented and noncomplemented cells, one clone of each was subcloned. Subclones were expanded from single cells to a confluent T25 flask; roughly another twenty cell divisions. Table 3 shows results for twenty subclones expanded from primary clone irs1SF-C14 #1, and eighteen subclones expanded from primary clone irs1SF-C7 #1. In the irs1SF-C7 (noncomplemented) subclones, 1052 metaphases bearing an aberrant marker chromosome were found out of 1800, a frequency of 58.4%; markedly higher than the 18% seen in the parent primary clone irs1SF-C7 #1 (Table 2). Although some of the irs1SF-C7 subclones had lower CII than the primary clone from which

Table 2
Chromosome rearrangements among irs1SF-C14 (*XRCC3*-complemented) and irs1SF-C7 (noncomplemented) primary clones

Primary clones	Metaphases scored	Metaphases with aberrations (%)	Chromosome instability index	Type of chromosomal aberrations					
				Deletion	Break	TransI	TransII	Insertion	Others
irs1SF-C14									
#1	100	0	0.00	0	0	0	0	0	0
#2	100	0	0.00	0	0	0	0	0	0
#3	100	1	0.01	0	0	1	0	0	0
#4	100	0	0.00	0	0	0	0	0	0
#5	100	0	0.00	0	0	0	0	0	0
#6	100	2	0.02	0	0	2	0	0	0
#7	100	0	0.00	0	0	0	0	0	0
#8	100	0	0.00	0	0	0	0	0	0
#9	100	0	0.00	0	0	0	0	0	0
irs1SF-C7									
#1	100	18	0.07	1/8	1/4	2	3/4	0	0
#2	100	24	0.23	13	1	1	8/9	0	0
#3	100	63	0.63	31	13	0	10	8	1
#4	100	60	0.41	13/31	1	8	5/6	14	0
#5	100	20	0.20	10	1	2	4	3	0
#6	100	25	0.25	12	4	2	8	0	0
#7	100	26	0.22	8/9	4	4	2/5	2	2
#8	100	11	0.10	6	1	1	4/5	0	0

they were derived, the average CII of irs1SF-C7 subclones was 0.191 ± 0.032, increased from 0.070 for the primary clone. For the irs1SF-C14 (*XRCC3*-complemented) subclones, mean CII was 0.004 ± 0.001. A *t* test showed the mean CII of irs1SF-C7 subclones to be significantly higher than for irs1SF-C14 subclones ($p < 0.001$). None of the individual irs1SF-C14 subclones had CII differing from the control mean by more than 2.3 standard deviations. But among the eighteen irs1SF-C7 subclones, all eighteen had CII exceeding the control mean by more than 2.3 standard deviations. Again, by a chi-square test, the difference in proportions of unstable subclones among the noncomplemented versus complemented groups is significant to a level of $p < 0.001$.

Overall, human marker chromosomes were much more stable in *XRCC2*- and *XRCC3*-complemented clones than in noncomplemented mutant clones. The presence of *XRCC2* and *XRCC3* genes evidently stabilizes human chromosomes 7 and 14 in irs1-C7 and irs1SF-C14 cells. In the noncomplemented irs1-C4 and irs1SF-C7 cells, the absence of wild-type *XRCC2* or *XRCC3* genes is associated with sharply elevated frequencies of spontaneous aberrations in the human marker chromosome.

3.3. Transmissible chromosome aberrations occur in XRCC2- and XRCC3-deficient primary clones and subclones

The presence of persistent chromosome aberrations means that specific aberrations occurring in individual cells early in the expansion of a cell population have been transmitted to successive generations of daughter cells. This phenomenon is apparent in *XRCC2*- and *XRCC3*-deficient primary clones and *XRCC3*-deficient subclones, especially in the latter. For many of these clones, CII is not equal to the percentage of metaphases with aberrations involving the human marker chromosome. This is because metaphases containing the same aberrations are scored as a single event in determining CII. Persistent, or transmissible, chromosome aberrations were seen in four of eight *XRCC2*-deficient irs1-C4 primary clones examined (#4, #5, #7, and #8;

Table 1), and in five of eight *XRCC3*-deficient irs1SF-C7 primary clones (#1, #2, #4, #7 and #8; Table 2). Among subclones of irs1SF-C7#1, twelve of eighteen (subclones A, C, D, F, I, K, L, M, O, P, Q, and R; Table 3) also showed transmissible aberrations. The types of transmissible chromosome aberrations found were mainly deletions and unbalanced translocations. We have not seen persistent chromosome aberrations in the *XRCC2*- and *XRCC3*-complemented irs1-C7 or irs1SF-C14 primary clones or irs1SF-C14#1 subclones, indicating that the few chromosome aberrations seen in these populations

Table 3
Chromosome instability among irs1SF-C14 (*XRCC3*-complemented) subclones of and irs1SF-C7 (noncomplemented) subclones

Secondary clones	Metaphases scored	Metaphases with aberrations (%)	Chromosome instability index	Type of chromosomal aberrations					
				Deletion	Break	TransI	TransII	Insertion	Others
irs1SF-C14#1									
A	100	0	0.00	0	0	0	0	0	0
B	100	2	0.01	0	0	1/2	0	0	0
C	100	1	0.01	0	0	0	1	0	0
D	100	0	0.00	0	0	0	0	0	0
E	100	1	0.01	0	0	0	1	0	0
F	100	1	0.01	0	0	1	0	0	0
G	100	1	0.01	0	0	0	1	0	0
H	100	0	0.00	0	0	0	0	0	0
I	100	1	0.01	1	0	0	0	0	0
J	100	0	0.00	0	0	0	0	0	0
K	100	0	0.00	0	0	0	0	0	0
L	100	0	0.00	0	0	0	0	0	0
M	100	0	0.00	0	0	0	0	0	0
N	100	0	0.00	0	0	0	0	0	0
O	100	1	0.01	1	0	0	0	0	0
P	100	0	0.00	0	0	0	0	0	0
Q	100	0	0.00	0	0	0	0	0	0
R	100	1	0.01	1	0	0	0	0	0
S	100	0	0.00	0	0	0	0	0	0
T	100	0	0.00	0	0	0	0	0	0
irs1SF-C7#1									
A	100	100	0.03	0	0	0	3/100	0	0
B	100	46	0.46	13	14	6	12	1	0
C	100	100	0.02	0	0	0	1/99	1	0
D	100	99	0.03	1	0	0	1/97	1	0
E	100	11	0.11	7	0	1	4	0	0
F	100	93	0.20	3	0	6/13	10/72	1/2	0
G	100	18	0.18	8	0	5	7	0	0
H	100	13	0.13	2	1	5	5	0	0
I	100	98	0.05	0	0	0	5/98	0	0
J	100	21	0.21	6	6	2	7	0	0
K	100	31	0.28	8	1/4	10	9	0	0
L	100	33	0.32	16	2	1	10/11	1	2
M	100	48	0.46	20	4	4	18/20	0	0
N	100	24	0.24	12	3	3	5	1	0
O	100	22	0.20	9/10	0	1	10/11	0	0
P	100	100	0.09	0	0	0	7/98	1	1
Q	100	95	0.29	10	0	3	16/79	3	0
R	100	100	0.14	0	0	3	11/97	0	0

Table 4
Human chromosome rearrangements in *XRCC2*-complemented and noncomplemented cells during extended passage

Passage no.[a]	Metaphases scored	Metaphases with aberrations (%)	Type of chromosomal aberrations					
			Deletion	Break	TransI	TransII	Insertion	Others
irs1-C7#4								
P0	100	0	0	0	0	0	0	0
P3	100	4	1	0	2	1	0	0
P6	100	1	0	0	1	0	0	0
P9	100	2	0	0	0	0	0	2
irs1-C4#3								
P0	100	5	0	0	4	1	0	0
P3	100	13	2	0	2	9	0	0
P6	100	15	2	4	9	0	0	0
P9	100	20	2	2	6	8	1	0

[a]Approximately 3.3 cell divisions for each passage.

generally represent new chromosome changes that have not been transmitted.

3.4. Chromosome instability is transmissible during extended passage of XRCC2- and XRCC3-deficient clones

The results of the subcloning experiment described above showed that chromosome instability was transmissible in one clone of noncomplemented *XRCC3*-deficient cells. To more generally assess the persistence of chromosome instability in *XRCC2*- and *XRCC3*-deficient cells, one clone each of *XRCC2*- or *XRCC3*-complemented and noncomplemented cells was continuously subcultured for several weeks, with harvests for chromosome spreads at intervals. The results are shown in Tables 4 and 5. For *XRCC2*-deficient (clone irs1-C4 #3) cells, the frequency of chromosome aberrations increased from 5% at passage 0 to 20% at passage 9. Here, passage 0 means the primary colony expansion. Similarly in *XRCC3*-deficient cells (clone irs1SF-C7 #1), the frequency of aberrations increased from 18% at passage 0 to 65% at passage 9. In contrast, the human marker chromosomes in *XRCC2*- and *XRCC3*-complemented cells (irs1-C7 #7, irs1SF-C14 #1) were quite stable over extended passage.

Table 5
Human chromosome rearrangements in *XRCC3*-complemented and noncomplemented cells during extended passage

Passage no.[a]	Metaphases scored	Metaphases with aberrations (%)	Type of chromosomal aberrations					
			Deletion	Breaks	TransI	TransII	Insertion	Others
irs1SF-C14#1								
P0	100	0	0	0	0	0	0	0
P3	100	1	0	0	1	0	0	0
P6	100	0	0	0	0	0	0	0
P9	100	0	0	0	0	0	0	0
irs1SF-C7#1								
P0	100	18	8	4	2	4	0	0
P3	100	47	22	4	4	20	0	0
P6	100	55	29	2	6	24	0	0
P9	100	65	29	0	5	29	1	1

[a]Approximately 3.3 cell divisions for each passage.

3.5. Interstitial telomere-like repeats may be preferentially involved in rejoining of chromosome breaks

It has been proposed previously that interstitially located telomere-like repeat sequences ('interstitial telomere bands', ITB) can act as 'hot spots' in the rejoining of chromosome breaks [16–18]. Using FISH with the telomeric repeat $(TTAGGG)_7$ as a probe, we have analyzed interchromosome rejoining events, in which a portion of a human marker chromosome became translocated onto or inserted into a hamster chromosome, for proximity to ITB. ITB signals occurred at subcentric regions as well as other intrachromosomal locations in the endogenous Chinese hamster chromosomes (Fig. 3). We found that interchromosome joints were often in close proximity to ITB (i.e., no separation between them could be resolved by fluorescence microscopy at 1000× magnification) in *XRCC2*- or *XRCC3*-complemented and in noncomplemented cells (Fig. 3). This was the case in four out of seven events (57%) in irs1-C7 cells, in 78 of 314 events (25%) in irs1-C4 cells, in four of nine events (44%) in irs1SF-C14 cells, and in 648 of 1100 events (59%) in irs1SF-C7 cells. The frequent proximity of joints to interstitial telomere-like repeats supports the idea that a rejoining mechanism using these sequences is frequently involved in healing chromosome breaks. However, neither *XRCC2* or *XRCC3* appear to be needed for rejoining events of this kind. Although translocations and insertions were far more frequent in noncomplemented irs1 and irs1SF cells than in complemented controls, the relative proportions of joints in close proximity to telomere-like repeats were not conspicuously different.

4. Discussion

4.1. The XRCC2 and XRCC3 genes are required for stable maintenance of an introduced human chromosome

It has previously been observed that the chromosomes of irs1 and irs1SF cells show elevated numbers of gaps, breaks and rearrangements relative to the respective parental cell lines V79 and AA8. In irs1 cells, Tucker et al. [7] reported a higher frequency of spontaneous breaks and exchanges, and further increases after treatment with gamma rays or MMC. Increased formation of ring chromosomes in irs1 cells after X-irradiation has been noted by Okayasu et al. [19]. Full or partial correction of the spontaneous chromosome instability in irs1 cells after transfection with cDNA or genomic clones of the *XRCC2* gene has been reported by Cartwright et al. [8] and by Liu et al. [9]. In irs1SF cells, an excess of both spontaneous and X-ray induced chromosome aberrations was noted in the original description of the cell line [2], and full correction of this defect after transfection with a cosmid clone of the genomic *XRCC3* locus was reported by Tebbs et al. [3].

In this clonal analysis of chromosomal instability in irs1 and irs1SF cells, three questions have been considered: (1) What features of the cytogenetic data best describe chromosomal instability? (2) Is a particular clone unstable? and (3) Do the mutations induce instability? In regard to the first question, we have evaluated chromosome instability on the basis of a CII, defined as the average number of unique rearrangements occurring during the expansion of a population from a single cell. This is a better measure of instability than total aberrations, since the latter contains a variable contribution from transmission of aberrations occurring earlier or later during the expansion. An often overlooked difficulty in regard to the second two questions is that all cell populations are unstable to some extent, in the sense that chromosome aberrations arise spontaneously even in repair-proficient controls. In the method of analysis used here, this complication has been explicitly taken into account both in the definition of clonal instability and in the criterion for classifying a mutation as an inducer of instability.

We have found that human chromosome 7 (which contains the *XRCC2* gene) and human chromosome 14 (which contains the *XRCC3* gene) complemented irs1 and irs1SF cells for chromosome stability in primary clones and during extended passage. Among noncomplemented irs1 and irs1 SF clones, we found significantly higher mean CII; about 21-fold higher than complemented controls for irs1 and about 50-fold higher for irs1SF, when adjusted for the differences in marker chromosome lengths. Every individual noncomplemented primary clone was unstable by our statistical criteria and, in aggregate, the noncom-

plemented clones were more unstable than complemented controls with a very high degree of statistical certainty. Persistent chromosome rearrangements were seen in many of the noncomplemented primary clones. Subcloning and extended passage confirmed that chromosome instability is transmissible in noncomplemented clones of irs1 and irs1SF.

The phenotypes of noncomplemented irs1 and irsSF clones are very similar, but analysis of the data in Tables 1 and 2 indicates that they are not identical. There is a statistically significant difference ($p < 0.001$) in the proportions of balanced translocation and unbalanced translocation, with a greater proportion of balanced translocation in irs1-C4 clones, and of unbalanced translocation in irs1SF-C7 clones. There are also significantly more deletions in irs1SF-C7 clones ($p < 0.001$). While there was a tendency toward a higher proportion of breaks in irs1SF-C7 clones, the difference falls short of significance ($p \sim 0.06$) mainly due to an exceptionally large number of breaks in one irs1-C4 clone. Taken together, the cytogenetic data suggest a greater tendency for incomplete repair in the irs1SF-C7 mutant line. This implies that, while *XRCC2* and *XRCC3* genes probably act in the same DNA repair pathway, the role of *XRCC3* may be more critical than that of *XRCC2*. However, it is also possible that the more severe phenotype of the irs1SF mutant relative to irs1 is due to differences in genetic background between their parental cell lines V79 and AA8.

4.2. *Chromosome instability in XRCC2- and XRCC3-deficient cells may be due to a defect in repair by homologous recombination associated with DNA replication*

In yeast (*S. cerevisiea*), members of the *RAD51* gene family have been shown to play critical roles in repair of DNA double-strand breaks (DSB) through homologous recombination (reviewed in Ref. [20]). This repair pathway is evidently most efficient during S and G2 phases of the cell cycle, when a sister chromatid is available as a template. In both the haploid and diploid states, yeast is more resistant to ionizing radiation during these parts of the cell cycle [20]. In the diploid state, where both a homologous chromosome and a sister chromatid are available as templates for repair, the sister chromatid is used preferentially [21]. Genes involved in homologous recombination are also required in yeast for chromosome stability. Null mutation of *RAD52* in diploid strains of yeast results in frequent loss of chromosomes, and the frequency of loss is dramatically increased by exposure to X-rays, such that chromosome number in survivors may fall to near-haploid levels [22].

Because of their DNA sequence similarity to *S. cerevisiea RAD51*, mammalian *RAD51*-family genes have been proposed to participate in analogous pathways for repair of DSB though homologous recombination [8,9,12]. Chromosome instability has now been correlated with deficiency in three of the mammalian *RAD51*-family genes: *XRCC2* and *XRCC3* (as discussed above), and *RAD51* itself. In mouse, null mutation of *RAD51* by gene targeting results in early embryonic death, and is apparently lethal at the cellular level [23,24]. In early $RAD51^{-/-}$ embryos, growth is severely impaired and the few metaphase cells that can be recovered have sharply reduced chromosome numbers [24]. In a conditional gene knockout in DT40 chicken lymphoblastoid cells, shutdown of *RAD51* expression resulted in arrest of most of the population in G2/M phase of the cell cycle, with numerous chromosome breaks, followed by massive cell death [11]. The defect in chromosome stability arising from *RAD51* knockout is clearly more severe than for the *XRCC2* and *XRCC3* mutations in irs1 and irs1SF (since the latter are viable).

One possible explanation for the phenotypes of *RAD51*, *XRCC2* and *XRCC3* mutations is an essential function for homologous recombination in higher eukaryotes, in a form of repair that is closely coupled to replication and required for its successful completion. That mammalian cells cannot survive without Rad51, even though they are extremely proficient in DSB repair by nonhomologous end-joining [25], implies that the critical function of Rad51 is something more than DSB repair per se. The *XRCC2*- and *XRCC3*-mutant cell lines irs1 and irs1SF show only moderate hypersensitivity to ionizing radiation and no measurable defect in post-irradiation DSB repair [1,2], but nonetheless suffer reduced cloning efficiency and chromosome instability even in the absence of exogenous genomic insults. The irs1 and irs1SF cell lines also show sensitivity to UV, alkylat-

ing agents and especially crosslinking agents [1,2], all of which produce covalent DNA modifications that are barriers to replication [26]. Single-strand damage or breaks have been proposed to require a form of homologous recombination if they are not otherwise repaired before the passage of a replication fork [27,28]. A deficiency in such homologous replication-coupled repair may result in double-strand chromatid breaks, which in turn result in deletions or translocations when repaired nonhomologously. It should be noted that in the irs1 and irs1SF hamster–human hybrids reported here, only one copy of a human marker chromosome was transferred; no homologous chromosome is present. If homologous repair of the human marker chromosome takes place, this could only happen in S/G2 phases of the cell cycle using a sister chromatid as template.

Acknowledgements

This study was supported by funding from the US Department of Energy and by an NIH grant (CA74046) to D.J. Chen, and by a grant from the Ministry of Health and Welfare (Japan) to M. Oshimura. The authors thank Ms. Paige Pardington and Mr. James Fulwyler for their assistance with microcell-mediated chromosome transfer, Dr. Stefan Burde for advice on electronic photo image presentation, and Dr. Jac Nickoloff for helpful discussions.

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Publication information: *Mutation Research, DNA Repair* (ISSN 0921-8777). For 1999 volumes 433–435 are scheduled for publication. Subscription prices are available upon request from the Publisher. Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by surface mail except to the following countries where Air delivery via SAL mail is ensured: Argentina, Australia, Brazil, Canada, Hong Kong, India, Israel, Japan, Malaysia, Mexico, New Zealand, Pakistan, PR China, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. For all other countries airmail rates are available upon request. Claims for missing issues should be made within six months of our publication (mailing) date.

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Accepted for publication in the Proc. Natl. Acad. Sci.

Classification: Biological Sciences, Cell Biology

Title: **DNA Double-Strand Break Repair Proteins Are Required to Cap the Ends of Mammalian Chromosomes**

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Manuscript information: # text pages = 20. # figures = 2. # tables = 3.

Word and Character counts: # words in abstract = 223. total # characters = 44,232.

Abbreviations footnote: dsb, double-strand break; NHEJ, non-homologous end-joining; TF, telomeric fusion; *scid*, severe combined immunodeficiency

ABSTRACT Recent findings intriguingly place DNA double-strand break (dsb) repair proteins at chromosome ends in yeast where they help maintain normal telomere length and structure. In the present study, an essential telomere function, the ability to cap and thereby protect chromosomes from end-to-end fusions, was assessed in repair-deficient mouse cell lines. Using fluorescence *in situ* hybridization (FISH) with a probe to telomeric DNA, spontaneously occurring chromosome aberrations were examined for telomere signal at the points of fusion, a clear indication of impaired end-capping. Telomeric fusions were not observed in any of the repair-proficient controls and occurred only rarely in a p53 null mutant. In striking contrast, chromosomal end fusions that retained telomeric sequence were observed in non-transformed DNA-PK_{cs} deficient cells where they were a major source of chromosomal instability. Metacentric chromosomes created by telomeric fusion became even more abundant in these cells following spontaneous immortalization. Restoration of repair proficiency through transfection with a functional cDNA copy of the human DNA-PK_{cs} gene reduced the number of fusions compared to a negative transfection control. Virally transformed cells derived from Ku70 and Ku80 knockout mice also displayed end-to-end fusions. These studies demonstrate that DNA double-strand break repair genes play a dual role in maintaining chromosomal stability in mammalian cells; the known role in repairing incidental DNA damage, as well as a new protective role in telomeric end-capping.

Telomeres are unique structures at the physical ends of linear eukaryotic chromosomes that were first described over sixty years ago by Muller in the fruit fly *Drosophilia melanogaster* based on their chromosome end protection function (1). Shortly thereafter, McClintock's cytogenetic studies in maize demonstrated that broken chromosomes were subject to end fusions (2). These studies demonstrated that a cell's ability to respond differently to natural chromosome ends than they do to ends created by spontaneous or induced breakage is critical to preserving a stable genetic inheritance (3-5). Modern molecular analysis has revealed that telomeric DNA consists of tandem arrays of short, repetitive G-rich sequence (6-7) oriented 5'-to-3' towards the end of the chromosome, terminating in a 3' single-stranded G-rich overhang (8). Together with associated telomeric binding proteins, a dynamic terminal structure is created that "caps" each end of linear chromosomal DNA molecules, providing protection from illegitimate recombination, exonucleolytic attack and degradation.

In contrast to natural chromosome ends, DNA dsb are highly recombinogenic and represent a major threat to the integrity of the cell's genome because of their potential for causing chromosome aberrations, mutagenesis and carcinogenesis if misrepaired or left unrepaired. In mammalian cells, the majority of dsb are rejoined through repair pathways known as non-homologous end-joining (NHEJ) (9). The Ku70, Ku80 and DNA-PK_{cs} genes are part of the most studied NHEJ pathway. The Ku heterodimer, composed of 70kDa and ~80kDa subunits, is the most abundant DNA end-binding protein in mammalian cells. It recognizes DNA ends whether blunt, overhanging or hairpin in structure, and binds with high affinity in a DNA-sequence independent manner. Mutant cells lacking Ku are deficient in the repair of dsb, as well as in recombination of the immunoglobulin V(D)J region (10). DNA-PK is a nuclear serine/threonine kinase made up of two components, an ~465kDa catalytic subunit (DNA-PK_{cs}) and the Ku heterodimer (11). Yeast also possess NHEJ pathways, crucial components of which appear to be conserved between yeast and mammalian cells (12). For example in *Saccharomyces cerevisiae* (baker's yeast), homologues of both human Ku70 (yKu70 or Hdf1) and Ku80 (yKu80 or Hdf2)

have been identified (13-14). Yeast has no close homologue of DNA-PK_{cs}, but other kinases may play a similar role.

In addition to functioning in the vital process of DNA repair, NHEJ proteins are required for yeast telomere function, maintenance and telomere-associated transcriptional silencing. The absence of the Ku heterodimer affects the perinuclear clustering of telomeres normally seen in wild type yeast cells (15). Furthermore, strains defective in Ku70 or Ku80 lose the majority, but not all, of their terminal telomere repeats (14,16). The roles these proteins play in telomere maintenance and function is currently unclear, but they may contribute to a telomere end-binding complex that recruits other proteins. The recent finding that yKu70 interacts with the yeast silencing protein Sir4p, which in turn interacts with Rap1p, a key regulator of telomere length, supports this idea (17-18). Sir proteins (2p, 3p and 4p) function in transcriptional silencing at telomeres through induction of a condensed, inaccessible heterochromatic state (19). Disruption of Ku function debilitates telomere-associated silencing, and interestingly, mutation of Sir genes disrupts Ku-dependent NHEJ. Ku and Sir proteins present at yeast telomeres relocalize to sites of DNA damage and this movement is controlled by DNA damage checkpoint genes (20-21). It also appears that Ku is critical for maintaining the Sir proteins, and thus transcriptional silencing, at telomeres.

Binding of Ku to mammalian telomeric DNA also has been demonstrated (22). Two human telomere-specific DNA binding proteins, TRF-1 (telomeric repeat binding factor-1) and TRF-2 have also been identified (23). TRF-2 has been shown to protect chromosome termini from end-to-end fusions and is the first telomere-associated protein implicated in the maintenance of the correct terminal DNA structure necessary for proper telomere function (24). An important clue as to what that structure might be was uncovered by the discovery that TRF-2 can remodel mammalian telomeric DNA into large duplex loops, termed t loops (25). A t loop is created when a telomere end loops back and the single-stranded G-rich tail invades an interior segment of duplex telomeric DNA. By sequestering natural chromosome ends, t loop formation may be an effective end-capping mechanism in mammalian cells.

Telomeres, as the physical ends of linear DNA duplexes, run the risk of being identified as dsb in need of repair and ligation. With this in mind, the recent evidence locating several proteins of the cell's repair arsenal at telomeres presents an interesting paradox. Why are DNA repair proteins, capable of binding and joining double-stranded ends, present at the telomere, and how are they involved in normal telomere function and maintenance?

MATERIALS AND METHODS

Cell culture. Early passage non-transformed lung fibroblast cultures were derived from the following male mice; a repair-proficient mouse (C57BL/6TacfBR-[KO]p53N4 WT), two mice having severe combined immunodeficiency (Tac:Icr:Ha(ICR)-scid), a p53 knockout mouse (C57BL/6TacfBR-[KO]p53N4 HO), and two mice with a double $p53^{-/-}$/*scid* mutation that were obtained by crossing p53 knockout mice with *scid* homozygous mice (26). These same cell lines were allowed to undergo spontaneous immortalization by simply maintaining cultures until they overcame senescence. Ku70 and Ku80 deficient mouse fibroblast cell lines were established from C57BL/6 knockout mice (27-28). These cell lines and an isogenic control were transformed with the Abelson murine leukemia retrovirus. Some cells derived from the lung of a *scid* mouse were transformed with SV40 as previously described (29). These *scid* cells were transfected with the pPGDP-8 plasmid, which has an intact cDNA copy of the human DNA-PK_{cs} gene, plus the

pSV2neo plasmid (30). SV40 transformed *scid* cells were also transfected with the pSV2neo plasmid only. As expected, resistance to radiation was restored in cells transfected with the DNA-PK_{cs} gene, but not in the negative transfection control (30). All cells were incubated at 37°C, 5% CO_2 and cultured in alpha MEM supplemented with 20% fetal bovine serum and antibiotics. A 1:10 split ratio was used at each passage.

Fluorescence *in situ* hybridization (FISH). Near confluent cultures were subcultured into fresh medium and incubated at 37°C for 24 hours. Colcemid (0.2μg/ml) was added for four hours to accumulate mitotic cells. Cultures were trypsinized and cells suspended in 75mM KCl for 15 minutes before fixing in 3:1 methanol/acetic acid. Fixed cells were dropped onto cold wet glass microscope slides. A probe to telomeric DNA was made by synthesizing an oligomer having the sequence $(CCCTAA)_7$ and was labeled by terminal deoxynucleotidal transferase tailing (Boehringer Mannheim) with Cy3-dCTP according to the manufacturer's instructions. A hybridization mixture containing 0.4μg/ml probe DNA in 30% formamide, and 2xSSC (1xSSC is 0.15M NaCl, 0.015M sodium citrate) was applied to slides that had been denatured in 70% formamide, 2xSSC at 70°C for two minutes. Following an overnight hybridization at 37°C, the slides were washed in 2xSSC at 42°C and mounted in a glycerol solution containing 1mg/ml of the antifade compound p-phenylenediamine HCl and 0.1μg/ml 4',6-diamidino-2-phenylindole (DAPI) (31-32). Cells were viewed with a Zeiss axiophot fluorescence microscope. Images were obtained using a Photometrics CH250 CCD camera.

Suspected telomere involvement in a chromosome aberration was scored according to three progressively more stringent criteria. The first criterion is that commonly used for scoring telomeric associations, *i.e.* two chromosome termini are separated by no more than the width of a chromatid (i.e., *close*). Since no separation would be expected in a true end fusion, we felt that this criterion might be too lax and so devised two more restrictive standards. The second criterion allowed no visible separation between FISH telomere signals, but did score as telomere associations instances where telomeres were *touching* but still visible as distinct bodies. The third criterion scored as true end fusions only those events where the telomeres of adjoining chromosomes had fused into a *single signal*. In our experience, events scored as telomeric associations occurred in all cell types examined. However, scoring by the second and third criteria resulted in the expected zero background level of events in repair-proficient control samples. Few cases were observed where telomere signals were touching. Therefore we chose to utilize the third, most stringent, criterion in order to reduce false positives to a minimum. We call aberrations meeting this standard "telomeric fusions (TF)" to distinguish them from events scored by other criteria. DAPI bright regions visible on most chromosomes correspond to the mouse major satellite (33) and are a useful indicator of centromere position.

RESULTS

The effectiveness of mammalian telomeric end-capping was evaluated with FISH. Using a telomere probe, spontaneously arising chromosome aberrations were inspected for telomere signal at the point of fusion between two chromosomes, the presence of which gives an unambiguous indication that telomeric end-capping has failed. The appearance of such aberrations, it was reasoned, in a DNA repair-deficient cell line would be a clear demonstration that the mutated gene was required for the normal capping function of telomeres. Mouse cells are especially suitable for this type of analysis since their chromosomes have exceptionally long telomeres, thus yielding bright signals, and are without cytogenetically detectable interstitial blocks of TTAGGG

repeats. The murine model has the additional advantage of genetic diversity, including several DNA repair gene mutants.

Examination of early passage non-transformed lung fibroblasts revealed chromosome aberrations having centrally located telomere signals, indicating a failure of telomeric capping, in each of the dsb repair-deficient cell lines analyzed. Examples of TF are shown in Figure 1. Both chromosome- and (more rarely) chromatid-type TF were observed. In contrast, TF were not observed in repair-proficient controls. At least two independent sets of experiments were conducted with similar findings. Additionally, *scid* and $p53^{-/-}$/*scid* cells were obtained from two different mice. Combined results are summarized in Table I.

In the *scid* and $p53^{-/-}$/*scid* cell lines, TF outnumbered ordinary exchange-type aberrations. Dicentric and metacentric TF occurred with about equal frequency. Oddly, chromosomal rings were never seen. This may indicate that interphase chromosome architecture is such as to keep the tips of the long and short arms of a chromosome well separated, thus reducing the probability of their fusing to form a ring. The metacentric chromosomes may have been either isochromosomes or Robertsonian translocations. In mouse, Robertsonian translocations are common, and the DNA sequence at the point of exchange has been studied previously (34-35). The point of joining always appears to lie within mouse minor satellite DNA, the sequence thought to serve as the mouse centromere (36), while the telomeres on the short arms of the two fusing telocentric chromosomes are invariably lost. Thus, the TF Robertsonians (or isochromosomes) observed in DNA-PK_{cs} mutant cells are distinctly different.

Hypothetically, TF might be expected to occur at a rate proportional to telomere length, and were observed in our experiments simply because repair-deficient cells spawn numerous aberrations, a significant fraction of which might be TF due to the extraordinary length of mouse telomeres. That this is not the case can be seen from the following argument. The average mouse telomere length has been estimated to be 20-150 kilo base pairs (kbp) by Southern blot analysis (37) and 10-60 kbp by quantitative FISH (38). Assuming an average mouse telomere length of 100 kbp and haploid G_1 genome size of $3x10^9$ bp, the fraction of the mouse genome that is composed of telomeric DNA is approximately $1.4x10^{-3}$. If spontaneous TF occur in proportion to total telomere length, then about 1 in 700 aberrations would be expected to show telomere involvement. Contrary to expectation, TF were observed ~500 times more frequently than predicted in the both *scid* and $p53^{-/-}$/*scid* cell lines, a difference that statistically is highly significant (χ^2 test, $p<<0.001$).

A single TF chromatid dicentric was seen in 100 $p53^{-/-}$ cells examined. Thus, p53 plays a far less significant role in telomeric capping. In comparison to the $p53^{-/-}$/*scid* double mutant, *scid* cells had more than twice as many exchange-type aberrations per cell. However, the proportion of exchange-type aberrations that were created by fusion between telomeres was not significantly different (χ^2 test, $p>.05$). Thus loss of p53 gene function does not enhance the effect of the *scid* mutation on end capping, implying that TF do not trigger p53-dependent apoptosis or a cell-cycle checkpoint.

Following spontaneous immortalization, these same cell lines were reexamined. Wild type and $p53^{-/-}$ transformed cells did not exhibit TF. However, the numbers of TF were found to have increased sharply in the transformed *scid* and $p53^{-/-}$/*scid* cells (Table II). This increase is attributable to a high frequency of a single type of fusion product, metacentrics created by fusion between telomeres (Figure 2). The number of these aberrations increased still further in *scid* cells observed seven passages later. On the basis of gross chromosomal morphology, two metacentrics

appeared to reoccur in nearly all of the *scid* cells examined. Fewer TF were seen in immortalized $p53^{-/-}$/*scid* cells as compared to *scid* cells, even after adjusting for the lower average number of chromosomes per cell. This finding further indicates that loss of p53 does not enhance the end capping deficiency of the *scid* mutation.

A new type of TF appeared in immortalized DNA-PK_{cs} deficient cells. This new aberration was distinguishable by its having a block of telomeric sequence located within a chromosome arm but not adjacent to a centromere. The frequencies of this aberration, which was called interstitial telomeric sequence (ITS), are recorded separately in Table II.

Spontaneously occurring aberrations were analyzed in a virally transformed *scid* cell line restored to repair proficiency by transfection with the DNA-PK_{cs} gene (SC-A1) and a negative transfection control (SC-EM). SC-A1 cells had statistically fewer TF than the repair deficient SC-EM cells (t test, $p<0.001$) (Table III). Six passages later, TF in repair proficient SC-A1 cells had decreased significantly ($p<0.001$) by nearly three fold while no significant change occurred in SC-EM cells ($p>0.05$). These results indicate that restoration of DNA-PK_{cs} function to *scid* cells improves the efficiency of end capping. Those TF observed in SC-A1 cells presumably were pre-existing in the population at the time of transfection.

A deficiency in either Ku70 or Ku80 clearly results in an overall increase in chromosome aberrations (Table III). These included TF. Thus both Ku genes are required for effective end capping. TF were not seen in the virally transformed repair-proficient control cells, indicating that virus transformation by itself does not compromise end capping.

DISCUSSION

Collectively, these experiments demonstrate a vital, if paradoxical, requirement for three members of the Ku NHEJ repair pathway in mammalian telomere biology. The need for both Ku70 and Ku80 suggests they function in end-capping as a heterodimer just as they do in dsb repair. Yet the Ku heterodimer alone is insufficient to protect telomeres. The data also are consistent with a requirement for DNA-PK_{cs}, which could serve either a structural or enzymatic role, e.g., post-translational modification by DNA-PK of proteins affecting telomeric end-capping.

There was no noticeable reduction in signal brightness between those telomeres that entered into fusions as compared to those which did not. In fact, the brightest observed telomeric signals frequently occurred at the points of joining, as might be expected if no telomeric DNA was lost during fusion. This observation implies that joining is tip-to-tip, and argues against telomere length erosion as the primary cause of impaired capping. This contrasts sharply with the end-to-end associations observed in mouse cells lacking the telomerase RNA component (mTR) (39). These cells have undetectable telomerase activity and exhibit a progressive decrease in telomere length. In $mTR^{-/-}$ mice, Robertsonian chromosomal rearrangements lack pericentromeric telomeric DNA as observed by FISH. An inability to create or maintain a special end structure may be involved in both cases, *i.e.* $mTR^{-/-}$ cells eventually lose too much of the DNA sequence from which the end structure is formed, while dsb repair mutants lack one or more proteins that participate in its construction.

The end-capping function of human telomeres is known to require at least one other protein, the telomere-specific DNA-binding protein TRF-2 (24), apparently through its ability to form t loops at the ends of chromosomes (25). Ku70, Ku80 and DNA-PK_{cs} may function together with TRF-2 in creating t loops. For example, DNA-PK may recruit, and/or influence the activity of, a 5'-to-3' exonuclease at chromosome ends in order to remake 3' overhangs following

DNA synthesis. The mammalian MRE11/RAD50/NBS1 complex is a candidate exonuclease since its yeast counterpart participates in telomere function, and it has been shown to influence 5'-to-3' exonucleolytic activity *in vivo* (40-42). TRF-2 might then complete the process by using 3' overhangs to remodel linear chromosome ends into a looped configuration.

Ku70 and Ku80 knockout mice are small in size and have a short lifespan, whereas *scid* mice have a normal lifespan. This observation implies that, in addition to its role in NHEJ, the Ku heterodimer may have another function not requiring DNA-PK_{cs}. Based on the recently discovered role of Ku in yeast telomere biology, it was proposed that Ku also is required in mammalian telomere biology (9). Our results confirm the proposed role of Ku, but further demonstrate a need for DNA-PK_{cs}, thus they do not support a Ku-related deficiency in telomere function as the sole cause of the shortened lifespan observed in Ku knockout mice.

By impairing end capping, DNA-PK_{cs} deficiency greatly increases illegitimate recombination involving telomeres. Ineffective telomeric capping is an important source of spontaneous instability in a DNA-PK_{cs} deficient background, as can be seen from the large contribution that TF make to total exchange-type aberrations. Fewer TF were observed in the virally transformed Ku knockout cell lines compared to the transformed *scid* and $p53^{-/-}$/*scid* cell lines. Though tempting, this finding does not necessarily indicate a lesser role for the Ku genes, but rather may reflect differences in the cell culture history of the respective cell lines, such as the number of population doublings that occurred before analysis, or genetic background.

The large increase in TF metacentric chromosomes that occurred in spontaneously immortalized *scid* cells was unexpected. These aberrations, which averaged about five per cell, are not lethal to the cells possessing them despite their being dicentrics. However, their progressive loss in *scid* cells restored by transfection to repair proficiency suggests TF metacentrics are only quasi stable. Whether or not a particular TF metacentric is maintained in a growing culture is likely to depend on the potential of that aberration to confer a growth advantage. Interestingly, telomeric associations have been observed previously in a transformed *scid* cell line (43). However, under the less restrictive scoring requirements employed, similar events were recorded in repair proficient controls as well. Under our more stringent scoring criteria, actual fusions were restricted to dsb repair deficient cells, thus clarifying the dependence of these events on genetic background.

The impairment of end-capping associated with dsb repair deficiency is similar to that caused by expression of a mutant form of TRF-2, but is much less severe. Curiously, the severity of TRF-2 dysfunction precludes long-term cellular consequences, since the condition is most likely lethal. In contrast, the milder phenotype associated with dsb repair deficiency allows cells to survive with a genomic instability that is transmitted to future generations. Thus, through their contribution to efficient telomeric end capping, NHEJ repair genes help to preserve the fidelity of genetic inheritance. It is clear that a complete understanding of the mammalian DNA repair-deficient phenotype, and in particular how this phenotype relates to cancer proneness, will need to encompass the new role of repair genes at the telomere.

We thank Drs. Stefan Burde for assistance with image analysis, and Merl Hoekstra for the gift of several DNA repair-deficient mice. This work was supported by grants from the U.S. Army (DAMD 17-97-1-7165), Department of Energy (ERWF645), and NIH (CA50519, CA56909 and CA78497).

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Figure 1. Chromosome aberrations illustrating telomeric fusion (TF). Top left; an image of a chromatid dicentric observed in a *scid* cell. Bottom left; a dicentric chromosome seen in a $Ku70^{-/-}$ cell. Right top and bottom; two examples of metacentric chromosomes seen in *scid* cells. In the upper left hand corner of the top image is a tetra-radial, an ordinary chromatid-type aberration.

Figure 2. Representative metaphase spread of a spontaneously immortalized *scid* mouse fibroblast. Five Robertsonian translocations created by end-to-end fusion are visible in the photograph and are indicated by arrows.

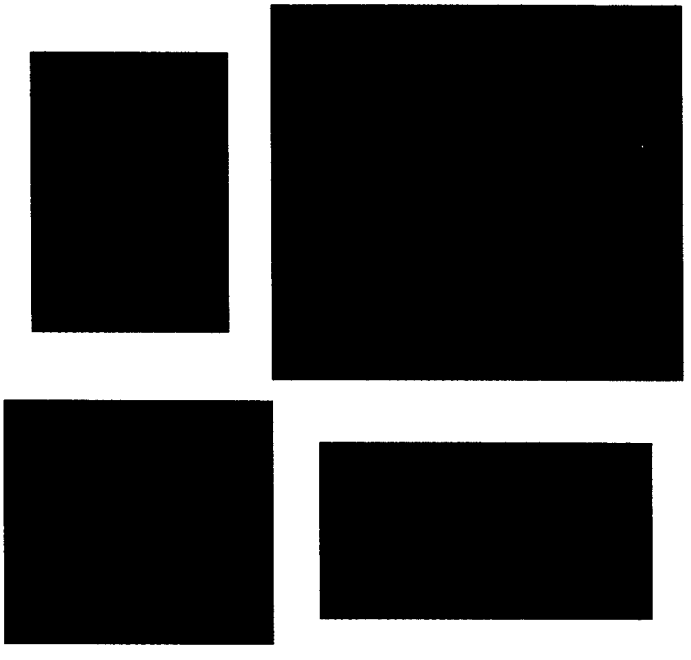

Table I. Spontaneous Chromosome Aberrations in Non-Transformed Cells

Cell type	No. cells scored	Gaps, breaks and deletions[1]	Total Exchange-type aberrations[2]	Telomeric fusions[3]	Telomeric fusions per cell
WT (p3,4)[4]	100	26	3	0	0.000
scid (p5,6)	91	47	22	15	0.165
$p53^{-/-}$ (p3,5)	100	57	10	1	0.010
$p53^{-/-}$/*scid* (p5,6)	100	12	10	8	0.080

[1]Non-exchange-type aberrations are combined into a single category.

[2]All identifiable exchange-type aberrations, *i.e.* chromosome and chromatid dicentrics, metacentrics and rings are included in this category. Many $Ku^{-/-}$ cells had small difficult to classify, yet clearly aberrant, chromosomes. Their frequency, and the fact that some appear to be clonal in origin, indicate they are not lethal to the cells possessing them. They are not included in aberration counts.

[3]Number of exchange-type aberrations having telomere sequence at points of fusion.

[4]Passage numbers.

Table II. Chromosome Aberrations in Spontaneously Immortalized Cells

Cell type	No. cells scored	Gaps, breaks and deletions	Total Exchange-type aberrations	Telomeric fusions	ITS[1]	TF per cell	Chromosomes per cell[1]
WT (p34)	50	52	7	0	0	0.000	73.3 ± 3.7
scid (p28)	50	12	226	225	1	4.50	71.2 ± 5.2
scid (p35)	50	13	264	254	8	5.08	74.0 ± 3.1
$p53^{-/-}$ (p26)	50	17	28	0	0	0.000	65.7 ± 7.5
$p53^{-/-}$/*scid* (p23)	50	26	69	58	3	1.16	57.5 ± 8.4

[1]ITS, *i.e.* interstitial telomeric sequence.

[2]Average number of chromosomes per cell ± standard deviation.

Table III. Chromosome Aberrations in Virally Transformed Cells

Cell type	No. cells scored	Gaps, breaks and deletions	Total Exchange-type aberrations	Telomeric fusions	ITS	TF per cell	Chromosomes per cell
WT	100	19	16	0	0	0.000	62.8 ± 7.6
Ku $70^{-/-}$	76	20	50	8	0	0.105	54.6 ± 5.1
Ku $80^{-/-}$	61	13	118	8	0	0.131	74.4 ± 9.1
SC-A1	50	13	68	54	10	1.08	56.5 ± 6.6
SC-EM	50	21	95	89	3	1.78	57.5 ± 6.2
SC-A1 (p +6)[1]	50	14	55	19	21	0.380	57.3 ± 6.5
SC-EM (p +6)	50	41	81	70	5	1.40	57.0 ± 4.8

[1] Reexamination six passages later.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
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1 JUN 2001

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DAMD17-96-1-6020	ADB244256
DAMD17-96-1-6023	ADB231769
DAMD17-94-J-4475	ADB258846
DAMD17-99-1-9048	ADB258562
DAMD17-99-1-9035	ADB261532
DAMD17-98-C-8029	ADB261408
DAMD17-97-1-7299	ADB258750
DAMD17-97-1-7060	ADB257715
DAMD17-97-1-7009	ADB252283
DAMD17-96-1-6152	ADB228766
DAMD17-96-1-6146	ADB253635
DAMD17-96-1-6098	ADB239338
DAMD17-94-J-4370	ADB235501
DAMD17-94-J-4360	ADB220023
DAMD17-94-J-4317	ADB222726
DAMD17-94-J-4055	ADB220035
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DAMD17-94-J-4391	ADB219964
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