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Effects of the *Saccharomyces cerevisiae* endo-exonuclease *NUD1* gene expression and poly(ADP-ribose) polymerase inhibition on homologous recombination in mammalian cells — potential application to gene targeting

by

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Abstract

In eukaryotes homologous recombination is the basis of such important biological events as meiotic recombination, generation of the antigen recognition molecule repertoire of the immune system and repair of DNA double strand breaks (DSBs).

Homologous recombination between an exogenous DNA sequence and a chromosomal sequence is termed "gene targeting". In the future, gene therapy will predictably become the treatment of choice for a number of inherited and acquired genetic diseases. The main obstacle to the application of gene targeting to gene therapy is the low frequency of homologous recombination in mammalian somatic cells. The ability to specifically increase the frequency of homologous recombination in mammalian cells could make gene targeting applicable to gene therapy of hereditary genetic disorders and cancer.

Here, we present the results of our studies in which we explored two approaches for transient increase of the frequency of homologous recombination in mammalian somatic cells: expression of the *S. cerevisiae* endo-exonuclease (EE) *NUD1* gene, and inhibition of poly(ADP-ribose) polymerase (PARP) with 1,5-isoquinolinediol (ISQ).

We have demonstrated that transient expression of *NUD1* in HeLa cells increased the resistance of the latter to ionizing radiation and cis-platin, two agents known to cause DNA DSBs, whereas it had no effect on cells' resistance to DNA-methylating agents. We have also shown that transient expression of *NUD1* enhanced the frequency of extrachromosomal homologous recombination in mouse Ltk- cells. These results provide additional evidence for the involvement of EEs in homologous recombination in mammalian cells. They also demonstrate that overexpression of EEs, such as Nud1p, can potentially be used as a means of selectively increasing the frequency of homologous recombination in mammalian cells without a concomitant increase in the frequency of illegitimate recombination.

Our studies of PARP inhibition showed that treatment of cells with 0.622 mM ISQ results in an average 4.6-fold increase in the frequency of extrachromosomal homologous recombination in mouse Ltk- fibroblasts. We have also demonstrated that treatment of mouse

Ltk- cells with ISQ leads to an increase of up to 8-fold in the absolute frequency of gene targeting of the stably integrated HSV *tk* gene. We believe that our results concerning the effect of ISQ on gene targeting may have potential application for the improvement of such technologies as the generation of genetic knock-outs and the *ex vivo* gene therapy. Our findings also appear to solve the conflict in the previously reported results that PARP inhibition stimulates chromosomal recombination but not extrachromosomal homologous recombination or gene targeting.

Résumé

La recombinaison homologue chez les eukaryotes est à l'origine d'un grand nombre de processus cellulaires importants, tels que la recombinaison méiotique, la production du répertoire des molécules du système immunitaire responsables de la reconnaissance d'antigènes et la réparation des cassures à double brin d'ADN.

La recombinaison homologue entre une molécule d'ADN exogène et une séquence d'ADN chromosomale est appellée "ciblage génique". Bientôt la thérapie génique va sans aucun doute devenir le moyen de prédilection pour le traitement de plusieures maladies génétiques, aussi bien héréditaires qu'acquises. Le principal obstacle à l'application du ciblage génique à la thérapie génique est la fréquence très basse de recombinaison homologue dans les cellules mammifères somatiques. Par conséquent, il est primordial de trouver des moyens d'augmenter de façon spécifique la fréquence de recombinaison homologue dans les cellules mammifères afin de rendre le ciblage génique applicable à la thérapie génique des maladies héréditaires et du cancer.

Ci-dessous nous présentons les résultats de notre étude, laquelle avait pour but d'explorer deux approches visant à augmenter de façon transitoire la fréquence de recombinaison homologue dans les cellules mammifères sommatiques : l'expression du gène *NUD1* codant pour l'endo-exonuclease (EE) de la levure *S. cerevisiae*, et l'inhibition de la poly(ADP-ribose) polymerase (PARP) avec le 1,5-isoquinolinediol (ISQ).

Nous avons pu démontrer que l'expression transitoire du *NUD1* dans les cellules HeLa augmentait la résistance de ces dernières à la radiation gamma et à la cis-platine, deux agents qui causent des cassures à double brin d'ADN, tandis qu'elle n'a eu aucun effet sur la résistance des cellules aux agents alkylants. Nous avons également démontré que l'expression transitoire du *NUD1* augmentait la fréquence de recombinaison homologue extrachromosomale dans les cellules Ltk- de la souris. Ces résultats constituent une preuve supplémentaire de l'implication des EEs dans la recombinaison homologue dans les cellules

mammifères. Ils indiquent également que l'expression des EEs, telles que la Nud1p, peut, en principe, être utlilisée pour augmenter de façon spécifique la fréquence de recombinaision homologue dans les cellules mammifères sans toutefois augmenter la fréquence de recombinaison aléatoire.

Notre étude sur l'inhibition de la PARP a démontré que, contrairement aux résultats rapportés par d'autres, l'inihibition de l'enzyme avec 0.622 mM d'ISQ donne lieu à une augmentation de la fréquence de recombinaison homologue extrachromosomale, en moyenne de 4,6 fois, dans les cellules Ltk- de la souris. Nous avons également démontré que le traitment des cellules avec 0.622 mM d'ISQ a pour effet d'augmenter jusqu'à 8 fois la fréquence absolue du ciblage génique du gène de la *tk* du VHS dans trois lignées cellulaires-cibles dérivées des cellules Ltk-. Nous pensons que nos résultats peuvent éventuellement servir à améliorer des techniques telles que la production des "KO génétiques" et la thérapie génique *ex vivo*. Nos observations permettent également de resoudre la contradiction apparente dans les résultats rapportés auparavant. Ces derniers indiquaient que l'inhibition de la PARP stimulait la recombinaison chromosomale, mais ne stimulait pas la recombinaison extrachromosomal, ni le ciblage génique.

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Rationale and objective of the present study

Gene targeting by homologous recombination represents, in theory, the ultimate and the ideal treatment of both hereditary and acquired genetic diseases. The applicability of gene targeting to gene therapy in humans is hampered by at least two problems. The first is the extremely low frequency of homologous recombination in mammalian cells. The second is the extremely high rate of random integration of the targeting vector by illegitimate recombination. Both types of recombination are controlled and carried out by a great number of enzymes.

The rationale behind this study is that by selectively stimulating the activity of some of these enzymes and/ or inhibiting the activity of others, one could achieve an overall increase in homologous recombination.

The objective of our study was to show that overexpression of the gene coding for an yeast endo-exonuclease, and inhibition of poly(ADP-ribose) polymerase could both lead to an increase in homologous recombination in mammalian cells.

Claims to originality

1. We were the first to demonstrate that overexpression of the *Saccharomyces cerevisiae NUD1* gene leads to increased resistance of HeLa cells to ionizing radiation and cis-platin, sensitizes the cells to UV irradiation, and at the same time has no effect on the cells' resistance to the methylating agents.

2. We have demonstrated that *NUD1* overexpression in mouse Ltk- fibrobalsts results in stimulation of extrachromosomal homologous recombination.

3. We have shown that inhibition of PARP with 1,5-isoquinolinediol results in a 2 to 7-fold increase in extrachromosomal homologous recombination in Ltk- cells.

4. We have shown that inhibition of PARP with 1,5-isoquinolinediol increases the absolute frequency of gene targeting up to 8-fold in Ltk- cells.

Contribution of authors

Three reprints from the original publications and one manuscript submitted for publication are included in this thesis :

Semionov A., D. Cournoyer, T.Y.-K. Chow (1999) The effect of the *Saccharomyces cerevisiae* endo-exonuclease *NUD1* gene expression on the resistance of HeLa cells to DNA-damaging agents, *Mutation Research*, 433, 169-181;

Semionov A., D. Cournoyer, T. Y.-K. Chow (1999) Transient expression *of Saccharomyces cerevisiae* endo-exonuclease *NUD1* gene increases the frequency of extrachromosomal homologous recombination in mouse Ltk- fibroblasts, *Mutation Research*, 435, 129-139;

Semionov A., D. Cournoyer, T. Y.-K. Chow (1999) Inhibition of poly(ADP-ribose)polymerase stimulates extrachromosomal homologous recombination in mouse Ltk- fibroblasts, *Nucleic Acids Research*, 27, 4526-4531;

Semionov A., D. Cournoyer, T. Y.-K. Chow (1999) 1,5-isoquinolinediol increases the frequency of gene targeting by homologous recombination in mouse fibroblasts, *Gene Therapy*, submitted.

The four articles have been co-authored by Denis Cournoyer and Terry Chow who were responsible for the initial planning of the experiments, provided technical training and assistance, and contributed to the editing of the manuscripts. For all four publications, I was responsible for the design and the carrying out of the experiments, and the preparation of the manuscripts.

Chapter 1: Introduction

Foreword

DNA is the blueprint of life. However metaphorical, this statement is true. The information encoded by the DNA content of almost any cell of any living organism is sufficient for the minute reconstitution of the whole organism no matter how complex. Therefore, the ability to manipulate the genetic code at the molecular level is equivalent to the ability to manipulate life itself. The technology for genetic manipulations is presently in its infancy. Nevertheless, its first successes are visible already. Genetically modified farm plants are no longer a fiction but something we can buy at a supermarket. Hundreds of gene therapy clinical trials are under way in hospitals worldwide. Thousands of genetically altered laboratory animals are used in biological research everywhere.

These few examples illustrate the enormous potential and benefits to men resulting from controlled genetic manipulations. However, the existing methods for subtle modification of the genes of interest are very inefficient and time consuming. The need for the development of more advanced techniques for efficient and specific genomic alterations is obvious and pressing.

Gene targeting by homologous recombination has the potential to give rise to such a technology. The term itself refers to the process by which an exogenous DNA sequence undergoes the exchange of genetic sequence with its genomic homologue. The process can be exploited for the precise disruption of the targeted genes (known as genetic knock-outs) or the correction of genetic mutations. The former application is already widely used in research to study gene structure and function, embryonic development, as well as to generate animal models of diseases; the latter will undoubtedly become one day the basis for gene therapy of genetic disorders and cancer.

For the time being, however, gene targeting by homologous recombination presents a number of challenging problems which have to be overcome in order to make the technique useful for such applications as gene therapy. Although, the mechanisms behind homologous

recombination are so far poorly understood, huge progress has been made in the study of the process over the past decade.

Chapter 1 gives a general overview of what is known about homologous recombination today with particular emphasis put on gene targeting in eukaryotes. The two last sections of Chapter 1 give a general overview of, respectively, endo-exonucleases and of poly(ADP-ribose) polymerase in order to put the content of the subsequent chapters in perspective.

In Chapter 2 we present the results of our study of the effect of yeast endo-exonuclease expression on homologous recombination in mammalian cells.

In Chapter 3 we present the results of our study of the effect of PARP inhibition on extrachromosomal homologous recombination and gene targeting in mouse fibroblasts.

1.1 Homologous and illegitimate recombination

Much of the current knowledge of the mechanisms of recombination comes from the studies done with bacteria and yeast (Smith, 1988; Orr-Weaver et al., 1981). The bacterial and fungal systems were privileged in the studies of recombination for the reasons of lower complexity of their genomes and the ease with which this organisms can be grown and manipulated. Most of the general concepts derived from these studies apply to mammalian homologous recombination as well, since the process of recombination appears to be evolutionary conserved (Taylor and Lehmann, 1998).

Homologous recombination can be defined as a process in which two DNA molecules with a high sequence homology interact and exchange genetic information by adding or replacing their sequence elements (Clark, 1971).

Gene targeting is a specific case of homologous recombination in which the recombinational interaction occurs between an exogenous extrachromosomal and an endogenous chromosomal sequence by means of human manipulation (Bertling, 1995). However, homologous recombination is a naturally occurring cellular process, which can take place between two homologous chromosomes (interchromosomal recombination), between homologous sequences of the same chromosome (intrachromosomal recombination), or between two extrachromosomal elements (extrachromosomal recombination).

All of the naturally occurring recombination processes can be divided into three major categories: general homologous recombination, site-specific recombination and illegitimate recombination.

1.1.1. General Homologous Recombination

General homologous recombination is distinguished from site-specific recombination in that, in homologous recombination, there is no requirement for a specific DNA sequence.

Rather, homologous recombination may occur between any two sequences, provided that the two share sufficient homology.

The exchange of genetic information during a homologous recombination can be either reciprocal or non-reciprocal. In the latter case the process, referred to as gene conversion, is associated with some loss of genetic information contained in the recipient molecule. In non-reciprocal recombination it is possible to identify the donor and the recipient of genetic information. A reciprocal exchange is referred to as crossover. Multiple crossovers can occur along a given segment of DNA. A single product of a double crossover event may resemble gene conversion, with the DNA segment between the two crossover points appearing as a conversion tract. The only way to distinguish a double crossover from a gene conversion event is to examine all the recombination products with respect to the conservation of the genetic information present prior to the reaction. But given that it is not always possible to recover all products of recombination, especially in mammalian systems, it is often impossible to distinguish between a double crossover and a gene conversion (Liskay and Stachelek, 1983).

Cellular functions of general homologous recombination

Meiotic recombination

Homologous recombination is a major feature of meiosis in sexually reproducing plants and animals. Meiosis is the central vehicle for the exchange of genetic information in eukaryotes, and it is in meiosis that recombination in eukaryotes achieves its highest frequency (Murti et al., 1994). As well as being responsible for the reassortment of the genes, homologous recombination appears to be essential for proper chromosomal disjunction during first meiotic division (Kleckner, 1996). Crossing-over is in fact essential for proper chromosome segregation and, from yeast to humans, "non-exchange" chromosomes — those that do not undergo crossing-over — exhibit a high rate of non-disjunction (Haber, 1998).

The classical paradigm of meiosis postulates that the process that brings about homologous alignment of chromosomes in close apposition during meiotic prophase I, occurs prior to, and is required for, meiotic recombination. However, recent studies of meiotic recombination have challenged this view. Evidence suggests that double strand break (DSB) formation in early meiosis prophase I initiates genome-wide searches for homology and homologous DNA exchanges, which precede and mediate homologous chromosome pairing and synaptonemal complex formation (Roeder, 1997; Dernburg et al., 1998; Yoshida et al., 1998).

Many of the unique properties of meiotic recombination are apparently enforced by the synaptonemal complex, a proteinaceous structure that lies between the synapsed homologous chromosomes and is specific to meiotic cells. The synaptonemal complex apparently regulates the exclusion of sister chromatids as partners for recombination as well as the positions and frequency of crossing-overs (Haber. 1998). Although meiotic recombination involves many meiosis-specific proteins, it is believed that mechanisms for general homologous recombination in somatic cells (such as the ones that operate in DSB recombinational repair) are also recruited during meiosis as a homology seeking mechanism (Game, 1992).

Repair of DNA double strand breaks

DSBs are important DNA lesions that can arise in mitotic cells spontaneously or in response to certain DNA-damaging agents. Some DNA-damaging agents, such as x-rays, can produce DNA DSBs directly, whereas others generate DSBs or gaps following processing of initial lesions by repair enzymes. The consequence of unprocessed DSBs are blockage of DNA replication and loss of genome integrity leading to lethality. The repair of DSBs and gaps occurs via recombinational mechanisms. The DNA molecular structures generated during meiotic and mitotic recombination of chromosomes are similar to those occurring during recombinational repair of DSBs.

In *S. cerevisiae*, DNA DSBs are primarily processed by a homologous recombination pathway (Resnick et al., 1995). In contrast, evidence suggests that in mammalian cells an illegitimate recombination mechanism, DSB end-joining, rather than homologous recombination is the prevailing mechanism (Derbyshire et al., 1994). Other eukaryotes appear to lie in between these two extremes with respect to relative efficiencies of homologous and illegitimate recombinational processing of DSBs. It is not clear whether these differences reflect additional capacity for non-homologous DNA DSB joining or diminished pathways for homologous recombination in other eukaryotes compared to *S. cerevisiae*.

In yeast, repair of DSBs requires the products of the *RAD51, 52, 54, 55* and *57* genes (Parkash et al., 1993; Jessberger et al., 1993), which are known to be required to carry out homologous recombination reactions. The ability to repair DSB is severely compromised in mutants of these genes. Although the major pathway for repair of DSB in mammals appears to be the NHEJ repair pathway catalyzed by the Ku70/80 of the DNA PK (Fishman-Lobell, 1992; Moore and Haber, 1996), it has been shown that overexpression of human *RAD52* confers enhanced radioresistance to cultured monkey cells (Park, 1995). Also, it has been reported that cells derived from the *rad54* knockout mouse are radiosensitive (Essers et al., 1997).

1.1.2. Site-specific Recombination

Conversely to general homologous recombination, site-specific recombination typically occurs between precisely prescribed sites on two partner DNA sequences that otherwise bear no overall homology to each other. These sites typically comprise short recognition sequences for a particular DNA-binding protein(s) that act(s) on these binding sequences to catalyze recombination (Craig, 1988). The two most studied examples of site-specific recombination, i.e. mating type switching in yeast and V(D)J immunoglobulin recombination, are briefly reviewed below.

Mating type switching in yeast

Homothallic switching of the mating-type genes in *S. cerevisiae* occurs by a highly regulated site-specific homologous recombination event (reviewed in Haber, 1992). The HO-endonuclease makes a DSB in the *MAT* locus at its recognition site near the *MAT-y/z* border. This stimulates gene conversion to replace the DNA sequence at the *MAT* locus with sequences copied from one of two unexpressed donor loci, *HML* or *HMR*, located on the same chromosome. *MAT* conversion is not accompanied by reciprocal crossing-over.

The expression of HO-endonuclease is normally confined to the G1 phase of the cell cycle and to cells that have previously divided.

An early intermediate is a single long 3' single-strand tail beginning at one end of the induced DSB. It invades the donor site and primes DNA synthesis, thus copying the donor sequence (White and Haber, 1990). Strand invasion generates hDNA that is rapidly mismatch repaired, nearly always in favor of the donor sequences (Haber et al., 1993).

Mating-type switching in *S. pombe* is similar to that in *S. cerevisiae* in that it involves initiation by a DSB at the *mat1* locus with sequences copied from one of two unexpressed donor loci, *mat2-P* or *mat3-M*, located on the same chromosome (reviewed in Klar, 1992).

Immunoglobulin recombination

In vertebrates, V(D)J recombination occurs during B and T lymphocyte development and is responsible for the tremendous diversity in antibody and T-cell receptor specificity (reviewed in Lewis, 1994). During V(D)J recombination, three gene segments, the variable (V), joining (J), and diversity (D) elements, positioned at distinct genomic locations in germ cells, become rearranged into a contiguous exon.

The recombination is initiated by site-specific DSBs, acts between specific signal sequences, and does not require extensive sequence homology. Each gene segment is flanked by a signal sequence consisting of a conserved heptamer and nonamer separated by a relatively non-conserved 12 or 23 bp spacer (12 signal or 23 signal). Small mutations in

either the heptamer or the nonamer can dramatically reduce recombination efficiency, demonstrating the importance of both of these elements (Hesse et al, 1989). A segment flanked by a 12 signal can only be joined efficiently to one flanked by a 23 signal (Tonegawa, 1983), a restriction referred to as the 12/23 rule. A 12 signal and a 23 signal together constitute the necessary and sufficient cis-acting elements for efficient recombination in model substrates (Lewis, 1994).

The coding ends are processed extensively prior to ligation to generate additional diversity. Individual nucleotides can be added to the coding end in a non-templated manner by terminal deoxynucleotidyl transferase (TdT), an enzyme expressed specifically in lymphoid cells (Weaver, 1995). Oligonucleotides may be added at the joint site even in the absence of TdT, presumably by oligonucleotide capture (Gilfillan et al., 1993). A few nucleotides palindromic to one or both of the coding ends may be added by a process called P-nucleotide addition (Lafaille, 1989). Nucleotides can also be lost from the coding ends.

The question of what proteins recognize signal sequences during V(D)J recombination has been recently answered by experiments demonstrating that the proteins encoded by the recombination activating genes, *RAG1* and *RAG2*, are sufficient to mediate signal dependent site-specific DNA cleavage *in vitro* (McBlane et al., 1995; van Gent et al., 1995). Both RAG proteins are essential for lymphocyte development and V(D)J recombination (Shinkai et al., 1992) and are the only lymphoid-specific factors required for the reaction (Oettinger et al., 1990).

Otherwise, it seems that there exist a significant overlap between V(D)J recombination and the ubiquitous non-homologous end-joining mechanism of processing of DSBs in all other cell types (Blunt et al, 1995). For example, the DNA-dependent protein kinase (DNA-PK) is indispensable in V(D)J recombination (Bogue and Roth, 1996). Mice deficient for the DNA-PK or for its regulatory subunit, DNA-binding protein Ku, completely lack antigen receptors (Kirchgessner et al., 1995; Zhu et al., 1996).

1.1.3. Models of Homologous Recombination

Fungi represent an ideal model system for the study of meiotic recombination (Orr-Weaver et al., 1981). Following a sexual cross between two strains carrying different genetic markers, the isolation and analysis of the spores of intact tetrads and octads allows the detection of each DNA strand of each chromatid present after meiosis. Thus, the initial investigations of meiotic recombination using yeast have rapidly generated a great amount of data. These investigations established the occurrence of not only crossovers (reciprocal exchanges) but also the non-reciprocal exchanges, such as gene conversions. These studies also showed that there was a definite correlation between non-reciprocal gene conversion within a gene and reciprocal crossing-over of flanking markers. Models were then postulated in order to explain the genetic data on meiotic recombination in molecular terms. The models had to define a mechanism for the association of gene conversion and crossing-over.

Holliday model

The first model of homologous recombination which could explain most of the observations from the recombination experiments was the one proposed by Holliday (Holliday, 1964). It presented a paradigm upon which most later models have been based (Figure 1).

An initial step in the Holliday model is the pairing of the homologous sequences. The model postulates that the two homologously paired DNA duplexes simultaneously acquire single-stranded DNA breaks in strands of like polarity. The following reciprocal invasion of the homologous duplexes by ssDNA ends results into a local strand exchange and, upon subsequent sealing of the ssDNA ends, gives rise to the so-called Holliday structure (junction).

The Holliday structure is considered as the central recombination intermediate. The formation of Holliday structures has been directly visualized in *E. coli* using electron microscopy (Potter and Dressler, 1979). Holliday structure can be viewed as being equivalent

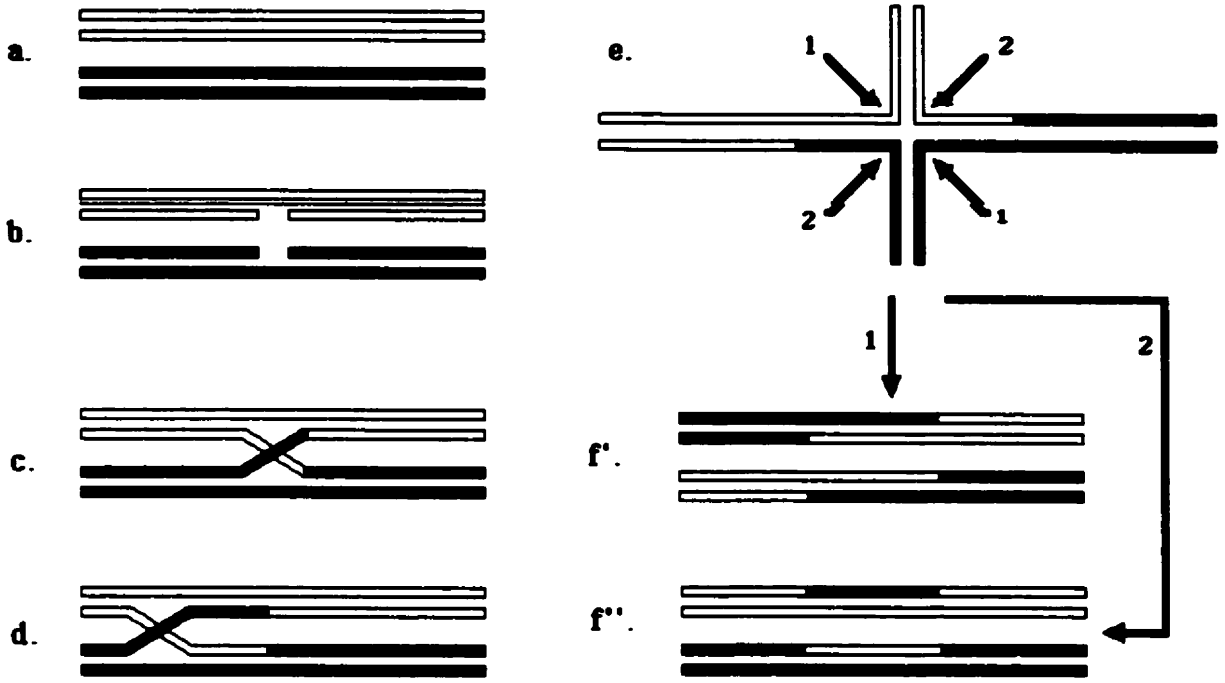

Figure 1. Holliday Model of Homologous Recombination. Homologous duplexes pair up (**a**). An internal nick (**b**) in the two participating double-stranded DNA molecules leads to a local strand exchange and to the formation of Holliday junction (**c**). The point of exchange can migrate (branch migration) (**d**). The Holliday structure can be resolved in two ways (**e**) giving rise to either splice (**f'**) or patch (**f''**) recombination products.

to a branched DNA structure, with four duplex arms containing a central four-way junction. This Holliday junction can migrate in both directions with respect to the site of the initial crossover, the so-called branch migration.

The final step of the recombination consists in resolution of the Holliday structure by enzymatic cleavage of the four-way junction through the central structure in one of two ways. One mode of resolution yields a non-crossover product, while the other cleavage yields a product with a crossover.

In this model gene conversion is explained as a result of the non-reciprocal repair of the heteroduplex DNA by the mismatch repair enzymes and both gene conversion and crossover products are generated from the same basic structure.

Meselson-Radding model

The Meselson-Radding model has been formulated based on the discovery of recA of *E. coli*. (Meselson and Radding, 1975; Radding 1982). According to the model recA promotes a pairing of an at least partially single-stranded DNA with duplex DNA in three steps (Figure 2).

In a presynaptic phase, the protein binds to single-stranded DNA and polymerizes to a nucleoprotein filament. In the following synaptic phase, this filament binds non-specifically to duplex DNA and migrates along the DNA until a homologous region is found. In the last step, the actual strand exchange occurs.

The invasion is postulated to be driven by DNA synthesis at the break site which displaces the invading strand. As strand invasion proceeds, the displaced strand of the invaded duplex forms an increasingly large D-loop. Eventually, the D-loop breaks and a free end of DNA from the broken D-loop invades the other duplex. Breaks in strands are eventually sealed, and the two duplexes are covalently joined in a Holliday structure. For the subsequent steps of the reaction the Meselson-Radding model does not differ from the Holliday model.

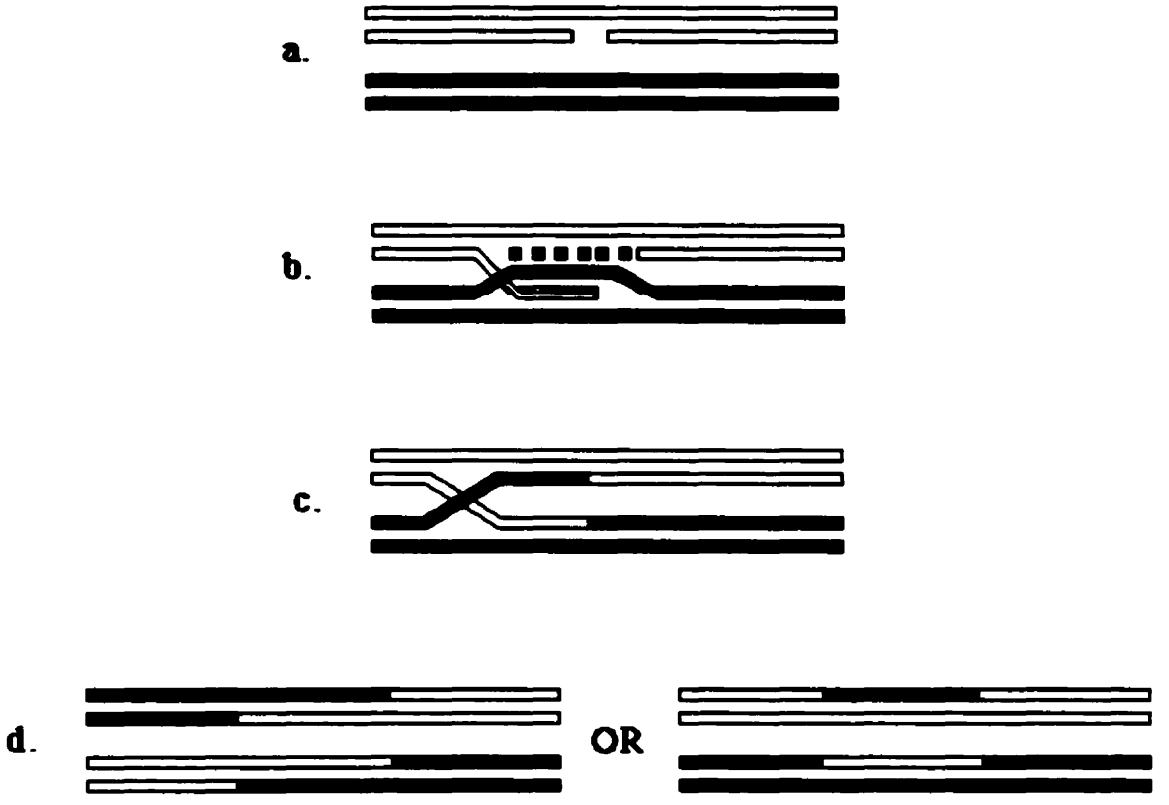

Figure 2. Meselson-Radding Model of Homologous Recombination. Only one of the homologous DNA duplexes acquires a single strand break (**a**). DNA synthesis is primed from the break. The synthesis displaces the 5' end which invades the homologous duplex (**b**) giving rise to a D-loop structure. Cleavage of the D-loop and its subsequent ligation to the homologous strand of the other duplex gives rise to the Holliday structure (**c**) which is then processed as in the Holliday model to form spliced or patched recombinant products (**d**).

Double-strand break model

The double-strand break model was proposed in order to account for the observation that double-strand breaks greatly increase recombination rates in yeast. Initially suggested by Resnick and later modified by Szostak et al., and others, this model postulates that the recombination starts with a double strand break of one of the participating homologous DNA molecules (Resnick, 1976; Szostak et al., 1983; Rosenberg and Hastings, 1991).

This initial break is expanded to a gap by exonucleases and is further processed enzymatically into 3' single-stranded (ss) DNA overhangs. Studies of the meiotic recombination initiator site at *ARG4* revealed that 3' ssDNA tails of a length of up to 800 bp were generated from the site of a DSB (Sun et al., 1991). The single-stranded DNAs invade the homologous duplex and are used as primers for DNA synthesis or trigger an exchange, leaving the other ends reactive and causing a cross-over. The original gap is thus repaired using the homologous duplex as a template. The repaired gap defines a conversion tract. Gene conversion in this model, may, therefore, be accomplished in the absence of heteroduplex repair (although short stretches of heteroduplex DNA may flank the gap). However, evidence suggested that chromosomal gene conversion resulted mainly from the mismatch correction of asymmetrical heteroduplexes (Nag et al., 1989; Lichten et al., 1990).

Also, although double strand gaps (DSGs) produced *in vitro* are repaired *in vivo* with information donated by an endogenous duplex (Orr-Weaver et al., 1981), there is no evidence that DSBs are processed into gaps *in vivo*. Instead, it appears that both mitotic and meiotic DSBs are processed not to a gap but directly to 3' ss DNA overhangs. Thus the DSBR model has been modified to postulate that both 3' ss tails generated from the initial DSB invade the homologous duplex to produce hDNA that is mismatch repaired to result in gene conversion. Another essential difference between the DSBR and Meselson-Radding models is that the broken sequence in the DSBR model acts primarily as a recipient of genetic information, whereas in the Meselson-Radding model the broken sequence acts as a genetic donor.

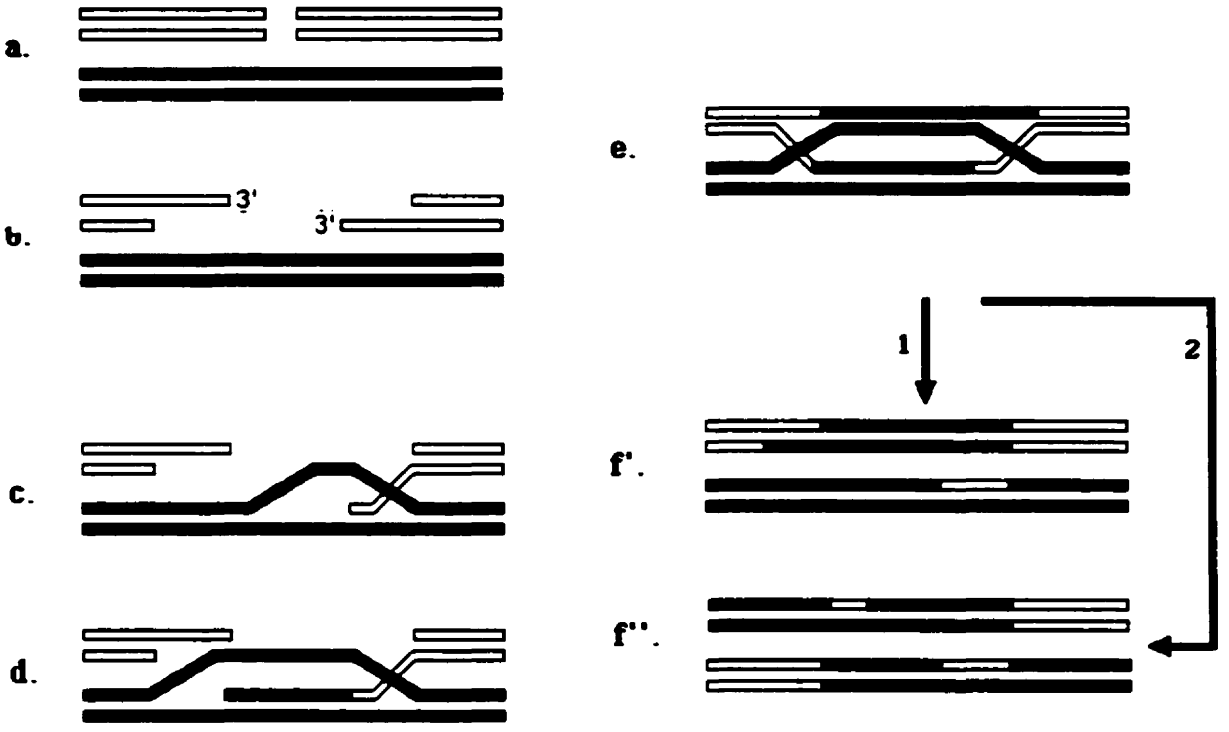

Figure 3. Double Strand Break Model of Homologous Recombination. The initial DSB (**a**) in one of the molecules is first enlarged to a gap. Exonucleases degrade the DNA at the ends of the break and expose single-stranded regions with 3'-overhanging ends (**b**). One of these ends invades the homologous duplex donor molecule and displaces a single strand creating a D-loop (**c**). DNA synthesis primed at this end results in expansion of the D-loop (**d**). DNA synthesis primed at the second 3' end completes gap repair. Branch migration results in the formation of two Holliday junctions and the establishment of asymmetric and symmetrical heteroduplexes flanking the repaired gap (**e**). This recombination intermediate can be resolved in two ways: if the two Holliday junctions are cut in the same orientation, a non-crossover configuration is produced (**f'**); if the two Holliday junctions are cut in opposite orientation, a crossover configuration is produced (**f''**).

During the course of DSBR, a double Holliday structure is formed, with the two Holliday junctions flanking the conversion tract. The coronation of the DSBR model came with the detection of such double Holliday junctions as recombinational intermediates in yeast (Schwacha and Kleckner, 1995; Stahl, 1996).

Each Holliday junction can be resolved in two ways, for a total of four possible modes of resolution. Two of these yield crossover products, whereas the other two yield non-crossover products.

One-sided invasion model

It has been observed that the two 3' tails generated by the action of a unidirectional 5'—>3' exonuclease from the site of initial DSB act independently of each other in finding and invading a homologous duplex (White and Haber, 1990; Adair et al., 1989; Belmaaza et al., 1990). Also there is no requirement for both ends to invade, as repair synthesis primed from one end can produce a region complementary to the opposite 3' tail. Taken together these observation led Balmaaza and Chartrand (1994) to postulate a modified version of the DSBR model, known as the one-sided invasion model.

This model proposes that following strand invasion by one of the 3' tails, DNA synthesis is primed from the 3' end using the invaded duplex as template. Annealing between the newly synthesized strands and the non-invading ss end would lead to the formation of hDNA on one side of the DSB, and a short heteroduplex could also be formed on the other side.

A Holliday junction could be generated by cutting at the front end of the D-loop with subsequent annealing with the non-invading end and DNA synthesis. As in previous models, associated crossovers would depend on the manner the Holliday structure is resolved. It has been observed that processing of DSBs at the *MAT* locus of *S. cerevisiae* occurs at only one end, with asymmetrical strand transfer resulting in hDNA only in the recipient molecule, as predicted from one-sided invasion model. However, Schwacha and Kleckner (1995)

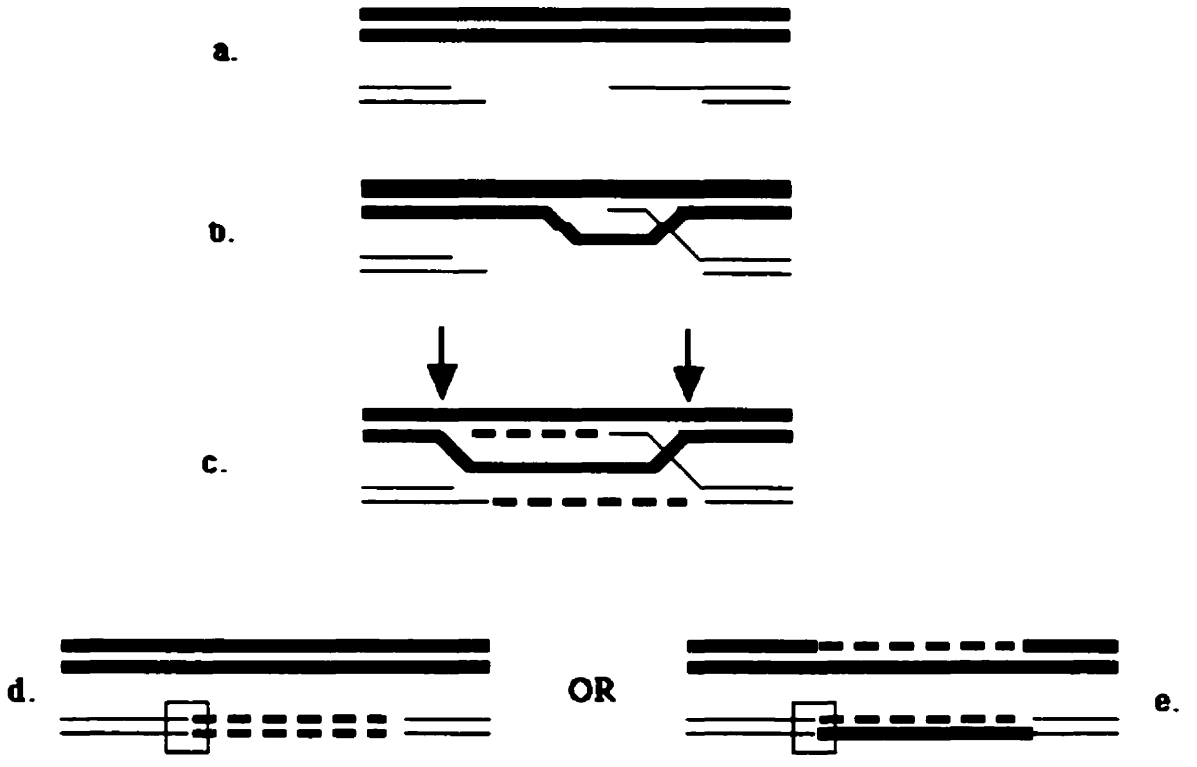

Figure 4. One-sided invasion Model of Homologous Recombination. A DSB in the recipient is followed by single-strand formation by the action of exonucleases or unwinding (**a**). Next, one end of the break is involved in single-strand invasion of a donor sequence which results in the formation of a D-loop (**b**). DNA synthesis primed at the invading strand extends the D-loop (**c**). Resolution occurs by unwinding of the newly synthesized strand or its displacement due to branch migration followed by ligation to the non-invading end of the recipient molecule (**d**). Alternately, resolution can occur as a result of single-strand nicks at the extremities of the D-loop (arrows in **c**), followed by ligation to the non-invading end of the recipient molecule (**e**). In both cases an illegitimate junction is produced (open rectangles in **d** and **e**).

described double Holliday junctions in yeast meiotic recombination intermediates, indicating that these events involve two-ended invasions.

Single-strand annealing model

This model has been based on the results from transformation experiments in mammalian cells (Lin et al., 1984; Lin et al., 1990). As in the DSBR model, recombination initiates at the site of a double-strand break. However the DSB is subjected to bi-directional single-strand-specific exonucleolytic digestion. This degradation proceeds until two complementary single-stranded sequences are exposed. The complementary strands then anneal. Single-stranded unannealed tails are excised by specific endonucleases, and any single-strand sequence gaps are repaired.

Thus, conversely to the homologous recombination models described above, in SSA there is no step that involves pairing of DNA duplexes, and so there is no true homology search involved, no Holliday structures are formed as intermediates, recombination products are exclusively crossovers, and recombination is non-conservative.

It is currently believed that SSA is the predominant mechanism for extrachromosomal homologous recombination (Lin et al., 1990). This model is supported by the analysis of products of DNA injected into *Xenopus* oocytes (Maryon and Carroll, 1991a,b). However, at least one work by Yang and Waldman (1992) challenges the notion that all, or even most, extrachromosomal recombination in mammalian cells proceeds via SSA.

None of the models for homologous recombination in eukaryotes is consistent with all the available data from the extensive studies on recombination in a variety of systems. This is probably because recombination in eukaryotes involves multiple pathways utilizing different mechanisms. Nevertheless, generally, most models postulate at least six stages : (1) initiation involving formation of a single-strand nick, a DSB or gap, followed by formation of a single-stranded DNA; (2) presynapsis involving activation of the single strand to allow

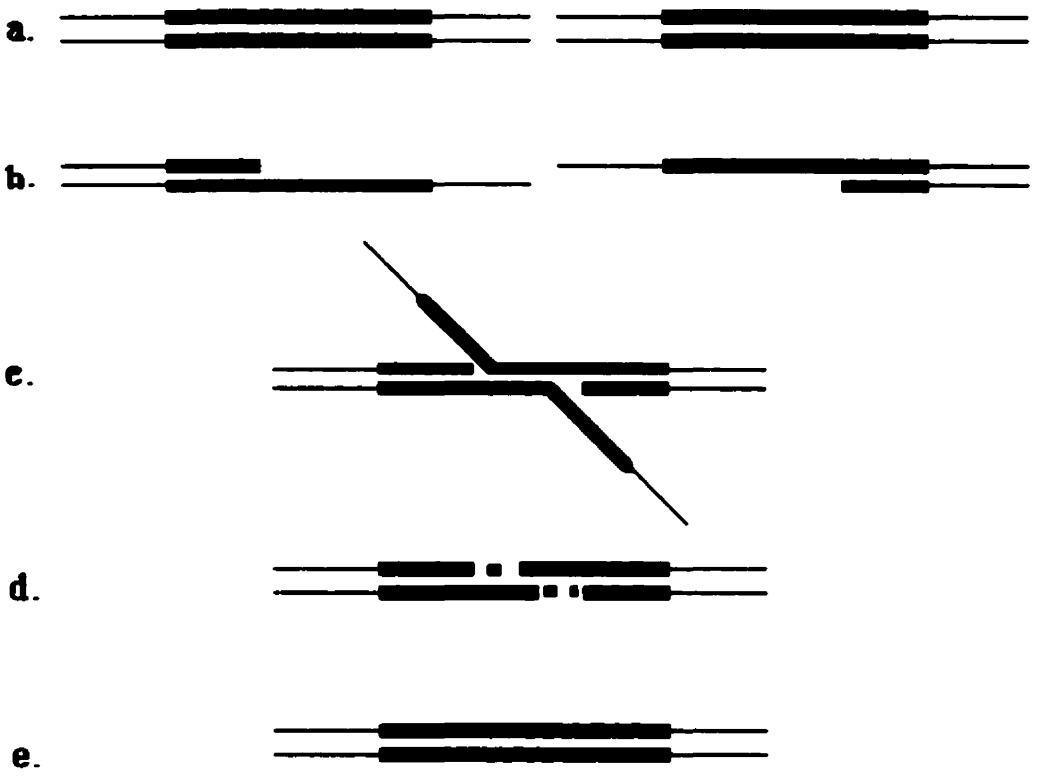

Figure 5. Single Strand Annealing Model of Homologous Recombination. The DNA ends at a DSB are degraded by a strand specific exonuclease to expose the flanking homologous sequences (**b**). Annealing of the complementary single-strand ends and the removal of non-homologous ends (**c**) occur followed by DNA synthesis and ligation (**d**). The recombination results in formation of a single molecule containing one homologous junction (**e**).

homology searching; (3) the search for homology and homologous DNA pairing; (4) strand exchange leading to hDNA formation; (5) Holliday junction formation and branch migration; and (6) resolution of Holliday junctions to yield recombinant products (Osman and Subramani, 1998).

1.1.4. Homologous recombination proteins

The great majority of the proteins involved in homologous recombination were identified through the isolation and characterization of mutants defective in some aspect of recombination. In most cases, recombination genes have been isolated by complementation of the mutant phenotype, particularly in lower eukaryotes. In recent years, some of the corresponding genes from higher eukaryotes have been defined following the identification of conserved sequences of homologues of recombination genes from lower eukaryotes.

Homologous recombination proteins of *E. coli*

In *E. coli* over 25 proteins are needed to carry out homologous recombination (Table 1). Their enzymatic activities include DNA strand exchange, DNA renaturation, DNA helicase, nuclease, ATPase, topoisomerase and DNA-binding activities. Most of these activities are also commonly encountered in other biological processes, but one activity, the DNA strand exchange activity of the recA protein is unique to recombination.

RecA protein will pair two homologous DNA molecules only if one of them is at least partially single-stranded. Since DNA is normally double-stranded, this requirement imposes a necessary initiation step: one of the DNA substrates must be processed to reveal ssDNA.

In nearly all models of homologous recombination, the initial event is the generation of a ssDNA or dsDNA break. In *E. coli*, a DSB is created in all of the processes (conjugation, transduction, transformation) that result in recombination (Thaler and Stahl, 1988; Smith, 1988).

The first step of the recombination reaction is the processing of one of the linear DNA molecules to create ssDNA. This can occur by a variety of biochemical means, but the primary method operative in wild-type *E. coli* is the combined unwinding and degradation of dsDNA by the recBCD enzyme (Smith, 1988; Smith, 1991; Kowalczykowski et al., 1994). This enzyme, in conjunction with the recombination hotspot Chi, acts to create ssDNA that can be used by recA protein for the subsequent homologous pairing phase. An alternative approach for the production of ssDNA uses either a strand-specific dsDNA exonuclease or a DNA helicase, or the action of both.

The recA protein, assisted by the ssDNA-binding (SSB) protein (and, perhaps, facilitated by the recF, recO, and recR proteins), promotes invasion of the homologous molecule. As the recombination process proceeds the Holliday junction is eventually formed. Also the Holliday junction is capable of undergoing DNA heteroduplex extension by a thermally driven branch migration a class of proteins catalyzes this extension process. RecA, ruvAB, and recG proteins all possess this activity (Lloyd and Sharples, 1992; Kowalczykowski et al., 1994).

The final step of a homologous recombination reaction is resolution of the Holliday junction by symmetric cleavage. In *E. coli* this step is carried out by ruvC protein.

RecA

Probably the most important single protein factor in homologous recombination is RecA protein. It was first identified by Clark and Margulies (1965) in *E. coli* recombination mutants. Conjugational recombination was reduced by 10 000 times in recA mutants.

RecA is a small protein of 38.5 kDa that is capable of polymerizing on single-stranded DNA to form a right-handed helical filament (Stasiak and Egelman, 1988). This nucleoprotein filament is about 100 Å in diameter, has a characteristic 94 Å pitch, and extends the DNA by about 50% over the normal dimension of B-form dsDNA. About one RecA monomer is required for every four nucleotides to fully coat ssDNA (Cox and Lehman,

Table 1. Proteins involved in genetic recombination in *E. coli*

Protein	Activity
RecA	DNA strand exchange; DNA renaturation; DNA-dependent ATPase; DNA- and ATP-dependent coprotease
RecBCD (exonuclease V)	DNA helicase; ATP-dependent ssDNA and dsDNA exonuclease; ATP-stimulated endonuclease; Chi-hotspot recognition
RecBC	DNA helicase
RecE (exonuclease VIII)	dsDNA exonuclease, 5'—>3' specific
RecF	ssDNA and dsDNA binding; ATP binding
RecG	Branch migration of Holliday junctions; DNA helicase
RecJ	ssDNA exonuclease, 5'—>3' specific
RecN	Unknown, ATP binding consensus sequence
RecO	Interaction with recR and (possibly) recF proteins
RecQ	DNA helicase
RecR	Interaction with recO and (possibly) recF proteins
RecT	DNA renaturation
RuvA	Holliday-, cruciform-, and 4-way junction binding; interaction with ruvB protein
RuvB	Branch migration of Holliday junctions; DNA helicase; interaction with ruvA protein
RuvC	Holliday junction cleavage; 4-way junction binding
SbcB (exonuclease I)	ssDNA exonuclease, 3'—>5' specific; deoxyribophosphodiesterase
SbcC	Associated with SbcD to form an ATP-dependent dsDNA specific exonuclease
SbcD	Associates with SbcC
SSB	ssDNA binding
DNA topoisomerase I (*topA*)	type I topoisomerase
DNA gyrase (*gyrA*, *gyrB*)	DNA gyrase; type II topoisomerase
DNA ligase (*lig*)	DNA ligase
DNA polymerase I (*pol A*)	DNA polymerase; 5'—>3' exonuclease; 3'—>5' exonuclease;
Helicase II (*uvrD*, *uvrE*, *recL*, *mutU*)	DNA helicase
Helicase IV (*helD*)	DNA helicase

Adapted from Kowalczykowski et al. (1994)

1981). ATP binding but not hydrolysis is required for this step (Cox and Lehman, 1981; Menetski and Kowalczykowski, 1985).

The RecA filament promotes homologous recombination by pairing with homologous duplex DNA and catalyzing strand exchange, which leads to the formation of heteroduplex DNA (West 1992). RecA is capable of renaturation of DNA *in vitro*. It is capable of mediating synapsis between a supercoiled circular DNA and a single-stranded DNA, where at least one of the ends is homologous to part of the circular DNA, leading to the formation of a D-loop. RecA can catalyze the pairing of a single-stranded circular DNA and a double-stranded homologous linear DNA, as well as the pairing of a single-stranded DNA to a homologous gapped portion of the circular duplex DNA. RecA is also capable of mediating strand exchange between duplex molecules.

Although many details of the homology search remain unclear, several of the reaction characteristics are known. The process is extremely rapid, occurring *in vitro* in just a few minutes; specific, needing less than 50 nucleotides of homology (Conley and West, 1990; Hsieh et al., 1992); discriminating, rejecting as great as a 200 000-fold excess of non-homologous sequences (Honigberg et al., 1986); and completely independent of ATP hydrolysis (Cox and Lehman, 1981; Menetski et al., 1990).

The search process is first order in DNA concentration, implying that the rate-limiting step in the pairing process occurs within non-homologously paired complexes of ssDNA and dsDNA (Gonda and Radding, 1986). Homologous recognition does not occur by repeated denaturation of the dsDNA (West et al., 1981; Bianchi et al., 1985; Hsieh and Camerini-Otero, 1989), but rather, through transient non-Watson-Crick hydrogen bonding interactions between ssDNA and dsDNA, leading to a 3-strand-containing structure (Stasiak, 1992; Camerini-Otero and Hsieh, 1993), at least transiently (Menetski et al., 1990). During the homology search, the dsDNA is topologically unwound, presumably due to distortion required to transiently align the dsDNA with the extended dimensions of the presynaptic filament (Stasiak et al., 1983; Rould et al., 1992).

RecA is also capable of promoting DNA heteroduplex extension of the joint molecule recombinational intermediate at a rate of about 10 to 20 bp/sec (Cox and Lehman, 1981). This process requires ATP hydrolysis and is unidirectional, occurring 3'—>5' relative to the ssDNA strand displaced. It can proceed for at least 7 kb and can traverse heterologies in the DNA as large as 1308 nucleotides (Bianchi and Radding, 1983).

RecBCD and DNA helicases

DNA helicases are essential to the homologous recombination reaction, acting at two different steps of the process: early in initiation and later in DNA heteroduplex extension.

For the major pathway of recombination in *E. coli*, recBCD enzyme plays a major role as the initiator of DNA strand exchange. The recBCD enzyme is a multi-functional heterotrimeric enzyme encoded by the *recB*, *recC* and *recD* genes. It possesses both DNA helicase and nuclease activities and, hence is unusual among helicases. A more detailed description of recBCD and of its function is presented further in the text in the section discussing the endo-exonucleases.

In addition to recBCD enzyme, there is at least one other DNA helicase that potentially functions at the initiation step of recombination. This is the recQ helicase, and its need in recombination is manifested in cells defective for recBCD (Nakayama et al., 1984). As for all helicases, hydrolysis of either ATP of dATP is required for dsDNA unwinding by recQ protein (Umezu et al., 1990). In the absence of SSB protein, the recQ protein is a relatively poor helicase, unwinding only about 143 bp and requiring high concentrations of protein. However, SSB protein stimulates its DNA helicase activity, presumably by binding to the unwound strands. Under these conditions, up to 343 bp are unwound and unwinding approaches catalytic behavior (Umezu and Nakayama, 1993). Presumably this limited level of DNA unwinding suffices for initiation of DNA strand exchange by recA protein. Alternatively, the recQ protein, in concert with a nuclease such as recJ protein can produce longer regions of ssDNA.

Helicases can also act at the DNA heteroduplex extension step (Lloyd and Sharples, 1992). Extension of the region of heteroduplex DNA can stabilize joint molecules by preventing their loss through random branch migration in the reverse direction. Although recA is capable of promoting such a uni-directional reaction, two additional DNA helicase, ruvAB and recG, are also important for the process. These two enzymes possess the unique attribute of acting on Holliday junctions (West and Connolly, 1992). The ruvAB protein complex can unwind up to 558 bp with an apparent 5'—>3' polarity (Tsaneva et al., 1993). RecG is more efficient than ruvAB in the branch migration reaction (Lloyd and Sharples, 1993). Unlike ruvAB, however, the recG protein appears to be specific for Holliday structures, since it is unable to unwind conventional helicase substrates (Lloyd and Sharples, 1993).

Nucleases

Aside from the initial step of homologous recombination, where nucleases (such as recBCD, recE or recJ proteins) are required to produce ssDNA suitable for use by recA protein, nucleases are also needed for the resolution of the Holliday junction. In *E. coli* this function is carried out by the ruvC protein. RuvC is a homodimeric protein composed of subunits with an estimated molecular weight of 18.7 kDa.

In the absence of Mg^{2+} or ATP, ruvC protein will bind to Holliday junctions, but not cleave the DNA (Dunderdale et al., 1991). In the presence of Mg^{2+}, ruvC protein will cleave the junction, provided that at least six bp are fully homologous (Dunderdale et al., 1991). Cleavage is symmetric, occurring on the 3' side of thymine residues, and shows no bias, producing equal amounts of patched and spliced recombinant products (Bennett et al., 1993). In the presence of recA protein, however, the spliced products are produced more often than patched products (Dunderdale et al., 1991). The reaction is apparently not catalytic, requiring as much as one ruvC monomer per 80 nucleotides of DNA (Dunderdale et al., 1991).

SSB protein

SSB protein stimulates the DNA strand exchange promoted by recA protein. SSB belongs to the class of ssDNA-binding proteins that have no enzymatic activity, but bind ssDNA cooperatively and non-specifically. SSB protein has a mass of 18.8 kDa and binds ssDNA and RNA (Kowalczykowski et al., 1981).

The stimulation of presynaptic complex formation by SSB protein results from SSB protein melting the secondary structure of the ssDNA (Kowalczykowski and Krupp, 1987). Post-synaptically, SSB protein serves to prevent formation of homologously paired networks of DNA that result form intermolecular re-invasion events and limits the reversal of DNA strand exchange (Lavery and Kowalczykowski, 1992): it binds to the displaced linear ssDNA, thus preventing its utilization by recA protein (Chow et al., 1988). The binding of SSB protein to the displaced ssDNA directly stimulates the rate of joint molecule formation (Lavery and Kowalczykowski, 1992).

Homologous recombination proteins of eukaryotes

The identification of genes involved in recombination in the yeast *S. cerevisiae* resulted from isolation of several sets of mutants, *rad* series being one such set. The *rad* mutants were identified based on their sensitivity to ionizing radiation. A subset of these genes, referred to as the *RAD52* epistasis group (*RAD50-RAD57*), so named because *RAD52* is the gene that confers the greatest X-ray sensitivity when mutated or deleted, were shown to be involved in DSB repair and homologous recombination (Edelmann and Kucherlapati, 1996). Mutations in any of the genes in the *RAD52* epistasis group lead to defects in the homologous recombination pathways of meiotic recombination, mitotic recombination, and mating type switching. Additionally, these mutations cause reduced homologous recombination in extra- and intrachromosomal assays and decreased DSB repair.

Rad51 eukaryotic homologue of recA protein

RAD51 is a member of the above group and the cloning of the gene revealed that its product is the eukaryotic homologue of bacterial RecA protein (Shinohara et al., 1992; Aboussekhra et al., 1992). *Rad51* mutants fail to efficiently repair DSB, and feature reduced sporulation in meiosis (Shinohara and Ogawa, 1995).

Another yeast gene whose product has homologies to the bacterial recA protein, *DMC1*, has been isolated by Bishop et al. (1992). *DMC1* is expressed only during meiosis and plays a role in recombination. Both *RAD51* and *DMC1* are expressed during meiosis and seem to have non overlapping functions.

The protein product of *RAD51* shows homology to the central core region of the bacterial RecA protein (Shinohara et al., 1993). In addition to structural similarities, the two proteins also share functional similarities (Ogawa et al., 1993). The Rad51 protein binds to double- and single-stranded DNA in an ATP-dependent manner. The protein also has an ATP-independent binding activity to single-stranded DNA. It can form right handed helical filaments on ssDNA, as well as catalyze DNA strand exchange in the presence of RPA (Sung, 1994). RPA is a functional equivalent of bacterial SSB protein, and may be involved in removing secondary structures within ssDNA (Alani et al., 1992).

Recently, mouse and human *RAD51* genes were isolated by virtue of their homology to the corresponding yeast genes (Shinohara et al., 1993). The mouse *RAD51* was able to complement the methyl methanesulfonate (MMS) sensitivity of yeast *rad51* mutants (Shinohara et al., 1993; Morita et al., 1993). The mouse gene is expressed at high levels in thymus and testis and at lower levels in a number of other tissues, suggesting that mouse *RAD51* may function in mitotic and meiotic cells. Moreover, mammalian Rad51 protein localizes to synaptonemal complexes and forms foci in somatic cells after DNA damage (Haaf et al., 1995).

In contrast to yeast *rad51* mutants, which are viable (Shinohara et al., 1992), mice embryos bearing a homozygous loss of function mutation in *RAD51* rarely survive past the

four-cell stage (Lim and Hasty, 1996). Therefore in mammals, *RAD51* is an essential gene required for cellular proliferation.

RAD52, RAD54, RAD55 and *RAD57*

RAD52, RAD54, RAD55 and *RAD57* are the remaining genes of the *RAD52* epistasis group which are involved in homologous recombination.

Rad52 is a ssDNA binding protein which is capable of stimulating DNA strand exchange by targeting the Rad51 protein to a complex of RPA with ssDNA (New et al., 1998; Benson et al., 1998). *S. cerevisiae rad52* mutants have severely impaired homologous recombination, much worse than in the case of *rad51* mutants, and are extremely sensitive to radiation (Schiestl et al., 1994). However, the homologous *S. pombe* mutant, *rad22*, is much less sensitive (Muris et al., 1996).

Human homologue of *RAD52* gene has been isolated (Muris et al., 1994). Among the homologues of the genes from the *RAD52* epistasis group, *hRAD52* is least conserved between yeast and mammals (Taylor and Lehmann, 1998).

RAD52 knockout mice are viable, fertile and have no apparent defect in immunoglobulin gene rearrangement (T. Rijkers and A. Pastink, unpublished). Interestingly, cells from *rad52* knockout mouse are not at all sensitive to ionizing radiation (Taylor and Lehmann, 1998). However, when human *RAD52* was overexpressed in wild type monkey cells, it conferred increased resistance to ionizing radiation and increased levels of homologous recombination (Park, 1995). Also, *RAD52* null mutants of chicken DT40 cells show decreased frequencies of gene targeting rearrangements (Y. Uamaguchi-Iwai and S. Tadeka, unpublished).

The Rad54 protein belongs to the family of SNF2/SW12 DNA dependent ATPases (Eisen et al., 1995). The proteins of this family are implicated in chromatin remodeling in the context of transcription, DNA repair and recombination. Rad54 contains distinct motifs found

in replicative DNA helicases such as *E. coli* DnaB, and branch migration proteins such as *E. coli* RuvB (West 1996). Rad54 interacts with Rad51 (Clever et al., 1997). Therefore, it has been suggested that Rad54 may be a processivity factor for Rad51-mediated strand exchange (Kanaar and Hoeijmakers, 1997).

RAD54 knockout mice are viable and exhibit an increased sensitivity to ionizing radiation and DSB-inducing agents (Esser et al., 1997). Although cells from *RAD54* knockout mice exhibit a 5-10 fold reduction in the frequency of gene targeting, V(D)J recombination is not impaired (Esser et al., 1997). *RAD54* null mutants of the chicken B cell line DT40 are also hypersensitive to ionizing radiation, and feature 7-fold reduced gene conversion frequencies. In contrast, gene targeting frequencies are reduced by at least two orders of magnitude in *RAD54* null chicken cells (Bezzubova et al., 1997).

Rad55 and Rad57 have very similar structures to that of Rad51 (Lovett, 1994). When mutated in *S. cerevisiae* they produce similar phenotypes to *rad52* and *rad54*, but less severe (Sugawara et al., 1995). Rad55 and Rad57 proteins have been found to interact *in vivo* (Johnson and Symington, 1995) and can be isolated as a complex from wild type yeast extracts (Sung, 1997b). The bacterial proteins RecO and RecR help "load" RecA onto ssDNA when RecA is in competition with the SSB protein (Shan et al., 1997), and the yeast protein Rad55 and Rad57 have been reported to play a similar role *in vitro*. It has been suggested that Rad55 and Rad57 function to promote DNA strand exchange by Rad51 by overcoming the inhibitory effect of RPA (Sung, 1997).

1.1.5. Illegitimate recombination

Illegitimate or non-homologous recombination occurs between DNA sequences with no prescribed sites and with no homology, or at best only a few base pairs of homology. Illegitimate events are typically non-conservative in that they result in the loss or gain of a

small number of nucleotides at the site of recombination (Williams and Fried, 1986; Wilkie and Palmiter, 1987).

Illegitimate recombination in yeast

In *S. cerevisiae*, most of recombination proceeds via homologous recombination pathways. However, non-homologous recombination has been detected as well, albeit at low frequency (at least 100-fold less frequent than homologous recombination events). When *S. cerevisiae* cells are transformed with linearized plasmids bearing no homology to genomic DNA, recircularization can occur via direct rejoining of the ends (Mezard and Nicholas, 1994), or via interaction of the ends of two linear molecules, resulting in the formation of head-to-head plasmid dimers (Kunes et al., 1990). These illegitimate recombination events are associated with short deletions or insertions around the ends of the DSB. Non-homologous integration of the linearized plasmids at low frequency has also been observed to occur in *S. cerevisiae* (Schiestl et al., 1993; Zhu and Schiestl, 1996). These integration events utilize little or no (≤4 bp) end-sequence homology and are also associated with small deletions and insertions at the joining sites.

Illegitimate recombination is much more efficient in other eukaryotes compared to *S. cerevisiae*. In *S. pombe*, although linearized plasmids bearing sequence homology to each other are efficiently recircularized by homologous recombination, 1 in 26 (compared to less than 1 in 100 of *S. cerevisiae*) are recircularized by non-homologous end-joining (Goedecke et al., 1994). Similarly the frequency of illegitimate integration of linearized plasmids is higher in *S. pombe* than in *S. cerevisiae* (Grimm et al., 1988; Tatebayashi et al., 1994).

Illegitimate recombination in mammals

In mammalian cells, illegitimate recombination is the predominant mechanism for processing of DSBs in extrachromosomal substrates. High frequency of random integration of targeting vectors into the genome of the transfected cells by illegitimate recombination is

seen as the major limitation for efficient gene targeting in mammalian cells. Most of the transfected DNA molecules will insert at random sites rather than at the predetermined homologous site with frequencies anywhere between 10^{-2} to 10^{-3} (Thomas et al., 1986; Doetschman et al., 1988; Deng and Capecchi, 1992; Waldman, 1992). Considering that gene targeting in mammalian cells occurs at frequencies of 10^{-5} - 10^{-7}, the ratio of homologous recombination to illegitimate integration is at best, 1 to 100.

The role of free DNA ends

Free DNA ends appear to be essential for the process of illegitimate recombination. For instance, illegitimate integration is stimulated over 40-fold by linearization of the transfected plasmid (Folger et al., 1982). DSBs resulting from the inhibition of the religation step of topoisomerases by VM26 and VP1 proteins has been reported to stimulate transfection frequencies (Woynarowski et al., 1988). Similarly, illegitimate integration can be increased 1000-fold by the introduction of a predefined DSB within the chromosomal DNA using the rare cutting yeast endonuclease I-SceI (Sargent et al., 1997). Blocking of free DNA ends by terminal addition of dideoxynucleotides reduces the frequency of illegitimate integration in mammalian genome (Chang and Wilson, 1987).

NHEJ is the basis of illegitimate recombination

Mammalian cells are extremely proficient in DNA end joining (Wake et al., 1984) and the high rate of illegitimate integration observed in mammals is the direct consequence of this ability. The primary mechanism responsible for this is the so-called non-homologous end joining (NHEJ) repair pathway, for which the basic function is the repair of chromosomal DSBs by direct ligation of the free DNA ends.

NHEJ appears to be the major pathway for repair of DSBs in mammals, as shown by the very marked radiosensitivity of mutant cells defective in this pathway (Jeggo et al., 1995). Several proteins of the NHEJ have recently been identified and characterized.

DNA-dependent protein kinase

DNA-dependent protein kinase (DNA-PK) is absolutely required for the process of NHEJ. It is composed of the 70 kDa and 86 kDa (encoded by the *XRCC5* gene) subunits of the DNA-end binding Ku protein, and the 460 kDa catalytic subunit (product of the *XRCC7* gene), which is recruited when Ku binds to DNA ends (Taylor and Lehmann, 1998).

Ku binds tightly to double-stranded ends, having equal affinity for 5'-protruding, 3'-protruding, and blunt ends. Ku also binds to DNA nicks, gaps, bubbles, and DNA ending in stem-loop structures (Blier, 1992).

Sequence analysis of the catalytic subunit of DNA-PK has shown that the kinase domain of DNA-PK shares strong homology to the phosphatidylinositol 3-kinase superfamily (Hartley et al., 1995). DNA-PK phosphorylates a number of *in vitro* substrates including histones, topoisomerases, various transcription factors, the tumor suppressor p53, RPA, and both subunits of Ku (Anderson, 1993). On phosphorylation by DNA-PK, Ku acquires an ATPase activity (Cao et al., 1994). Ku also possesses an ATP-dependent helicase activity (Tuteja et al., 1994), suggesting that autophosphorylation by DNA-PK confers helicase activity to Ku (Kimryn and Chu, 1998). Recently Ramsden and Gellert (1998) have demonstrated that Ku protein stimulated DNA end joining by mammalian DNA ligases, thus showing the direct role of Ku in repair of DNA DSBs.

Homologues of the two Ku subunits have been identified in yeast (Feldmann and Winnacker, 1993; Boulton and Jackson, 1996). Mutants in yeast *Ku70* (*HDF1* gene) and *Ku86* are barely sensitive to ionizing radiation (Siede et al., 1996). However, in a *rad52* background, deletion of the *Ku* genes results in a substantial sensitization to radiation (Siede et al., 1996).

Disruption of the yeast *Ku70* leads to a drop in illegitimate and restriction enzyme-mediated integration (Manivasakam and Schiestl, 1998).

XRCC4

Another protein required for NHEJ in mammals is XRCC4 (X-ray crosscomplementing). Mutants in the *XRCC4* gene are very radiosensitive (Li et al., 1995). It has been shown that the XRCC4 protein was tightly bound to DNA ligase IV, indirectly implicating this ligase species in NHEJ in mammalian cells (Critchlow et al., 1997; Grawunder et al., 1997).

Rad50, Mre11, and Xrs2 proteins

Another important player in the NHEJ repair of DSBs in yeast is the enzymatic complex formed by the Rad50, Mre11, and Xrs2 proteins (Taylor and Lehmann, 1998). It shares structural and functional similarities with the SbcCD protein complex of *E. coli* which is an ATP-dependent double-strand specific exonuclease (Sharples and Leach, 1995). All three proteins were shown to be essential for DSB repair by non-homologous end joining (Moore and Haber, 1996). The yeast null mutants of either *mre11*, *rad50* or *xrs2* feature poor mitotic growth, a delay in mating type switching, elevated rates of spontaneous mitotic recombination, short telomeres, and a defect in the formation of meiosis-specific DSBs (Ivanov et al., 1994; Kironmai and Muniyappa, 1997; Tsubouchi and Ogawa, 1998). Deletion of *RAD50*, *XRS2* or *MRE11*, leads to chromosome loss and reduces illegitimate recombination 70-fold, yet has little effect on the frequency of homologous recombination (Moore and Haber, 1996).

Human homologues of *RAD50* and *MRE11* have been isolated (Petrini et al., 1995; Dolganov et al., 1996). The human homologue of *XRS2*, *NBS1*, is mutated in Nijmegen breakage syndrome, a disease associated with radiation sensitivity, immunodeficiency, and chromosomal instability (Carney et al., 1998). Rad50 and Mre11 colocalize in mammalian cell to foci in response to DSB-inducing agents (Maser et al., 1997). Mre11 alone or in association with human Rad50 features exonuclease activity of 3'—>5' orientation *in vitro*

(Paull and Gellert, 1998). In murine embryonic stem cells, conditional deletion of *MRE11* is lethal, indicating that this gene is essential for proliferation (Xiao and Weaver, 1997).

Complex genomes can tolerate the imperfect DSB repair by NHEJ

DSBs are potentially lethal DNA lesions that are generated during normal cellular processes such as DNA transcription and replication (Roth and Wilson, 1988). When breaks do occur, illegitimate recombination provides a means for rapidly joining free DNA ends and thus maintaining the integrity of the cell genome.

Mammalian genomes contain enormous amounts of non-coding DNA sequences, such as LINEs, SINEs, centromeric repeats and introns. The overall amount of DNA in mammalian genomes is several times that of yeast. The chance that a DSB will occur in non-coding DNA is much more likely in a complex genome containing introns and repetitive elements than in a simple one. Breaks that occur in non-coding DNA, when repaired imprecisely, will produce silent mutations, without compromising the viability of the cell.

Hence, the efficient illegitimate recombination may be tolerated by mammalian cells due to the complexity of their genome. Moreover, multicellular organisms can tolerate imprecise or aberrant recombination because most of the cells in these organisms are somatic and genetic mutations in these cells are not heritable (Roth and Wilson, 1988).

1.1.6. Gene targeting

Gene targeting or a homologous recombination reaction between a transfected DNA sequence and a homologous genomic target sequence is an experimental application of a cell's ability to perform homologous recombination in order to bring about a desired alteration to an endogenous sequence. Much of the current knowledge about the mechanism of gene targeting derives from studies of extrachromosomal and intrachromosomal recombination in mammalian cells. There exist, however, several differences between the two latter modes of

recombination and it is still unclear whether gene targeting more closely resembles extra- or intrachromosomal recombination.

To facilitate the recovery of rare targeting events, early studies of gene targeting used artificially introduced loci with a defective marker gene to make them selectable targets (e.g. neomycin-resistance gene or the HSV *tk* gene). In these studies, the ratio of gene targeting to random integration was found to be between 10^{-2} to 10^{-5} (Waldman, 1992). Subsequent targeting of natural loci, both selectable and non-selectable, showed the same frequency of recombination as targeting with artificial loci (Doestschman et al., 1988; Adair et al., 1989; Baker et al., 1988).

Mechanisms of gene targeting

Gene targeting can take place via a single crossover event (Hinnen et al, 1978; Orr-Weaver et al., 1981), resulting in an insertion of the new piece of DNA into the genomic sequence (Figure 6a). Alternatively, a targeted replacement can occur via a double crossover (Rothstein, 1983) (Figure 6b). The latter event can result in a deletion if the replacement vector is shorter than the homologous genomic sequence, or if it is longer, it can give rise to an insertion.

Similar to the replacement process is gene conversion, which leads to the adaptation of the sequence of one strand to the sequence of another strand. Gene conversion can be defined as locally restricted copying of genetic information from one strand to another. It may be considered as a different form of homologous recombination, not necessarily being associated with cross-overs and, therefore, being non-reciprocal.

In gene replacement, an endogenous sequence is precisely replaced with a homologous sequence of the targeting vector, and the total number of nucleotides in the genome is not altered. In a targeted insertion, the entire transfected targeting vector, including the non-homologous sequences, is inserted at the target locus. After targeted insertion, two copies of a target sequence reside in the genome flanking an integrated copy of the vector.

For a number of applications, such as gene therapy, gene replacement is preferred over targeted insertion, for it need not augment the genome with foreign DNA sequences and consequently will not cause any undesirable alterations of the overall chromatin structure at the locus. Also, the closely linked duplicated target sequences resulting from targeted insertion are genetically unstable since the repeated sequences may undergo intrachromosomal recombination with one another.

At present it is still unclear whether gene targeting is mechanistically more close to extra- or intrachromosomal homologous recombination. There exist two major differences between the two latter modes: sensitivity to heterology embedded within the region of shared homology and the conservation of the recombining sequences. The former point is discussed below. As far as the question of sequence conservation is concerned, it is generally believed that extrachromosomal recombination occurs largely via the SSA mechanism and, therefore, is non-conservative (Lin et al., 1984; Chakrabarti and Seidman 1986; Lin et al., 1987). On the other hand, intrachromosomal recombination is conservative. Analysis of the structure of recombinants has revealed that spontaneous intrachromosomal recombination is mainly a conservative mechanism in mouse L-cells, with gene conversion representing approximately 80% of the recombination events (Liskay et al., 1984). The results from the study by Pennington and Wilson (1991), who attempted to determine whether gene targeting is a conservative or non-conservative process in CHO cells, indicate that gene targeting is conservative.

Targeting in the latter study was performed using an insertion type targeting vector, i.e. the targeting vector was linearized within targeted homology of the *aprt* gene. It was observed that genetic markers in the vicinity of the DSB were replaced by markers from the genomic target. This result is consistent with a DSBR model. Further confirmation for gene targeting occurring via DSBR in mammalian cells comes from the observation that transfected molecules containing large gaps within the targeted homology were capable of correcting a defective *hprt* gene in mouse ES cells and that the gaps on the transfected sequence were

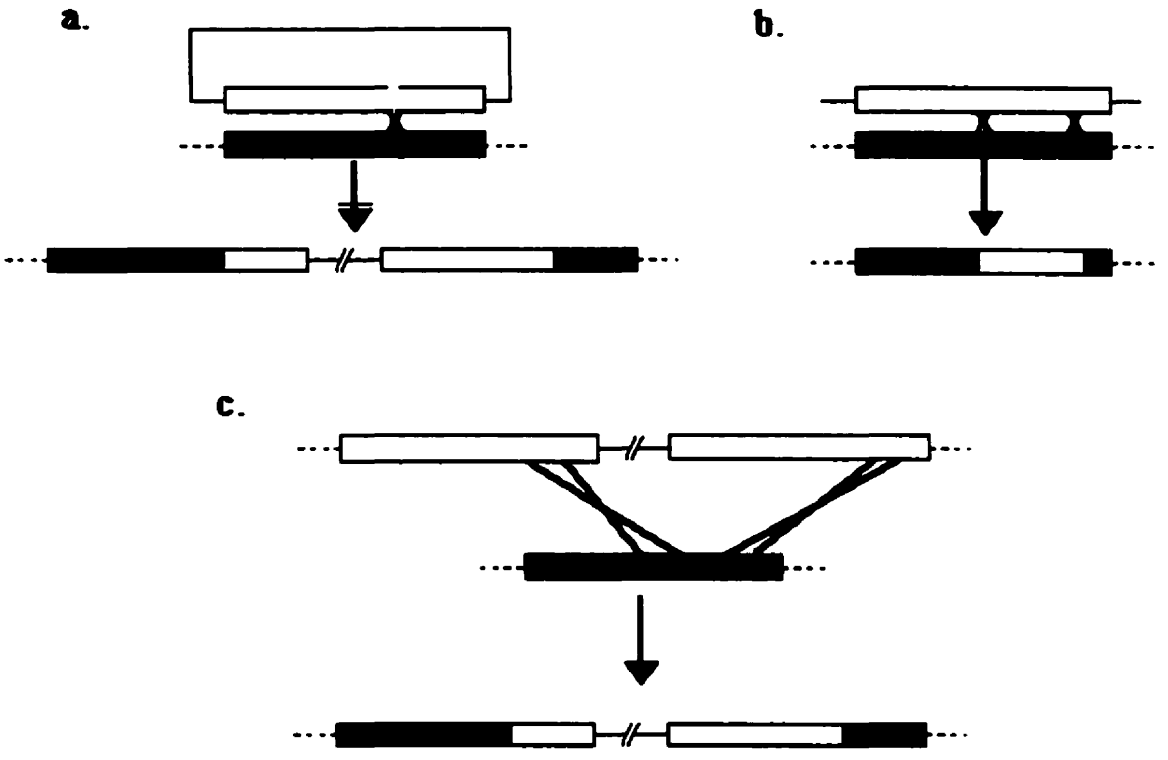

Figure 6. Modes of Gene Targeting. The target sequence is denoted by the black rectangle, with flanking genomic sequences denoted by the broken line. The targeted homology contained on the transfected construct is represented by the white rectangle, with vector sequences depicted by thin lines. (**a.**) Targeted insertion by a construct cleaved within targeted homology. Insertion results in two copies of the target sequence flanking an integrated copy of the vector. Insertion may occur by a single crossover at the break in homology, as shown, or by a single crossover involving a circular construct. (**b.**) Gene replacement by a construct cleaved within vector sequences. Part of the genomic target is replaced by the transfected sequence, while the fate of the transfected construct is usually unknown. Gene replacement can occur by a double crossover or by gene conversion. (**c.**) Apparent targeted insertion via gene replacement involving a concatemer of the transfected construct. As illustrated, such a gene replacement may yield a product that is indistinguishable from a targeted insertion.

repaired using genetic information contributed by the genomic target sequences (Valancius and Smithies, 1991). Additional studies by Deng et al. (1993), using mouse ES cells, also revealed results consistent with DSBR model.

The targeted alteration of a genomic sequence implies that the flow of genetic information proceeds from the targeting vector to the chromosomal sequence. However, the events where the defective gene of the targeting molecule was corrected by the chromosomal sequences have been reported as well (Jasin et al., 1985; Shaul et al., 1985; Subramani, 1986). It is not clear what factors are responsible for directing the flow of genetic information during gene targeting. Thomas et al. (1986) suggested that mammalian cells might have a propensity to correct deletions rather than insertions, that is, it might be wiser to try to introduce an insertion rather than a deletion mutation into a genomic target so that information flows to, rather than from, the genome.

Parameters of efficiency

Homology requirements

Extra- and intrachromosomal recombination as well as gene targeting are sensitive to the length of homology shared by the recombining sequences. In at least one report, the rate of targeting was essentially exponentially sensitive to homology length, approaching a plateau at approximately 14 kb (Deng and Capecchi 1992). This is different from homology requirements for recombination in bacteria and yeast, where homologous recombination shows only linear dependency on the length of homology (Szostak and Wu, 1979; Smolik-Utlant and Petes, 1983).

Two alternative strategies were used to address the question of homology requirements for homologous recombination in mammalian cells. The first made use of two truncated molecules with different lengths of overlapping, uninterrupted homology. When the two truncated molecules share homologies between 295 bp and 1.8 kb, the rate of gene conversion is directly proportional to the length of uninterrupted homology. Compared to 295

bp of homology, the rate is reduced 7-fold with 200 bp of homology and 100-fold with 95 bp of homology (Liskay et al, 1987).

The alternative strategy used two molecules of approximately the same size but containing sequence polymorphism. Waldman and Liskay (1987) used a mutated HSV *tk* genes recombinational system in which one complementary *tk* copy came from HSV type 1 and the other copy from HSV type 2. These *tk* genes share 81% of homology. It was observed that with 19% divergence, the rate of intrachromosomal homologous recombination was reduced 1000-fold relative to the rate of homologous recombination between two identical HSV *tk* sequences. In contrast, the rate of extrachromosomal homologous recombination was only reduced by a factor of 3 to 15 (Waldman and Liskay, 1987).

Thus, the minimum homology required for efficient homologous recombination is between 163 and 240 bp in mammalian cells (Rubnitz and Subramani, 1984; Liskay et al., 1987, Waldman and Liskay). It has been recommended that at least 1 kb of targeted homology flank any embedded heterology residing in a gene replacement type of construct. This is to ensure that gene targeting is not only efficient but accurate as well, since limited homology has been associated with aberrant gene targeting events (Thomas et al., 1992).

The homology requirements for homologous recombination reactions appear to differ depending on whether the recombination is intra- or extrachromosomal. Intrachromosomal recombination has been found a great deal more sensitive to small degrees of heterology than extrachromosomal recombination. Intrachromosomal recombination in mouse cells was generally found to be inversely proportional to the length of a heterologous sequence embedded within one of the recombining sequences (Letsou and Liskay, 1987). Waldman and Liskay (1988) reported that about 150 bp of contiguous homology are required for efficient intrachromosomal recombination in mouse fibroblasts, and that even a single nucleotide mismatch embedded in a 200 bp stretch of homology can reduce intrachromosomal recombination rates by more than an order of magnitude. No such exquisite sensitivity to heterology was found for gene targeting in mammalian cells (Deng and Capecchi, 1992).

It is generally believed that the frequency of gene targeting decreases with increasing length of heterologies embedded into the homologous frequency (Thomas and Capecchi, 1987; Bollag et al., 1989; Frohman and Martin, 1989). However, Mansour et al. (1990) have reported that varying length of non-homologous interruption (up to over 10 000 bases) had no effect on gene targeting frequency as long as they are surrounded by sufficiently long stretches of homologous sequences. Berinstein et al. (1992) have reported efficient gene targeting even with one-sided homology. Thus, it seems that it is not the absolute length of heterologous sequences but rather their location within the homologous region which is crucial for an efficient gene targeting. Hence, a location close or at the end of a homology region will initiate no or only one single crossover (leading to an insertion), rather than a double crossover required for replacement gene targeting.

Dependence on copy number

Neither the number of targets nor the amount of the incoming DNA appear to change the gene targeting efficiency.

Thomas et al. (1986) injected increasing amounts of DNA into recipient nuclei (from 5 to 100 copies). This increase of the number of incoming DNA did not improve the targeting efficiency. Similar result has been obtained by Rommerskirch et al. (1988). These results imply that the initial interaction of targeting vector with chromosomal DNA is not the rate-limiting step in a targeting reaction. Alternatively, it is possible that as the amount of incoming DNA increases, so does the length of tandem arrays of the linearized vector, probably due to the strong ligase activities of mammalian cells. So, the vector DNA gets concatamerized faster than recombination can occur. Yet, another explanation might be that as the number of transfected molecules increases they start to recombine between themselves rather than with the chromosomal target. This latter explanation is supported by the observation that extrachromosomal recombination is dependent on the number of recombining molecules (Waldman and Liskay, 1987).

The effect of the different number of target sites on gene targeting frequency have been investigated by Zheng and Wilson (1990). When targeting a locus with 400 copies of the DHFR gene, only a single targeting event typically occurred, suggesting that one targeting event may exclude additional targeting. However, it can be argued that the 400 copies of the gene were present in the target cells in tandem arrays, and consequently occupied only a relatively small region within the genome, and unlike unlinked copies, did not contribute to the number of additional entry sites for homologous exchange. Also, targeting of tandem repeats may involve mechanisms different from targeting of non-redundant loci, which allows the former to remain stable in the chromosome.

Effect of GT, GC repeats

A variety of DNA sequences may play direct or indirect roles in recombination by their effects on the DNA structures. Treco and Arnheim (1986) have reported that the evolutionary conserved repetitive sequence d(TG, AC)$_n$ promoted reciprocal exchange in yeast. It was proposed that GT and GC repeats, which can give rise to Z-DNA, may influence recombination (Blaho and Wells, 1989). Several groups have reported that GT, GC repeats and minisatellite repeats, stimulated extrachromosomal recombination of transfected DNA in mammalian cells (Bullock et al., 1986; Wahls and Moore, 1990; Wahls et al., 1990). However, a $(GT)_{29}$ repeat was unable to stimulate intrachromosomal homologous recombination (Sargent et al., 1996).

Local chromatin structure

It has been suggested that local chromatin structures and local variations in accessibility and functional levels of DNA regions to be targeted were responsible for variations in gene targeting rates (Lin et al., 1985, Thomas et al., 1986).

Effect of transcription

Transcription stimulates homologous recombination in *S. cerevisiae* (Thomas and Rothstein, 1989; Voelkel-Meiman et al., 1987). It is not clear whether transcription of the target locus affects homologous recombination in mammalian cells.

Targeted modification of the β-globin gene in human EJ cells (Smithies et al., 1985) and of the *adipsin* and adipocyte P2 genes in mouse ES cells (Johnson et al., 1989) suggest that transcription of the target gene is not required for gene targeting.

On the other hand, stimulation by transcription of extrachromosomal (Nickoloff and Reynolds, 1990) and intrachromosomal recombination (Nickoloff, 1992) has been reported in CHO cells. In the latter case, alleles transcribed at high levels recombined about 2 to 7-fold more frequently than identical alleles transcribed at low levels. Transcription has also been reported to stimulate gene targeting in HT1080 cells (Thyagarajan et al., 1995).

However, transcription appears to have no effect on recombination induced by DNA DSBs (Taghian and Nikoloff, 1997). It is possible that in the later case the stimulation of recombination by the DSB *per se* masks the effect of transcription.

Effect of cell cycle position

In yeast, chromosomes mitotic conversion events were shown to occur primarily in the G1 phase before DNA replication occurs (Esposito, 1978; Esposito and Wagstaff, 1982).

Wong and Capecchi (1987) examined the ability of Rat-20 (adenine phosphorybosyl-transferase-negative) cells to mediate extrachromosomal homologous recombination between two plasmids carrying truncated but overlapping fragments of the *aprt* gene at various stages of the cell cycle. They found that homologous recombination activity was low in early G1, rose 10 to 15-fold by early to mid-S phase, and then declined as cells proceeded through the S phase and reentered G1. Thus, the peak of recombination activity was in early to mid-S phase, but replication of the substrate was not necessary for recombination.

Dependence on cell type

It is generally believed that embryonic stem (ES) cells undergo gene targeting at much higher rates than do the transformed somatic cells which have been extensively used in the early studies of gene targeting. However, there are few studies which have consistently compared gene targeting frequencies in ES cells with those in other mouse cell lines.

Two such studies suggest that F9 embryonic carcinoma cells (Coll et al., 1995) and two pre-B cell lines, BASC6-C2 and 18-8tk- (Charron et al., 1990) undergo gene targeting as efficiently as ES cells. In another study (Arbones et al., 1994), primary myoblasts featured an absolute targeting frequency higher than did ES cells targeted with the same construct.

The only known case of a higher eukaryote cell line with reproducibly high gene targeting efficiency is the chicken B cell line DT40 (Buerstedde and Takeda, 1991). The immunoglobulin light chain locus and other unrelated loci can be efficiently modified by gene targeting in this cell line with homologous recombination events representing 6.5-100% of stably transfected clones. Absolute targeting frequencies in DT40 cells do not appear to be unusually high ($3.1 - 8.4 \times 10^{-6}$) (Buerstedde and Takeda, 1991), suggesting that non-homologous recombination might be inefficient in these cells.

Gene targeting in human cell lines, including fibroblastoid, lymphoid, hepatic, epitheloid, myeloid, bladder and colon cell lines, and several types of primary human cells, featured absolute targeting frequencies, and gene targeting/ illegitimate recombination ratios similar to those described for murine cells (reviewed in Yáñez and Porter, 1998).

It appears that gene targeting can be performed with similar efficiencies in a variety of cell types from different species, whether transformed or primary, pluripotent or differentiated.

Transfection methods

The method applied to introduce exogenous DNA into target cells can largely affect the rate of gene targeting. Thus, the highest ratios of homologous to random integration are obtained with nuclear microinjection, 10^{-2} according to Thomas et al. (1986). Whereas ratios of only 10^{-5} are usually obtained with the calcium phosphate coprecipitation method (Lin et al., 1985).

Two studies report that for targeting of the *APRT* (Nairn et al., 1993) and *HPRT* (Waldman et al., 1996) genes in CHO cells, electroporation can give higher absolute targeting frequencies than calcium phosphate precipitation, even though the two methods gave similar random integration frequencies.

One study compared the efficiency of gene targeting in the human fibrosarcoma cell line HT1080 using electroporation versus lipofectamine transfection (Yáñez and Porter, 1998) and concluded that gene targeting was 10-fold more efficient by electroporation than by lipofection.

Some ways of transfecting DNA were reported to cause more damage to the exogenous molecules than others (Lebkowski et al., 1984; Wake et al., 1984). This can explain the variations in recombination frequencies between different transfection methods. Another suggested explanation for the variation is that the transfected DNA lacks protection by nucleic acid-binding proteins and that even the accessibility of the DNA by such proteins varies for the different transfection methods. That could explain why the calcium phosphate coprecipitation method often shows lower rates than the methods that release naked DNA into the nuclei, such as microinjection or electroporation (Zimmermann, 1982).

Dependence on DSB

Gene replacement can be promoted by cleaving the targeting construct within vector sequences prior to transfection, so that the targeted homology on the transfected molecule is collinear with the genomic target (Thomas and Capecchi, 1987). Targeted insertions can be

promoted by cleaving the transfected molecule within the targeted homology to produce adjacent DNA termini within homology. Depending on the manner in which the transfected molecule is cut, it is referred to as either a gene replacement construct or an insertion construct.

As mentioned above, gene targeting is believed to proceed via the DSBR model. The model has been developed based on the observation that DSBs within homology could stimulate recombination. Interestingly DSBs outside of homology region, i.e. DSBs introduced in the non-homologous region of the targeting vector were found by some to stimulate recombination as well (Thomas and Capecchi, 1987). However, other investigators have reported that a double-strand break placed within the targeted homology is crucial for efficient targeting in mouse ES cells and that gene replacement vectors (cut outside of homology region) are intrinsically inefficient and their use leads to many aberrant events (Hasty et al., 1991).

Based on these observations, a model for gene targeting has been proposed by Hasty et al. (1992), in which adjacent DNA ends within homology play a critical role in the targeting process in a variation of the DSBR model. In this targeting model, two adjacent free DNA ends from the same broken strand of DNA from an insertion construct simultaneously invade the target sequence, forming a single D-loop. The ends of the invading strands often ligate within the D-loop. The event is then processed as in the DSBR model. The inefficiency of targeting using gene replacement constructs is seen as stemming from the need for such events to proceed via the formation of two D-loops. However, as mentioned above, according to the Holliday and Meselson/Radding models, gene conversions, which may be responsible for gene replacements, can proceed via the formation of only a single D-loop. The vast majority of gene targeting events observed by Hasty et al. were insertions. Although their model predicts that transfected molecules cut within targeted homology should work more efficiently than ones cut within vector sequences, it does not explain why targeting events should be resolved in favor of insertion over replacement.

Further controversy regarding the importance of the DSB position within the targeting vector stems from studies in which a targeting vector cut within targeted homology, and therefore, presumably primed for insertion, actually gave rise to a majority of gene replacement events (Adair et al., 1989).

In spite of controversy around the precise role of DSBs and of DNA ends in gene targeting, it is an established fact that linear molecules are more efficient targeting vectors than are circular molecules.

Since the broken DNA molecule tends to be a recipient rather than a donor of genetic information, the introduction of DSB within genomic targeted sequence rather than within the targeting vector, should dramatically increase gene targeting at this locus. Indeed, it has been shown recently that the frequency of gene targeting can be increased by over 50-fold by the induction of a specific DSB within the target locus (Smith et al., 1995). The experiment made use of I-SceI, a mitochondrial intron-encoded, site-specific endonuclease from yeast (Jasin, 1996). A modified version of I-SceI can be expressed in the nucleus. I-SceI recognizes a cleavage site of 18 bp, hence its expression is not toxic for mammalian cells. Induction of a site-directed chromosomal DSB by I-SceI stimulates homologous recombination between 50 and 100-fold (Taghian and Nickoloff, 1997; Rouet et al., 1994; Sargent et al., 1997). However, illegitimate recombination at the site of the DSB is stimulated 1000-fold (Sargent et al, 1997).

Improving gene targeting in mammalian cells

Conversely to homologous recombination in yeast, homologous recombination in mammalian cells is a highly inefficient process. The average frequency for gene targeting in mammalian cells is estimated at 1 for every 10^6 transfected cells. Another major obstacle that stands in the way of applying gene targeting to such useful ends as gene therapy is the extreme proficiency of the mammalian cells in illegitimate recombination. The ratio of random integration to gene targeting in transfected cells is typically 1000 to 1 (Waldman, 1992).

Hence, ways must be found to selectively stimulate homologous recombination in the target cells without, however, increasing the frequency of illegitimate recombination.

Increasing the length of homology

As mentioned above, all types of homologous recombination events that have been investigated in mammalian cells are sensitive to the length of shared homology between the recombining sequences. This knowledge has already been put to use to improve targeting efficiency and accuracy. It has been suggested that the use of exceptionally long homology (> 50 kb) would improve gene targeting even further. The latter can in principle be achieved through the use of artificial chromosomes, but this approach remains mostly theoretical (Waldman, 1995).

Use of isogenic DNA

The efficiency of gene targeting is very sensitive to heterologies within the homologous domain of the recombining sequences. DNA polymorphisms seem to be detrimental to efficient targeting presumably because of interference form the mismatch repair pathway (Deng and Capecchi, 1992). This problem can be solved by using isogenic DNA, i.e. DNA isolated from cells of the same individual as those to be targeted. A DNA clone is only isogenic to one of the two alleles in a diploid cell. Preferential targeting of the isogenic allele has been shown to occur in chicken DT40 cells (Bezzubova et al., 1997).

Alternatively, the efficiency of gene targeting using non-isogenic DNA can be increased by inhibiting the gene product of the mammalian *MSH2* gene. *MSH2* is a homologue of bacterial *MutS*, whose protein product binds to base mispairs and loops out up to four unpaired nucleotides, triggering the *mutSL* pathway of DNA mismatch repair (Grilley et al., 1990). The mouse *MSH2* has been disrupted in ES cells, and extracts from *MSH2* null mutants are defective in DNA mismatch-binding proteins *in vitro* (de Wind et al., 1995). Gene targeting of the *Rb* locus was found to occur as efficiently with a non-isogenic targeting

construct as with an isogenic one in the *MSH2* null ES cells (de Wind et al., 1995). However, no specific inhibitors of mismatch repair are currently available.

Linearization of the targeting vector

It is common knowledge nowadays that for efficient targeting the targeting vector must be linearized (Smithies et al., 1984; Jasin et al., 1985; Thomas et al., 1986). The site of linearization within the vector must be judiciously chosen according to the desired type of targeting: inside the region of shared homology for the insertion vector; outside for the replacement vector.

Optimization of transfection technology

As mentioned above, gene targeting efficiency is greatly influenced by the method used for the introduction of the targeting vector into the cells. The best gene targeting frequencies are so far obtained with nuclear microinjection (Thomas et al., 1986; Zimmer and Gruss, 1989). Although microinjection is being successfully used for gene targeting in ES cells, it is not practical for such application as *in vivo* gene therapy.

Over the past years a number of alternative transfection technologies were developed. These include different liposome formulations, several viral vectors, gene gun, polyamidoamine dendrimers and synthetic peptides. Few of these, however, were consistently studied to assess their comparative efficiency with respect to gene targeting.

Although extensively tested in gene supplementation gene therapy protocols, viral vectors have not been widely used for gene targeting because of restrictions in the size and design of the homologous DNA, potential interference by virally encoded proteins and because simpler delivery methods have been adequate for gene knockout studies. The high transfection frequency generally observed with viral vectors, however, make them very attractive as potential delivery systems for gene targeting *in vivo*.

In recent studies targeting of the *FGR* locus with isogenic DNA in mouse ES cells using a replication-incompetent adenoviral vector produced ratios of gene targeting to random integration as high as 1:2.5 (Mitani et al., 1995), while the same targeting cassette gave a ratio of 1:20 when transfected by electroporation. An earlier report described the use of replication-competent adenovirus vectors for gene targeting of the *APRT* locus in CHO cells (Wang and Taylor, 1993) with the observed gene targeting to random integration ratios ranging from 1:5 to 1:14, a 400-fold improvement over that reported upon calcium phosphate transfection of plasmid targeting construct.

RNA-DNA chimeric vectors

Chimeric oligonucleotides composed of DNA and RNA residues can be used to repair single base pair mutations. This strategy was prompted by the discovery that RNA-DNA hybrids were highly active in homologous pairing reactions *in vitro* (Kmiec et al., 1994).

These observations suggested a strategy for targeted correction in which a short, double-stranded oligonucleotide vector is activated for recombination by incorporating RNA residues. Such chimeric molecules are designed to hybridize to a target site within the genome and induce a single base mismatch at the residue targeted for mutation. The DNA structure created at this site is recognized by the host cell's mismatch repair system which mediates the correction reaction. This approach gave promising results in *in vitro* experiments correcting a single base pair mutation in the 6th codon of the β-globin gene in the lymphoblastoid cells with 50% efficiency (Cole-Strauss et al., 1996) and reversing the foci-forming phenotype in NIH3T3 cells by altering a single point mutation in the 12th codon of the *H-ras* gene (Kmiec, 1996).

Use of small ssDNA fragments

Small DNA fragments have been used to target the 3 bp deletion (DF 508) of the cystic fibrosis transmembrane regulator (CFTR) gene in transformed lung epithelial cells from a patient with cystic fibrosis (Kunzelmann et al., 1996). The targeting construct was a denatured 491 bp PCR product derived from a functional CFTR gene. A targeting efficiency of 10^{-2} has been observed. The reasons for this remain unclear. The use of ss vectors for gene targeting had been reported before, and it did not seem to produce an improvement (Fujioka et al., 1993).

Ex vivo targeting of stem cells

The paradigm for targeting of somatic stem cells in culture with the subsequent selective amplification of the targeted cells for return to the donor, are the mouse knockout experiments. Established mouse embryonic stem (ES) cell lines can be subjected to gene targeting and amplification in culture without losing their stem cell properties. Hence, after the genetic disruption by gene targeting is confirmed, the modified ES cells can be reintroduced into the embryonic inner cell mass where they contribute to the developing embryo (Melton, 1994).

Recent developments in the characterization and manipulation of human somatic stem cells, such as hematopoietic stem cells (Emerson, 1996) and keratinocytes (Watt, 1988) suggest that analogous procedures might be adopted for gene correction in specific tissues. These procedures would involve the growth and gene targeting of a patient's somatic stem cells in culture, including the expansion of targeted derivatives for return to the patient.

The limiting factor of such an approach is not so much the efficiency of gene targeting, but the ability to isolate and grow the somatic stem cells in culture. Given the low natural abundance of somatic stem cells and the difficulty in maintaining them in culture without inducing their differentiation, the partial purification of the stem cells and their manipulation in culture for only limited periods of time before returning them to the donor

appear as more realistic. In this case, an improvement in gene targeting efficiency would be required to make the *ex vivo* gene targeting approach work.

1.2 Endo-exonucleases

Endo-exonucleases are multifunctional nucleases as the name implies. They have true endonuclease activity which cleaves both linear and closed circular substrates and true exonuclease activity which cleaves linear substrates from their termini. It is now becoming apparent that these nucleases, from bacterial to human cells play an important role in recombination and recombinational DNA repair, leading to maintenance of the genome with some diversification (reviewed in Fraser, 1996). Evidence also exists that eukaryotic endo-exonucleases might be involved in apoptosis (reviewed in Fraser, 1994; Fraser, 1996), but this aspect of endo-exonuclease is not further discussed in the present text.

1.2.1 RecBCD of *E. coli*

The prime example of a prokaryotic endo-exonuclease is the ATP-dependent DNase of *E. coli*, the recBCD nuclease, also known as exonuclease V (reviewed in Kowalczykowski et al., 1994).

RecBCD was first isolated and characterized by Wright et al. (1971) and by Goldmark and Linn (1972). It is a heterotrimeric enzyme composed of subunits encoded by the *recB*, *recC* and *recD* genes, with respective molecular weights of 134, 129 and 67 kDa. Products of each *rec* gene have been individually purified and used in reconstitution of the holoenzyme *in vitro* (Hickson et al., 1985; Masterson et al., 1992). Only the recB protein had a detectable enzymatic activity *in vitro*, a low DNA-dependent ATPase activity (Hickson et al., 1985; Masterson et al., 1992), thus demonstrating that the activities of the recBCD enzyme are critically dependent upon subunit interactions.

The recBCD nuclease has true ssDNA specific endonuclease activity as revealed by its ability to degrade ss-circular but not covalently closed double strand circular DNA, including

supercoiled and relaxed forms (Goldmark and Linn, 1972; Karu et al., 1973). The activity with ss-circular DNA is dependent on Mg^{2+} but not on ATP, although it is stimulated 7-fold with the addition of 2 mM ATP (Goldmark and Linn, 1972). The endonucleolytic breaks have 3'-OH and 5'-P termini. One consequence of not having activity with circular dsDNA is that the recBCD nuclease does not promote plasmid DNA recombination in *E. coli* (Smith, 1988). Endonucleolytic cleavage of nicked circular dsDNA by the recBCD is seen, however, when the ss-break is converted into a ss-gap of at least 10 nucleotides by the action of other exonucleases at the nick (Karu et al., 1973), presumably by allowing the large heterotrimeric enzyme to access the ss-region.

The exonuclease activity of the recBCD nuclease requires termini from which it degrades the DNA. It acts on both long linear ss- and dsDNA to release small 5'-P and 3'-OH-terminated oligonucleotides in a strictly ATP-dependent manner (Goldmark and Linn, 1972; Karu et al., 1973). No polarity in the progression of the enzyme from a terminus has been observed. The exonuclease activity in the presence of 10 mM Mg^{2+} is optimal at approximately 0.05 mM ATP, and is only 20% of optimal activity at 1-2 mM ATP (Goldmark and Linn, 1972). The activity is highly processive and can generate duplexes with ss-tails thousands of nucleotides long provided that the enzyme and DNA are present in equimolar concentrations. In the presence of excess enzyme, the ss-tails are cleaved.

A third activity of the recBCD nuclease is DNA-dependent ATPase activity. ATP is hydrolyzed by the enzyme to ADP and inorganic phosphate in the presence of Mg^{2+} and either ss- or dsDNA. It has been estimated that up to 20 ATP molecules were hydrolyzed for each phosphodiester bond cleaved in the DNA substrate (Goldmark and Linn, 1972). ATP hydrolysis can be uncoupled form nuclease action, for example in the presence of the non-hydrolyzable nucleic acids, DNA-RNA hybrid and cross-linked ds-DNA (Karu et al., 1973). A mutation in the recB gene encoding the ATPase subunit renders the mutant deficient in recombination (Eggleston and Kowalczykowski, 1993).

In the presence of inhibitors of the nuclease activities, such as an excess of SSB protein or 1 mM Ca^{2+}, the recBCD enzyme becomes a Mg^{2+} and ATP-dependent helicase (Rosamond et al., 1979). The enzyme unwinds the DNA duplex at a speed of about 300 bp per second, as it progresses from a ds-terminus, faster than the ss-regions behind the enzyme renature to reform the duplex (about 100 bp per second). This results in the unwinding of the duplex at about 200 bp per second to form twin ss-loops in the ds-DNA, appearing like rabbit ear structures in electron micrographs (Taylor and Smith, 1980). These transient structures were also detected in the absence of the SSB protein (Taylor and Smith, 1980).

The isolation of *E. coli recD* mutants that lacked appreciable nuclease activities but retained the ATP-dependent helicase activity and were fully recombination proficient (and even hyper-recombinogenic), led to the idea that the major role of the recBCD nuclease in recombination was not nucleolytic but rather DNA-unwinding (Amundsen et al., 1986). As well as the *recD* null mutants, special point mutants of *recC* feature similar phenotype (Smith, 1988), suggesting the importance of interaction between the recC and recD subunit for the nucleolytic activities of the holoenzyme. Direct comparison of the nuclease activities of the recBCD and recBC enzymes concluded that the recBC form of the enzyme retained some DNA-dependent ATPase and helicase activities but no nuclease activities could be seen in the absence of the recD subunit (Palas and Kushner, 1990).

Chi recombinational hot spots

The Chi recombinational hot spot has been identified in the studies of recombination of lambda bacteriophage in its *E. coli* host (Smith, 1988). The lambda phage *red- gam-* mutants, used in these studies, are deficient in the normal phage recombination proteins and thus, rely on the host recBCD nuclease for recombination. It has been observed that the recBCD dependent recombination rate of the phage double mutants was significantly enhanced by spontaneous point mutations occurring in the phage. The recombination would occur at and around the sites at which the mutations arose. Four such "hot spots" for

recombination were detected in the lambda mutants, 1 per 12 kb on average. These were called Chi sites and turned out to have a common octanucleotide sequence, 5'-GCTGGTGG-3'. Some other closely related octanucleotide sequences will also promote recombination dependent on the recBCD nuclease. An estimated 1000 Chi sites are present in the *E. coli* genome, 1 per 5 kb on average.

Chi dependent nick of lambda dsDNA by recBCD nuclease has been demonstrated *in vitro* (Ponticelli et al., 1985). Single endonucleolytic breaks are made 4 to 6 nucleotides 3' to the Chi sequences as the enzyme tracks in the 3' to 5' direction with respect to the Chi sequence. The Chi-dependent nicking occurs in the presence of ATP excess when all of the Mg^{2+} is chelated by the ATP and there is no appreciable degradation of the DNA to small products. In this case, Chi-dependent nicking occurs just behind the Chi site and the nuclease continues to track along the duplex and unwind the 3'-OH terminated strand. However, in the presence of Mg^{2+} excess, Chi-dependent production of the 3' ends occurs on the Chi-containing strand within the octameric Chi sequence. In addition, Chi-dependent ss-breaks occur on the complementary strand in and near the complementary Chi region. In this case also, the nuclease degrades the 3'-terminated strand to small oligonucleotides up to the vicinity of the Chi site (Dixon and Kowalczykowski, 1995; Taylor and Smith, 1995).

There are at least two well known models for Chi activity (Myers and Stahl, 1994). In a nick-initiated model for Chi, the activities proposed for recBCD are those observed *in vitro* when Ca^{2+} is added to inhibit ExoV activity. RecBCD enters a duplex DNA end and travels as a helicase. Occasionally recBCD manifests a strand-specific, ss endonuclease activity, nicking the strand that ends 3' at the recBCD entry site. Such nicks are more frequent at Chi, just 3' of the terminal G3'. The model proposes that the 3' end of such a nick is displaced by continued recBCD travel after nicking, that the displaced stand is coated with RecA which helps the 3' tail invade a homologue, eventually producing a Holliday junction.

In the split-end model, recBCD enzyme loads onto DNA at a DSB as ExoV. It travels in this mode, degrading both DNA strands to small oligonucleotides. When RecBCD

encounters a Chi site from the correct G3' side of the sequence, the enzyme loses its RecD subunit and is thereby transformed from ExoV into helicase mode. Helicase is postulated to be the recombinogenic activity of recBC. Continued leftward travel unwinds the DNA, creating a recombinogenic split-end. Both strands are invasive after being coated with RecA.

1.2.2 Mitochondrial endo-exonucleases

The first mitochondrial nuclease to be successfully purified and characterized at the molecular level was that from *S. cerevisiae* (Dake et al., 1988). It was found to be a 37 kDa endo-exonuclease acting on both DNA and RNA with activities that were very similar to a 33 kDa endo-exonuclease that had been purified previously from *Neurospora crassa* mitochondria (Chow and Fraser, 1983).

The gene for the *S. cerevisiae* mitochondrial endo-exonuclease, *NUC1*, is encoded by the nuclear DNA (Vincent et al., 1988). It is located on yeast chromosome X and contains an open reading frame that encodes a sequence of 329 amino acids, the expected length for a 37 kDa protein. The sequence is proline-rich (Vincent et al., 1988) and shows some low homology with the C-terminal end of the recC polypeptide (Fraser et al., 1990). *NUC1* is poorly expressed (100-300 molecules of enzyme per cell) and the expression does not vary more than 2-fold in cells grown on fermentable versus non-fermentable carbon source and in cells in logarithmic versus stationary phase. Deletion of *NUC1* results in mitochondria without detectable nuclease activity (Zassenhaus et al., 1988).

All of the detectable nuclease activity in fresh extracts of both *Neurospora* and *Saccharomyces* mitochondria can be completely immunoprecipitated by antibody to the purified *Neurospora* endo-exonuclease, suggesting that the endo-exonucleases are the major nucleases in the mitochondria of these species (Dake et al., 1988; Fraser et al., 1986).

The yeast mitochondrial endo-exonuclease is Mg^{2+} dependent, but can also utilize Co^{2+} and Mn^{2+} as activating metal co-factors. It possesses a ss-specific endonuclease activity on linear and closed circular ssDNA and on ssRNA. It also has 5'—>3' exonuclease activity

with linear ss and dsDNA (Dake et al., 1988). Unlike the exonuclease activity of the *Neurospora* endo-exonuclease which is processive (Chow and Fraser, 1983), the exonuclease activity of the yeast mitochondrial nuclease is distributive. The native enzyme is a homodimer of 68 kDa. Unlike the recBCD nuclease of *E. coli*, the endonuclease activity is unaffected by ATP and can act on small covalently closed circular DNAs to first relax and then convert them to unit-length liner dsDNA. The linear dsDNA fragments are then rapidly degraded to small acid-soluble oligonucleotides. The breaks made have 5'-P and 3'-OH termini. Unlike the recBCD nuclease, the exonuclease activity of the yeast mitochondrial endo-exonuclease with linear dsDNA is not dependent on ATP.

One of the first mammalian mitochondrial nucleases to be purified was a 37 kDa single-strand specific endonuclease form mouse plasmacytoma cells (Tomkinson and Linn, 1986). Subsequently, two 26-29 kDa endonucleases were purified from bovine tissues (Cummings et al., 1987) and a similar 44 kDa endonuclease from embryos of *Drosophila melanogaster* (Harosh et al., 1992).

The 26 kDa nuclease, called endonuclease G, was first isolated from calf thymus nuclei (Côté et al., 1989), but is now known to be mitochondrial nuclease which contaminated the preparation of nuclei from which it was isolated (Gerschenson et al., 1995). The gene for bovine endonuclease G has been cloned and sequenced (Côté and Ruiz-Carrillo, 1993). The identified open reading frame is predicted to encode a polypeptide of 299 amino acids with a mass of 34 kDa.. This result indicates that endonuclease G undergoes some proteolytic processing during purification.

All of the mitochondrial nucleases studied have been shown to be associated with the inner membrane of the mitochondrion (Dake et al., 1988; Cummings et al., 1987; Fraser and Cohen, 1983).

Despite differences in activities, specificity and size, it seems that all of these enzymes are related. The 37 kDa *S. cerevisiae* mitochondrial endonuclease and the 37 kDa

mitochondrial ss specific endonuclease from mouse plasmacytoma cells both showed cross-reactivity with a polyclonal antibody raised to the purified *Neurospora* endonuclease (Dake et al., 1988; Tomkinson and Linn, 1986). This same antibody had shown cross-reactivity with the recBCD nuclease of *E. coli* (Fraser et al., 1990).

The 44 kDa mitochondrial endonuclease isolated from embryos of *Drosophila* was found to be cross-reactive with antibodies raised to both *S. cerevisiae* and the bovine heart mitochondrial nucleases (Harosh et al., 1992).

The predicted amino acid sequence for the bovine mitochondrial nuclease shows 42% identity over a stretch of 227 amino acids to the amino acid sequence predicted from the yeast *NUC1* gene (Côté and Ruiz-Carrillo, 1993). Considerable homology was also detected with the amino acid sequence predicted for the endonuclease secreted from the bacterium *Serratia marcesens* (42% identity). This homology is consistent with the bacterial origin of mitochondria.

The *Serratia* endonuclease acts non-specifically on both DNA and RNA (Ball et al., 1987), and is active as a homodimer. Its putative active site contains the His and Glu residues proposed to act as catalytic groups (Miller et al., 1994). The identification of a His residue as a catalytic group is consistent with the observations that the yeast mitochondrial and *Neurospora* endo-exonucleases are irreversibly inactivated by treatment with ethoxyformic anhydride (Dake et al., 1988; Chow and Fraser, 1983).

Except for the ss-specific mouse plasmacytoma enzyme, the mammalian mitochondrial nucleases are Mg^{2+} or Mn^{2+} dependent endonucleases and degrade dsDNA extensively (Cummings et al., 1987; Côté et al., 1989). Their activity is salt sensitive, showing strong inhibition in the presence of 50 mM NaCl.

The 29 kDa bovine heart mitochondrial endonuclease has been purified to near homogeneity and shown to act, like the yeast, mouse and *Serratia* enzymes, in dimeric form to release 5'-P and 3'-OH terminated DNA fragments (Cummings et al., 1987). Unlike the mouse ss-specific enzyme, for which activity is optimal at pH 7.5 (Tomkinson and Linn,

1986), the bovine nuclease cleaves ss and dsDNA at both neutral and low pH. The same property has been observed for the partially purified yeast and the *Neurospora* endo-exonucleases (Rosamond, 1981; Chow and Fraser, 1983), but not for the pure yeast endo-exonuclease (Dake et al., 1988). At more physiological ionic strength, the bovine mitochondrial nuclease nicks dsDNA in a highly non-random fashion showing a strong preference for attack between two guanine residues (Low et al., 1987). The bovine mitochondrial nuclease does not have exonuclease activity with dsDNA.

The biological role of the yeast mitochondrial endo-exonuclease remains unclear since neither deletion nor overexpression of *NUC1* gene leads to any altered phenotype affecting DNA (Vincent et al., 1988; Zassenhaus et al., 1988). The observation that *NUC1* mutants hosting certain types of dsRNA genomes feature a 10-fold overproduction of virus-like dsRNA particle (Liu and Dieckmann, 1989), which becomes completely suppressed in strains that also host the killer-toxin-encoding dsRNA, had led to the suggestion that the mitochondrial endo-exonuclease may have a protective function in fighting viral infections.

The mammalian mitochondrial endonucleases are unlikely to play any role in recombination of the mitochondrial DNA since the mitochondrial DNA is not known to undergo recombination. It has been suggested that mitochondrial endo-exonuclease might play a role in the DNA repair of oxidative damage (Houmiel et al., 1991) and in replication of the mitochondrial DNA (Low et al., 1987).

1.2.3 Extra-mitochondrial endo-exonucleases of fungi and yeast.

The first eukaryotic endo-exonuclease to be partially purified was from *Neurospora crassa* (Fraser et al., 1976). The enzyme was found to possess a Mg^{2+}-dependent ss-specific endonuclease activity with ssDNA and ssRNA, and exonucleolytic activity with dsDNA. The exonuclease activity was found to be preferentially inhibited by 0.1-0.2 M NaCl, Zn^{2+}, Ca^{2+} and EDTA. Both activities were also partially inhibited by 0.1-0.5 mM ATP. The exonuclease activity could be abolished by trypsin treatment, with the resulting ss-specific

activity strongly resembling that of the ss-specific endonuclease previously purified from *Neurospora* (Linn and Lehman, 1965a,b).

Subsequently, a Mg^{2+} (or Mn^{2+})-dependent 31 kDa ssDNA-binding form of the endo-exonuclease was purified to homogeneity and characterized from sonicates of a mixture of small vacuoles and mitochondria (Chow and Fraser, 1983; Fraser et al., 1989). Its exonuclease activity was also abolished by trypsin treatment (Chow and Fraser, 1983). Later on, a trypsin-activatable endo-exonuclease precursor was partially purified and tentatively identified as a hydrophobic native protein of 94 kDa (Kwong and Fraser, 1978). Active and trypsin-activatable forms of endo-exonuclease were localized in the mitochondria, on the membrane of the endoplasmic reticulum, in the cytosol, in vacuoles and in nuclei of wild-type *Neurospora* mycelia (Fraser and Cohen, 1983; Ramotar et al., 1987). Attempts to isolate the inactive endo-exonuclease precursor led to the recovery of multiple active polypeptides of 33-40 kDa (Hatahet and Fraser, 1989) arising as a result of limited proteolytic cleavage during purification. It has been generally observed that endo-exonuclease is very sensitive to proteolysis. Thus, the activities of the purified 31 kDa enzyme, outlined below, may not be fully representative of the larger enzyme forms acting *in vivo*.

Like the mitochondrial endo-exonuclease, discussed above, the *Neurospora* endo-exonuclease was shown to act as a dimer *in vitro*. It possesses Mg^{2+} (or Mn^{2+})-dependent ss-specific endonuclease activity with DNA and RNA and a highly processive 5'—>3' exonuclease activity with linear dsDNA (Chow and Fraser, 1983; Fraser et al., 1989). Both ss-circular phage DNA and denatured DNA, as well as denatured RNA are good substrates for the enzyme. Although it can nick and relax supercoiled replicative forms of phage DNA, it has no detectable action on topoisomerase relaxed circular dsDNA (Fraser et al., 1989).

The endonuclease activity was found to be optimal at 1-2 mM Mg^{2+}, while the exonuclease activity was optimal at higher, 8-10 mM Mg^{2+} concentrations. At low Mg^{2+} , the enzyme simply nicks supercoiled DNA, whereas at high Mg^{2+} it can linearize circular supercoiled dsDNA and rapidly degrade the linear molecules by the exonuclease action

(Chow and Fraser, 1983). The cleavage product has 5'-P and 3'-OH termini and ultimate products of digestion are small acid-soluble oligonucleotides. When used at limiting concentrations with the bacteriophage T7 DNA, the enzyme was able to generate ssDNA tails and internal ss-gaps thousands of nucleotides long detectable by electron microscopy (Chow and Fraser, 1983). The ability of the *Neurospora* endo-exonuclease to generate internal ss-gaps indicates that the enzyme can act exonucleolytically and processively from nicks in the DNA. The *Neurospora* endo-exonuclease shows two pH optima for its ds-DNase activity, one at pH 8.0 an one at pH 5.6. While ss-DNase activity is also seen over this wide pH range, its single optimum is at pH 6.5 - 7.5 (Chow and Fraser, 1983).

The endonucleolytic activity of *Neurospora* endo-exonuclease is dependent on the presence of 5'-P termini in its linear DNA substrates, since the dephosphorylated linearized plasmid DNA is not cleaved by the enzyme, independently of whether the substrate DNA is generated by a blunt-end, 5' or 3' ss-overhang producing restriction enzyme (Fraser et al., 1989). It was concluded that the endo-exonuclease must track along the linear dsDNA from the 5'-P terminus to make the internal breaks.

Plasmids linearized by treatment with restriction nucleases and then exposed briefly to the *Neurospora* endo-exonuclease in the presence of low Mg^{2+} showed a "laddering" pattern upon agarose gel electrophoresis. This indicated that the enzyme made site-specific DSBs in the dsDNA. The site specific DSB are done much more less frequently than the random ss breaks. The precise mechanism for making the specific DSBs is not yet known. Sequencing at the break sites made by the *Neurospora* endo-exonuclease revealed preference for cutting in the middle of the hexanucleotide sequence 5'AGCACT and related sequences (Fraser et al., 1989).

Neurospora endo-exonuclease can be inhibited by the two non-specific nuclease inhibitors: low level aruin tricarboxylic acid and heparin (Chow and Fraser, 1983). Unlike the partially purified enzyme, the activities of the purified endo-exonuclease were not affected by ATP, and the ds-DNase activity was stimulated 2-fold, rather than inhibited by Ca^{2+}.

Endo-exonucleases are widespread in fungi. Very similar Mg^{2+} (or Mn^{2+})-dependent active and inactive forms of endo-exonuclease have been partially purified and characterized from *Aspergillus nidulans* (Koa et al., 1990), *Flammulina velutipes* (Sen et al., 1991) and *Schizosaccharomyces pombe* (Szankasi and Smith, 1992). A ss specific endonuclease, nuclease α, was purified previously in 42 kDa and 55 kDa forms from *Usilago maydis* (Holloman and Holliday, 1973a, b; Holloman et al., 1981). It has properties similar to the ss-endonuclease originally isolated from *Neurospora* (Linn and Lehman, 1965a,b). A 43 kDa endo-exonuclease was also isolated from the mushroom *Coprinus cinereus* (Lu and Sakaguchi, 1991), but its activities resemble more closely those of ss-exonucleases.

Antibody raised to the *Neurospora* endo-exonuclease is known to cross-react with the endo-exonucleases of both *Aspergillus* and *Saccharomyces* (Koa et al., 1990; Chow and Resnick, 1987). This antibody was actually used to purify the *S. cerevisiae* endo-exonuclease by immunoaffinity chromatography (Chow and Resnick, 1987) and to clone the corresponding gene from a lambda gt11 expression library (Chow et al., 1992).

The gene coding for the *S. cerevisiae* endo-exonuclease, named *NUD1*, encodes a protein of 617 amino acids with a predicted mass of 70 kDa (van Vliet-Reedijk and Planta, 1993), which corresponds closely to the purified yeast 72 kDa endo-exonuclease with Mg^{2+}-dependent activities (Chow and Resnick, 1987). No inactive forms were reported for *S. cerevisiae* endo-exonuclease and only a single RNA transcript of 2 000 nucleotides was detected by Northern analysis (van Vliet-Reedijk and Planta, 1993). The sequence of *NUD1* is unrelated to that of *NUC1*, the gene coding for the yeast mitochondrial endo-exonuclease.

Except for minor differences, the activities of all the fungal endo-exonucleases are identical to those of the *Neurospora* endo-exonuclease, with the exception of the *Coprinus* endo-exonuclease (Lu and Sakaguchi, 1991). The latter endo-exonuclease is unique in that it makes endonucleolytic breaks in ssDNA and supercoiled plasmid DNA with 3'-P and 5'-OH termini and degrades linear ssDNA, but not linear dsDNA, exonucleolytically to 3'-

mononucleotides in the 3' —>5' direction (Lu and Sakaguchi, 1991). Thus, the *Coprinus* endo-exonuclease is a ss-specific enzyme with both endo- and exonucleolytic activities.

1.2.4 Extra-mitochondrial endo-exonucleases of higher eukaryotes

The first endo-exonuclease to be purified and characterized from higher eukaryotes was a ssDNA-binding endo-exonuclease from *Drosophila melanogaster* (Fraser, 1996; Shuai et al., 1992). Like the *Neurospora crassa* and *Saccharomyces cerevisiae* endo-exonucleases, this 33.6 kDa enzyme (also known as nuclease III) possessed Mg^{2+}(or Mn^{2+})-dependent endonucleolytic activity with both ssDNA and RNA as well as an exonuclease activity with dsDNA. The optimal activity was at pH 7.0-8.5. Like the fungal and yeast endo-exonucleases, it converted supercoiled circular dsDNA to the relaxed and linear forms in a stepwise fashion. Unlike the fungal endo-exonuclease the *Drosophila* enzyme released 5'-mononucleotides, but the small oligonucleotides with 5'-P and 3'-OH could be detected as well. No evidence was found for inactive precursor forms. The amino acid composition of the purified enzymes showed no apparent homology either to the yeast mitochondrial endo-exonuclease (*NUC1* gene product) or to the *NUD1* gene product (Shuai et al., 1992).

The first mammalian endo-exonuclease was purified and characterized from cultured monkey CV-1 cells (Couture and Chow, 1992). It was isolated by affinity chromatography on an antibody-protein A-Sepharose column with antibody raised to the purified *Neurospora* endo-exonuclease. A single monomeric 65 kDa protein with both DNase and RNase activities was eluted from the column with 3.5 M $MgCl_2$. There was no evidence for the presence of larger inactive forms of this enzyme in CV-1 cell extracts. The activities associated with the protein were very similar to those of *Neurospora* endo-exonuclease, but were activated by a broader spectrum of divalent metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}). In the presence of both Mg^{2+} and Ca^{2+}, both the ss and ds DNase activities were synergistically activated. The 5'—>3' ds-exonuclease activity showed some processivity, but the endonuclease activity was distributive.

The same immunochemistry approach using the antibody raised against the purified *Neurospora* endo-exonuclease was used to demonstrate the presence of cross-reactive proteins in Chinese hamster ovary cell lines, human HeLa cells and human ataxia telangiectasia cell lines (Liu et al., 1995).

Both inactive (trypsin-activatable) and active forms of endo-exonuclease ranging from 37 kDa to 145 kDa in size have been identified in nuclear and extranuclear fractions of human leukemic CEM and MOLT-4 cells (Fraser et al., 1996). An active endo-exonuclease-related polypeptide of 97 kDa, which shows cross-reactivity to the anti-*Neurospora* endo-exonuclease antibody, has also been detected in low salt wash fractions of nuclei of both rat and human tumor cell lines (Pandey et al., 1994)

Nuclear matrix-associated endo-exonuclease present in two forms, 65 kDa and 32 kDa, has been detected in plant *Curcubita pepa* (Rudnicki et al., 1988; Szopa and Adamiec, 1993). The 65 kDa polypeptide was reported to nick supercoiled plasmid DNA, while the 32 kDa species had degradative DNase activity.

1.2.5 Evidence for role of eukaryotic endo-exonucleases in DNA repair

Given the similarities between the bacterial recBCD and eukaryotic endo-exonucleases, together with the requirement for enzymes able to generate ssDNA tails postulated by most models of homologous recombination, the involvement of endo-exonucleases in eukaryotic recombination had been anticipated early on.

The first pieces of evidence implicating endo-exonucleases in recombination and DNA repair in eukaryotes came from the observation that ss-endonuclease, nuclease α, was deficient in a *rec* mutant of *Ustilago maydis* (Holloman and Holliday, 1973a). Subsequently, it has been reported that the DNA repair deficient *uvs-3* mutants of *Neurospora crassa* featured endo-exonuclease levels of about 10% of those of the wild type (Fraser and Cohen, 1983; Ramotar et al., 1987). The *Coprinus cinereus* ss-specific endo-exonuclease has also

been implicated in meiotic recombination since it was specific to meiotic tissues of the basidiomycete which undergoes synchronous meiosis (Lu and Sakaguchi, 1991).

The best evidence implicating endo-exonucleases in eukaryotic recombination and DNA repair comes from studies in *S. cerevisiae*. The *rad52* mutants of *S. cerevisiae*, which are completely deficient in recombination and recombinational DSB repair, were shown to express 72 kDa endo-exonuclease at a level of only 10% of that in the wild type (Resnick et al., 1984; Moore et al., 1993).

An *in vitro* recombination system employing extracts of wild-type *S. cerevisiae* and *rad50*, *rad52* and *rad54* mutants was established to examine the recombination between circular and linear plasmid dsDNA with circular plasmid ss and dsDNA (Moore et al., 1993). The results showed that the endo-exonuclease was not required for recombination when one of the partner substrates is single stranded, but is essential for the majority of recombination events when both partner substrates are double stranded. An ELISA assay with anti-*Neurospora* endo-exonuclease antibody showed that extracts of the *rad50* and *rad54* mutants had wild type levels of the yeast endo-exonuclease, and confirmed that the *rad52* mutant had only 10% of wild type endo-exonuclease activity (Moore et al., 1993). Extracts of these *rad* mutants complemented one another in the *in vitro* recombination system. Thus, it has been concluded that the *RAD52* gene regulated expression of *NUD1*.

When *NUD1* was overexpressed in yeast, the increase of endo-exonuclease activity in the transfected cells was accompanied by an increase in cell survival after irradiation with gamma rays and an increase in spontaneous and radiation-induced mitotic intrachromosomal recombination frequencies between duplicated genes (Sadekova and Chow, 1996).

The yeast endo-exonuclease has been observed to interact with HO-endonuclease in a yeast two-hybrid assay (T.Y.-K. Chow unpublished data). Moreover, in a recent publication Asefa et al. (1998) have reported that the *nud1* yeast strain is highly sensitive to the HO-endonuclease-induced DSBs at the *MAT* locus, with cell survival being inversely proportional to the duration of HO-endonuclease expression. Analysis of the surviving

colonies from the isogenic *nud1* strain indicated that many of the survivors were sterile and the proportion of these sterile survivors increased with the time of HO-endonuclease expression. The same study also reports that the *nud1* mutation has a suppressor effect on DSB-induced lethality in *rad52* mutants.

Reduced levels of mammalian endo-exonuclease activity were reported in radiosensitive Chinese hamster ovary (CHO) xrs-5 and human ataxia telangiectasia (AT) cells as compared to their radionormal counterparts (Liu et al., 1995); these cell lines were shown to have reduced homologous recombination activities *in vitro* (Wahls and Moore, 1990).

The results of our own studies of the effect of *NUD1* expression on homologous recombination and DNA repair in mammalian cells are presented in Chapter 2 below.

1.3 Poly(ADP-ribose) polymerase

1.3.1 Poly(ADP-ribose) synthesis and degradation

As its name implies, poly(ADP-ribose) polymerase (PARP) catalyzes the ADP-ribose polymerization reaction. PARP is activated by DNA nicks and double-strand breaks and catalyzes the formation of ADP-ribose polymer (pADPR) primarily on itself but also on a number of nuclear proteins *in vivo*, such as topoisomerases I and II, DNA polymerases α and β, DNA ligase II and histones (Cleaver and Morgan, 1991). Polymer synthesis has also been observed on a variety of protein and non-protein acceptors *in vitro* (Yoshihara and Tanaka, 1981; Rapaport et al., 1981; Thomassin et al., 1985; Baker et al., 1987).

NAD+ is the sole substrate of PARP. Extensive pADPR synthesis and its rapid turnover in response to DNA damage may lead to the depletion of cellular pools of NAD+.

Prior to the polymerization step, PARP catalyzes the addition of an ADP-ribose residue (from NAD+) to the acceptor amino acid. Further ADP-residues are added successively to the 2" hydroxyl of the adenine ribose. ADP-ribose polymers may reach a length of more than 200 residues (Alvarez-Gonzalez and Jacobson, 1987; Hayashi et al.,

1983). A helicoidal conformation has been proposed for the long pADPR chains (Minaga and Kun, 1983) and immunological studies suggest a similarity between the secondary and tertiary structure of the polymer and ssDNA (Sibley et al., 1986). Intermittently, PARP adds an ADP-ribose residue to the 2" hydroxyl of what was the nicotinamide ribose, thus engendering a branch point. Branch points up to a proportion of 3% can be found within the polymer (Alvarez-Gonzalez and Jacobson, 1987).

pADPR half-life varies in relation to the length of the polymer chain and to the nature of the acceptor protein, but remains relatively short (about 1 min) due to high polymer turnover *in vivo* at high levels of DNA damage (Alvarez-Gonzalez and Althaus, 1989). The latter is attributed to high pADPR glycohydrolase activity (Jonsson et al., 1988). It is generally believed that pADPR glycohydrolase acts exoglycosidically by cleaving ribose-ribose bonds of the polymer to yield monomers of ADP-ribose. The rate of polymer degradation by pADPR glycohydrolase is a function of the length of the polymer (Lagueux et al., 1994). In undamaged cells the polymer turnover is much slower with pADPR half life of about 7 hours (Alvarez Gonzalez et al., 1994). Following glycohydrolase action, one molecule of ADP-ribose remains bound to the protein. This last ADP-ribose is removed from the protein by ADP-ribosyl protein lyase (Okayama et al., 1978).

1.3.2 Structure and function

PARP has been found in all eukaryotes examined with the exception of the yeasts *Saccharomyces* and *Torulae* (Uchida and Miwa, 1994). More than 95% of the enzyme is localized in the nucleus and association of PARP with nuclear membrane and nuclear matrix has been reported (Cardenas Corona et al., 1987; Lagueux et al., 1994). The nuclear PARP seems to localize preferentially to the nucleoli (Kaufmann et al., 1991). The number of PARP molecules present in the cell has been estimated at $2x10^5$ - $2x10^6$ depending on the cell type (Yamanaka et al., 1988), that is 1 PARP molecule for each 1.5-15 kb of DNA.

Human PARP is a monomeric enzyme with an estimated molecular weight of 113 kDa (Kurosaki et al., 1987). The activity of the enzyme is dependent upon the presence of DNA and is stimulated by Mg^{2+} and polycations such as histones or polyamines (Ueda and Hayashi, 1985).

The PARP gene is 43 kb in length, contains 23 exons, and localized on chromosome 1q41-42 in humans (Auer et al., 1989). Two pseudogenes have been found on chromosomes 13 and 14 (Cherney et al., 1987). The total number of PARP pseudogenes in human cells is now estimated at five (Guy Poirier, personal communication). Human PARP mRNA is 3.7 kb in length with an ORF coding for a protein of 1014 amino acids (Cherney et al., 1987).

PARP can be readily digested by partial proteolysis with papain or α-chymotrypsin into three functional domains: a 42 kDa N-terminal DNA binding domain, a 55 kDa C-terminal NAD+ binding domain and a 16 kDa middle piece that is the site of much of the automodification (Kameshita et al., 1984).

de Murica et al. (1994) have shown that all three enzymatic activities of PARP reside in the C-terminal fragment. However, the activity of the C-terminal fragment is only 0.2% of the activity of the complete protein. It would seem that the DNA-binding domain of the enzyme not only recognizes the DNA breaks, but also activates the enzyme by increasing its intrinsic activity about 500 times when the protein is bound to DNA. The C-terminal domain of PARP contains a highly conserved dinucleoide binding motif (Uchida and Miwa, 1994), which is very similar to the NTP binding motif or Rossman fold, typically associated with proteins that recognize and bind to phosphoanhydride bonds of mono- and dinucleotides, such as the one in NAD+ (Rossmann et al., 1974).

The N-terminal DNA binding domain is rich in basic lysine residues and contains two conserved zinc-finger-like motifs of the form $C\text{-}X_2\text{-}CX_{28.30}\text{-}H\text{-}X_2\text{-}C$ (Lautier et al., 1993). The Zn-finger that is closer to the N-terminus is essential for the enzyme activity in response to both DNA nicks and DSBs, while the second Zn-finger appears to be responsible for the activity triggered by nicked DNA but not DSBs (Gradwohl et al., 1990).

PARP is a metalloenzyme that requires Zn for activity. It has been determined that each PARP molecule contains two Zn^{2+} ions (de Murcia et al., 1991). The N-terminal domain also contains the nuclear localization signal that is conserved from frogs to mammals (Uchida and Miwa, 1994).

The automodification domain contains 15 highly conserved Glu residues, which are thought to be the auto-poly(ADP-ribosyl)ation sites (Uchida et al., 1993). In *Drosophila* the automodification domain also contains a leucine zipper motif (Uchida et al., 1993) which could be involved in the homodimerization of PARP as well as heterodimerization with other chromatin proteins. The former has been suggested by *in vitro* studies by Mendoza-Alvarez and Alvarez-Gonzalez (1993) which have suggested that the automodification reaction is intermolecular and involves homodimerized PARP.

1.3.3 Role of PARP in recombination

A variety of cytogenetic and molecular genetic studies suggest a role of PARP in preventing DNA homologous recombination.

PARP inhibition was reported to increase the frequency of sister chromatid exchange (SCE) in undamaged mammalian cells (Oikawa et al., 1980; Park et al., 1983; Morgan et al., 1986). Cell lines treated with various inhibitors of PARP show a dose-dependent increase in SCE frequency. The precise mechanisms of SCE are unknown, but it is generally believed that SCE proceeds via homologous recombination (Petes and Pukkila, 1995). Cell lines deficient in PARP also show a nearly 10-fold increase in SCE frequency (Chatterjee et al., 1989). Cell lines capable of growing in nicotinamide-free medium are deficient in substrate NAD+, and under this growth condition, they exhibit nearly a 10-fold increase in SCE. However, on growth in regular nicotinamide-containing medium, NAD+ and SCE levels return to normal (Chatterjee et al., 1989). Furthermore, if PARP inhibition is combined with DNA damage, then, in addition to an increased frequency of SCE, there is also observed a substantial frequency of chromosomal aberrations (Cleaver and Morgan, 1991).

Waldman and Waldman (1991) have reported that PARP inhibition increased the frequency of intrachromosomal homologous recombination between two defective Herpes thymidine kinase genes almost 4-fold in mouse Ltk- fibroblasts. The same authors have reported, however, that PARP inhibition had no effect on the frequency of extrachromosomal homologous recombination (Waldman and Waldman, 1990), and resulted in decrease in gene targeting frequency in CHO cells (Waldman et al., 1996).

PARP has also been demonstrated to play a role in modulation of gene amplification (Bürkle, 1989). PARP inhibition substantially decreases the frequency of successful Variable Antigen Type switching in African trypanosome, a process which involves gene conversion events (Shall, 1995).

Unequivocally, PARP inhibition results in a dramatic drop in illegitimate recombination of transfected DNA. There have been a number of reports indicating that PARP inhibition results in decrease of random integration of exogenous DNA into the mammalian genome (Farzaneh et al., 1988; Waldman and Waldman, 1990). In some cell types, an almost complete inhibition of DNA transfection has been observed as a result of PARP inhibition (Farzaneh et al., 1988). Furthermore, it has been observed that integration of retroviral provial DNA into the chromosomal DNA may be prevented by the inhibitors of PARP, as well as by the antisense RNA for the PARP gene (Shall, 1995).

The mechanism for these phenomena is unknown. It may be that normally PARP cooperates with DNA-PK to promote NHEJ of the DNA breaks. Recent studies by Ruscetti et al. (1998) show that the catalytic subunit of DNA-PK can be ADP-ribosylated by PARP *in vitro*, and that the protein kinase activity of DNA-PK can be stimulated by PARP in the presence of NAD+ in a reaction that is blocked by the PARP inhibitor 1,5-isoquinolinediol. The same study demonstrated that PARP can be phosphorylated by purified DNA-PK *in vitro*.

PARP knockout mice have recently been generated. Although mice that lack PARP display no gross phenotype, they exhibit high levels of SCE, thus confirming the anti-recombinogenic function of PARP (Le Rhun et al., 1998).

Mutation of the gene for the DNA-PK catalytic subunit leads to defective V(D)J recombination and arrests B- and T-lymphocyte development leading to a sever combined immunodeficiency (SCID) (Blunt et al., 1995). Morrison et al. (1997) have generated double knockout mice lacking both PARP and DNA-PK and showed that thymocytes of these mice were able to bypass the SCID block. Furthermore, the double-mutant mice develop a high frequency of T-cell lymphomas. These results demonstrate that increased recombination activity after the loss of PARP anti-recombinogenic function can rescue V(D)J recombination in SCID mice, implying that PARP and DNA-PK cooperate to minimize genomic damage caused by strand breaks.

1.3.4 PARP and DNA damage repair

PARP is activated in response to DNA nicks and DSBs but not in response to other types of DNA damage, such as UV-induced thymine dimers (Lautier et al., 1993). *In vitro* the above point has been demonstrated by the observation that closed circular SV40 minichromosomes, both intact or UV irradiated, did not activate PARP, whereas linear minichromosomes and UV-treated minichromosomes digested with *Micrococcus luteus* UV endonuclease did (Cohen and Berger, 1981). A similar conclusion has been reached in the studies of PARP activation in response to UV and MNNG damage in xeroderma pigmentosum (XP) cells (Berger and Sikorski, 1981) and many other studies (Berger, 1985; Cleaver and Morgan, 1991; Shall, 1994).

Thus, PARP is a damage-responsive enzyme, activated specifically by DNA strand breaks. However, PARP is different from the class of damage-inducible gene products in that PARP is a constitutive enzyme whose gene transcription and mRNA levels remain constant

after DNA damage, whereas transcription of damage-inducible genes is increased dramatically following DNA damage (Chatterjee and Berger, 1998).

Numerous studies have been conducted to assess the role of PARP in cellular responses to different DNA damaging agents, such as UV, alkylating agents, ionizing radiation and radiomimetic drugs. These include the studies utilizing competitive inhibitors of PARP, such as 3-aminobenzamide (3-AB) and 3-methoxybenzamide (3-MB); inhibition of PARP activity by the expression of PARP gene antisense RNA construct or overexpression of the PARP DNA binding domain; mutant cell lines deficient in PARP activity; cell extracts lacking PARP activity by depletion of PARP or NAD+; and PARP knockout mice (reviewed in Chatterjee and Berger, 1998). The results of these studies can be briefly summarized as follows.

NAD+ is required for efficient rejoining of DNA strand breaks in the presence of PARP, however, in the absence of PARP, rejoining occurs in an NAD+-independent mode (Creissen and Shall, 1982; Satoh et al., 1993).

PARP inhibition increases SCE frequency and exposure of cells to DNA-damaging agents with PARP inhibition results in a synergistic increase in SCE frequency (Oikawa et al., 1980; Heartlein and Preston, 1985).

Inhibition of PARP potentiates cytotoxicity induced by DNA-damaging agents (Ben Hur, 1984; Jacobson et al., 1985 a,b). The substantial increase in alkylating agent-induced DNA strand breaks observed in the presence of PARP inhibitors is not associated with the observed increased cytotoxicity (Ding et al., 1992; Stevnsner et al., 1994). Under exponentially growing conditions, PARP mutant cell lines are hypersensitive to gamma irradiation and MNNG, suggesting that PARP may be involved in the repair of DNA damage in replicating cells (Chatterjee et al., 1991). However, it is not the difference in strand break formation or their rate of repair that contributes to the enhanced cell killing in exponentially growing PARP-deficient cell lines. Under growth-arrested conditions, wild-type and mutant cell lines become similarly sensitive to both gamma-irradiation and MNNG (Chatterjee and

Berger, 1994), suggesting that PARP is not involved in the repair of DNA damage in growth-arrested cells.

1.3.5 PARP normal function is to prevent DNA recombination at DNA strand ends

In order to explain the above mentioned results a hypothesis has been formulated that PARP does not have a direct role in DNA repair, but does function normally to prevent DNA recombination and facilitate DNA ligation (Satoh and Lindahl, 1992; Chatterjee and Berger, 1994; Chatterjee and Berger, 1998).

Under normal growth conditions, a multitude of DNA strand breaks is produced as a result of normal DNA replication and the repair of spontaneous DNA damage. The postulated role of PARP is to bind transiently to these DNA strand ends and protect them from the action of nucleases as well as from entering into inappropriate recombination processes. Binding of PARP to DNA strand ends activates synthesis of pADPR, in particular on PARP itself. The polymers attached to the DNA-bound enzyme are more acidic than DNA, and thus, electrostatic repulsion dissociates the automodified PARP from the DNA. Subsequent to the dissociation of the enzyme from DNA, ligation reaction can take place to repair the DNA break.

Assuming that replicating cells contain an excess of strand ends relative to ligase, then many ends will undergo recombination unless they are shielded from this process until ligase becomes available. In this view, PARP protects the DNA strand ends, cycles on and off of these ends in a fashion that controls their rate of availability, and favors their utilization in the ligase reaction relative to the recombination process.

However, in the absence of normal PARP activity, the DNA strand ends will not be protected, and will undergo recombination at higher rates, leading to the observed increase in SCE. When replicating cells are exposed to DNA damaging agents the number of DNA strand ends generated by the combination of semiconservative replication and the excision

repair process exceeds the capacity of PARP to protect the DNA strand ends against recombination events. Hence, the observed dose-dependent increase in SCE that occurs in response to treatment with DNA damaging agents. Also consistent with this explanation, is the observation that replicating cells with impaired PARP activity show a 10 to 20-fold increase in SCE compared to normal, PARP-proficient cells.

In general, SCE represents homologous recombination events and are not cytotoxic. However, as the susceptibility of ends to nucleases and inappropriate recombination increases, it is likely that there is a proportional increase in imperfect recombination. The latter produces DNA rearrangements and deletions that are potentially mutagenic and cytotoxic. Thus, if only replicating cells can undergo recombination, impaired PARP activity will result in increased cytotoxicity in replicating cells treated with DNA-damaging agents, but not in growth arrested cells.

This hypothesis is in agreement with the suggestion by Creissen and Shall (1982) that pADPR synthesis facilitates the DNA ligation process. It provides an explanation for the observations that PARP inhibitors potentiate the effect of MNNG in proliferating cells, but not in quiescent cells (Jacobson et al., 1985a, b). It also provides an explanation for the observations that MMS-induced cytotoxicity can be maximally potentiated by 3-AB during S phase (Boorstein and Pardee, 1984) and that inhibitors of PARP in combination with DNA-damaging agents produce chromatid aberrations in a cell-cycle-dependent fashion with maximal aberrations occurring in cells treated during S phase (Wiencke and Morgan, 1987).

Role in apoptosis

Since activity of PARP is dependent on fragmented DNA and DNA fragmentation is associated with apoptosis, several studies have investigated the role of PARP in apoptosis. Conflicting results have been obtained regarding a direct role for PARP activation in apoptosis (Lautier et al., 1993; Chatterjee and Berger, 1998).

There is general agreement, however, that a specific PARP cleavage process occurs during apoptosis that may be a marker of cellular commitment to programmed cell death (Kaufmann et al., 1993). The biological role of this event remains so far unknown. Recent studies have identified a novel protease resembling interleukin 1-β-converting enzyme to be responsible for PARP cleavage (Lazebnik et al., 1994).

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Chapter 2: Evidence that overexpression of *Saccharomyces cerevisiae* endo-exonuclease *NUD1* gene stimulates homologous recombination in mammalian cells

In this chapter we present the evidence that overexpression of *S. cerevisiae* endo-exonuclease *NUD1* gene has stimulatory effect on various homologous recombination processes. Thus, we have demonstrated that transient expression of *NUD1* in HeLa cells increased the resistance of the latter to ionizing radiation and cis-platin, two agents known to cause DNA DSBs, whereas it had no effect on cells' resistance to DNA-methylating agents. Subsequently, we also showed that transient expression of *NUD1* enhanced the frequency of extrachromosomal homologous recombination in mouse Ltk- cells. These results provide additional evidence for the involvement of EEs in homologous recombination in mammalian cells. They also demonstrate that overexpression of EEs, such as Nud1p, can potentially be used as a means of selectively increasing the frequency of homologous recombination in mammalian cells without a concomitant increase in the frequency of illegitimate recombination.

2.1 The effect of the *Saccharomyces cerevisiae* endo-exonuclease *NUD1* gene expression on the resistance of HeLa cells to DNA-damaging agents (article published in Mutation Research, 1999; 433: 169-181. Reproduced with permission from Elsevier Science.)

The effect of the *Saccharomyces cerevisiae* endo-exonuclease *NUD1* gene expression on the resistance of HeLa cells to DNA-damaging agents.

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Abstract

HeLa cells transiently transfected with a mammalian expression DNA vector expressing the *Saccharomyces cerevisiae* endo-exonuclease *NUD1* gene have exhibited changes in cell survival frequencies after treatment with different DNA-damaging agents as compared to HeLa cells transfected with a control plasmid. The *NUD1*-transfected cells showed a dose-dependent increase in sensitivity to UV irradiation resulting in up to 58% decrease in cell survival. In response to gamma-irradiation, *NUD1* transfected cells featured an increased survival at doses equal to and greater than 2.0 Gy, reaching a maximum enhancement in survival frequency of 17%. At the same time, the *NUD1*-transfectants featured an increase in resistance to 0.25μM - 0.5μM cis-platin (up to 58% increase in cell survival) and 1.0 mM EMS (11% increase). At higher concentrations of EMS, *NUD1* expression resulted in a decreased cell survival of the transfected cells (17% decrease for 2.5 mM EMS). No difference in cell survival frequencies between the *NUD1*-transfectants and the controls was observed after treatment with different concentrations of chlorambucil and mechlorethamine. These results suggest possible roles played by endo-exonucleases in different DNA repair pathways — being stimulatory for the repair of certain types of DNA lesions, such as double strand breaks, and interfering with the endogenous DNA repair systems for the repair of other types of lesions. Furthermore, these results also provide additional indirect evidence for the role of endo-exonucleases in homologous recombination.

1. Introduction

In order to preserve the integrity of the genome, all living organisms from prokaryotes to mammals possess a panoply of enzymes evolved to repair damaged DNA. Some of these enzymes are specialized in repair of very specific types of damage such as DNA photolyase and O^6-methylguanine-DNA methyltransferase [1, 2], while other repair systems are more versatile and recognize several types of DNA lesions. Among the latter there are the base excision repair system that recognizes and repairs non-bulky base adducts, reduced or

fragmented bases [3], and the nucleotide excision repair (NER) system, which repairs bulky DNA adducts, such as cis-platin-guanine and psoralen-thymine adducts, thymine dimers, 6-4 photoproducts, as well as all of the lesions repaired by the direct and base excision repair systems above [4].

The major type of damage inflicted upon DNA by ionizing radiation and certain types of cross-linking chemical agents are DNA double strand breaks (DSBs) [5]. The major pathway for the repair of DSBs in lower eukaryotes, and specifically in yeast, is thought to be the recombination repair pathway [6, 7]. In mammalian cells the recombination repair pathway, however, is only one of the two pathways known to repair DSBs; the second (major) pathway being the non-homologous end-joining (NHEJ) repair catalyzed by the Ku70/80 of the DNA PK [8, 9]. The NHEJ DSB repair is dependent on the activity of the Rad50, Mre11 and Xrs2 proteins from a complex that has structural and functional similarities to the *sbcCD* proteins of *E. coli* [10], and is required to produce 5'-ended single strand DNA intermediates [11, 9]. On the other hand, the DSB repair via recombinational repair pathway absolutely requires a functional *RAD52* gene [11]. The proteins of the recombination repair pathway are believed to be the same ones that catalyze homologous recombination reactions during meiosis and sister-chromatid exchange in mammals and yeast, and the mating-type switching in *S. cerevisiae* [12, 13].

The precise mechanism of the homologous recombination process in mammalian cells is unknown [14]. A number of models of homologous recombination have been proposed based on the information obtained from studies using yeast [15, 16, 17]. Currently, the most robust and widely accepted model is the double strand break repair model [18, 19]. In this model, it is postulated that the initiation of homologous recombination requires the generation of a double stand break in the donor DNA molecule which (the break) is processed to generate a 3'-single stranded (ss) DNA tail that invades the homologous DNA duplex [20]. In *E. coli* the generation of the ssDNA tails is catalyzed by the RecBCD complex (reviewed in [21]). In eukaryotes, the nuclease(s) required to generate the 3'-single stranded DNA tails

has not been identified. Nevertheless, a group of nucleases with activities similar to RecBCD, collectively referred to as endo-exonucleases (EEs), have been isolated both from lower eukaryotes and from vertebrates, including mammals (for review see [22]). For several of these enzymes the corresponding genes have been cloned. One such EE, yNUCR (hereafter referred to as Nud1p), encoded by the *NUD1* gene, was isolated from *S. cerevisiae* [23, 24]. Nud1p is the only known nuclear EE in *S. cerevisiae*. The only other EE identified in *S. cerevisiae* is the mitochondrial 37 kDa EE encoded by the *NUC1* gene [25].

Nud1p is a 72 kDa protein with ssDNA- and RNA-specific endonuclease and dsDNA 5'—>3' processive exonuclease activities [26]. Immunochemically related EEs have been isolated from fungus, algae, fly and monkey CV1 cells [27, 28, 29, 30]. The cloning of the human homologue of *NUD1* is currently in progress.

There are several lines of evidence indicating the involvement of EEs in homologous recombination processes. **1)** Increased *NUD1* expression in *S. cerevisiae* is associated with increased intrachromosomal recombination and increased survival after irradiation with gamma-rays [31]. **2)** Reduced EE activity in *Neurospora crassa* is associated with meiotic sterility, sensitivity to UV, X-rays and alkylating agents [32]. **3)** A 90% reduction in Nud1p levels is observed in *rad52* yeast mutants [33]. **4)** Nud1p interacts with HO nuclease in a yeast two-hybrid assay, and *NUD1* mutants have abnormal mating-type switching following the introduction of a DSB at the *MAT* locus in *S. cerevisiae* [34]. **5)** Reduced levels of mammalian EE activity were reported in radiosensitive Chinese hamster ovary (CHO) xrs-5 and human ataxia telangiectasia (AT) cells [35]; these cell lines were shown to have reduced homologous recombination activities *in vitro* [36].

The cloning of the mammalian homologue of *NUD1* has been unsuccessful so far, and the only complete cDNAs for eukaryote EEs are available from yeast. Given the fact that mammalian and yeast EEs are immunochemically cross-reactive, it is possible that the two enzymes are sufficiently similar to be functionally interchangeable for some biological activity. Many enzymes of the DNA repair pathways are evolutionary conserved between

lower and higher eukaryotes. For example, the RAD52 protein is 30% identical and 58% homologous between human and yeast [37]. Furthermore, a number of prokaryote and lower eukaryote enzymes involved in DNA repair are capable of partially replacing their homologues in mammalian cells. For example, the expression of the *E. coli fpg* gene, coding for the FAPy glycosylase, in mammalian cells protects the latter against gamma-ray mutagenesis [38]; while the over-expression of *S. cerevisiae RAD52* gene in human fibrosarcoma-derived cell line resulted in increased frequency of inter-plasmid homologous recombination and increased resistance of the cells to diepoxybutane and methyl methanesulfonate [39]. To assess a possible involvement of EE in the repair of DNA damage in mammalian cells, we have examined the effect of transient expression of the Nud1p on the cell survival frequency of HeLa cells after their exposure to ionizing radiation, cis-platin, UVB, EMS, chlorambucil and mechlorethamine. We found that *NUD1* expression leads to an enhanced survival of the transfected cells after treatment with gamma-rays, cis-platin and low concentration of EMS, but does not improve the cell survival after treatment with nitrogen mustards, and reduces cell survival in the case of treatments with UVB or high concentrations of EMS.

2. Materials and Methods

pCI-*NUD1* expression vector.

The *Hind*III- *Xba*I 2.224 kb fragment of *NUD1* cDNA [40] was subcloned into the *Sal*I site of the pCI mammalian expression vector (Promega, WI USA) under the control of the constitutive CMV enhancer/ promoter. The expression from the vector was confirmed by Northern blot analysis of the RNA extracted from the transfected HeLa cells and by the nuclease activity assay.

Cell culture

HeLa cells were grown and maintained in DMEM supplemented with 10% FBS (GibcoBRL, NY USA) and Antimyotic-Antibiotic (GibcoBRL) at 37°C in 5% CO_2/ 95% air.

Transfections and treatment with DNA-damaging agents

About $3x10^5$ cells, plated on 60-mm dishes 20-24 hours prior to transfection, were transiently transfected with pCI-*NUD1* or pCI circular supercoiled plasmids using Superfect poly-cationic reagent from QIAGEN Canada, according to the specifications provided by the manufacturer.

At 20-24 hours post-transfection, the cells were trypsinized and an appropriate number of cells (250 - 20 000) were plated onto $25cm^2$ TC flasks (NUNC, IL USA) or onto 6-well dishes (Costar, MA USA). For each dose of radiation and for each drug concentration tested, the survival frequency determinations were done in triplicate. Each experiment was repeated at least 3 times.

At 5 hours after plating, the transfected cells were treated with DNA-damaging agents.

For chemical reagents, the reagents were added directly to the cell medium. Cis-platin, chlorambucil and mechlorethamine were obtained from Sigma Canada, EMS from Aldrich Canada.

UV-irradiation was performed using an inverted UV box (BioRad, CA USA) on 6-well dishes containing 1 ml of growth medium per well; after irradiation, 4 ml of medium were added to each well. The surface of the UV lamp was much larger than the size of the 6-well dishes, and since the center of the dish would be aligned with the center of the inverted UV lamp, every well would be exposed to the same amount of UV irradiation. The UV box was placed perfectly parallel to the bottom of the wells, such that no shadows could be cast by the sides of the wells. The *NUD1*-transfected and the control cells were seeded in the same 6-well dish, and, hence, were exposed to UV under exactly the same conditions.

Ionizing irradiations were performed with ^{60}Co gamma ray source (Theratron 780) at a dose rate of 155 cGy/min.

The treated cells and the untreated controls were then incubated in a CO_2 incubator for 10 days. The cells were then fixed with 10% formaldehyde phosphate buffered saline, stained with methylene blue and the colonies, formed by at least 20 cells, were counted.

Northern analysis

Total RNA was extracted from ~ $6x10^6$ HeLa cells transfected with either pCI-*NUD1* or pCI (control) plasmids, using TRIzol reagent from GibcoBRL. 20 µg of RNA was then treated for 30 min with DNAse I (Pharmacia) and run on MOPS 1% agarose gel. The RNA was then transferred to a Hybond-N membrane (Amersham, UK) and hybridized to the ^{32}P-dCTP labeled *Hind*III - *Xho*I fragment of the *NUD1* cDNA in 50% formamide for 16h at 42°C.

Nuclease activity determination

Nuclease activity assay was performed as previously described [31]. The transfected cells were trypsinized and resuspended in lysis buffer (0.02M Tris-HCl, 1mM EDTA, and 1mM phenylmethysulfonyl fluoride). The cells were lyzed by sonication. The lysate was centrifuged for 10 min at maximum speed. The protein concentration of the supernatant was determined by Bradford assay. The crude cell extracts, containing the same amount of protein, from *NUD1*-transfected and control cells were incubated with the same amount of anti-EE antibody at 4°C overnight. Following the incubation, protein A-Sepharose was added to the solutions and the mixtures were incubated for 30 min on ice. The immune-precipitate complex was isolated by centrifugation, washed with TE and resuspended in nuclease buffer [23]. The deoxyribonuclease activities were then determined by measuring the release of acid-soluble radioactivity from ^{32}P-labeled, heat-denatured, single-stranded pAdBM1 DNA, according to the method described in [23].

3. Results

NUD1 transient expression

The expression of *NUD1* from the pCI-*NUD1* mammalian expression plasmid upon its transfection into HeLa cells has been confirmed by Northern blot analysis. The Northern blot (Fig. 1) features a strong hybridization signal with the RNA extracted from *NUD1*-transfected HeLa cells, whereas none is seen with the RNA extracted from the control HeLa cells transfected with pCI plasmid. The smear-like appearance of the hybridization signal is due to the fact that the expression driven from the plasmid vector gives rise to incomplete transcripts of different sizes, and possibly to the degradation of the mRNA, since Northern blots from similar transfection experiments performed in Ltk- cells consistently feature a sharp hybridization band with very little smear (data not shown).

The enzymatic activity of the yeast *NUD1* gene product expressed from the transfected pCI-*NUD1* plasmid has been confirmed by the nuclease activity assay. This assay measures the rate of generation of the acid-soluble radioactivity from the denatured linear ^{32}P-dCTP labeled DNA substrate resulting from the nucleolytic activities of the fraction being assayed. Figure 2 shows the results of one representative nuclease activity assay. As can be judged from it, an increased rate of the nucleolytic activity is obtained for the fraction purified from the *NUD1*-transfected HeLa cells. The nucleolytic activity of the fraction purified from the control, pCI-transfected, HeLa cells lysate, is due to the fact that the antibody to the yeast EE cross-reacts with the endogenous human enzyme. From the initial part of the curves we can determine the rate of the nucleolytic reaction for the transfected and the control lysates as follows:

$$v(t) = dP/dt,$$

where v(t) is the rate of the reaction at time t, and P is the amount of the product of the reaction represented, in our case, by cpm of the acid-soluble radioactivity.

Hence,

$$v_{NUD} = [P_{NUD}(t=15') - P_{NUD}(t=0)] \div 15 \text{ min} = [17\,055 - 7\,739] \div 15 = 621;$$

$$v_{pCI} = [P_{pCI}(t=15') - P_{pCI}(t=0)] \div 15 \text{ min} = [13\,067 - 7\,018] \div 15 = 403 .$$

Assuming that the reaction is first order in EE,

$$v(t) = k[S][E],$$

where [S] is the concentration of the substrate and [E] the concentration of the enzyme. The concentration of the substrate [S] was initially the same in both reaction mixes, hence

$$v_{NUD} / v_{pCI} = (k[S][E]_{NUD}) / (k[S][E]_{pCI}) = [E]_{NUD} / [E]_{pCI} = 621 \div 403 = 1.54.$$

Hence, in this particular experiment *NUD1* transient expression led to approximately 54% increase in the nuclease activity in the EE-specific-antibody precipitable fraction. Three additional assays of the kind were performed, each featuring an increase in nuclease activity of the *NUD1*-transfectants ranging form 10% to 55%. The average increase in the nuclease activity of the *NUD1*-transfectants as compared to the controls was 36%. The observed increase is statistically significant with 97.5% confidence interval, as determined using the paired t-test method (n=4, P=0.0189).

No difference in plating efficiency between *NUD1*-transfectants, pCI-transfectants and untransfected HeLa cells was observed.

Effect of the *NUD1* expression upon cell survival after irradiation

The data for the survival of the *NUD1*-transfected and the control HeLa cells after treatment with gamma radiation is summarized in Table 1. For doses below 2 Gy (the cell death of the control cells < 9%) no consistent difference in cell survival of the *NUD1* - transfected cells as compared to the controls was observed (data not shown). However, for radiation doses equal to or greater than 2 Gy, *NUD1* expression resulted in a higher cell survival. At 2 Gy (66% mean survival of the control) the average difference in cell survival between *NUD1* and control cells is +16% (significant with 97.5% confidence interval), at 4 Gy (36% mean survival of the control) the average difference is +17% (significant with

99.5% confidence interval) and at 8 Gy (6.7% mean survival of the control) the average difference is +13% (significant with 95% confidence interval).

The most striking results were obtained, however, with the effect of *NUD1* expression on cell survival after UV treatment. The data is summarized in Table 2. For every UV dose above 0.25kJ/m^2, the expression of *NUD1* resulted in a reduction of cell survival. The difference in cell survival frequencies between the *NUD1*-transfectants and the controls increased with the increasing radiation dose : at 0.5 kJ/m^2 (mean cell survival of the control is 84%) the average difference in cell survival between *NUD1* and pCI transfectants is -18% (99% confidence interval), at 1 kJ/m^2 (65.3% mean survival of the control) the average difference in survival is -34% (99.95% confidence interval), and at 2 kJ/m^2 (42% mean survival) the average difference in survival is –56% (99% confidence interval).

Effect of *NUD1* expression upon cell survival after treatment with cis-platin

Cell survival frequency of *NUD1*-transfectants in the cell culture medium containing cis-platin was higher than that of the controls (Table 3). At [cis-Pt] = 0.25μM (46% mean survival of the control) the average difference in cell survival between *NUD1* and pCI transfectants was +24% (95% confidence interval); at [cis-Pt] = 0.5μM (30% mean survival of the control) the difference was +58% (95% confidence interval). For the cis-platin concentration of 0.75μM (15% mean survival of the control) no statistically significant difference in cell survival frequencies between the *NUD1*-transfectants and the controls was detected.

Effect of *NUD1* expression upon cell survival after treatment with alkylating agents

For EMS concentrations below 1.0 mM (mean survival of the control > 83%) and above 2.5mM (mean survival of the control < 28%) there is no significant difference in cell survival between *NUD1*-transfectants and controls (Table 4). At 1.0 mM EMS, *NUD1*

expression increases cell survival of the *NUD1*-transfectants by 11% as compared to the control (99% confidence interval). At 2.0 mM EMS, again, there is no significant difference in cell survival. However, at 2.5 mM EMS, *NUD1* expression results in a decrease of -17% in the cell survival (95% confidence interval).

No significant difference in cell survival between *NUD1*-transfectants and the controls upon treatment with mechlorethamine and chlorambucil at all concentrations tested, could be detected (Tables 5 and 6).

4. Discussion

Recombination repair pathway is one of the pathways for the repair of DNA double strand breaks in eukaryotes. DSBs are the predominant type of DNA lesions resulting from ionizing radiation and certain DNA cross-linking chemical agents [5]. The importance of this repair pathway in lower eukaryotes is exemplified by radiation hypersensitivity phenotype of the *rad52* yeast mutants [6, 7, 41]. In humans, inefficient repair of DSBs is responsible for ataxia telangiectasia (AT), a human genetic disease which is associated with cerebellar ataxia, high incidence of cancers and hypersensitivity to ionizing radiation [42], and for Fanconi's Anemia which is characterized by aplastic anemia, chromosomal aberrations and extreme sensitivity to cross-linking agents [43].

The enzyme critical for the initiation of homologous recombination at the DSB, which probably constitutes the rate limiting step of the recombination reaction, has been suggested to be the EE [44]. The EE is thought to recognize DSBs and process them to generate suitable substrates for the recA-mediated search of homology — 3' ssDNA overhangs. In yeast, mutations of the *NUD1* gene result in impaired repair of DNA DSBs, whereas the over-expression of *NUD1* leads to increased rate of radiation-induced interchromosomal recombination and increased resistance to gamma rays [32, 31]. A study of the EE levels in mammalian cells has shown that there exists a correlation between the level of EE activity in the cell and the cell's radiosensitivity [35].

To assess a possible involvement of EEs in the repair of DNA damage, we have examined the effect of the transient expression of the yeast EE on the cell survival frequency of HeLa cells after their exposure to ionizing radiation, cis-platin, UVB, EMS, chlorambucil and mechlorethamine.

Consistent with the suggested role of EE in homologous recombination, we have observed an increase of cell survival of the *NUD1*-transfected cells after the treatment with agents known to cause DSBs. Thus, the *NUD1*-transfected cells featured an up to 17% increase in cell survival after treatment with gamma-rays, and an up to 58% increase in cell survival after treatment with cis-platin.

We believe that the observed differences in cell survival of the *NUD1*-transfectants and the controls have presumably resulted from an enhanced ability of the *NUD1*-transfected cells to undergo repair via homologous recombination due to the enzymatic activity of the Nud1p.

At present, we have no direct proof that these effects are not due to Nud1p interaction(s) with NHEJ repair pathway. However, the dependence of *NUD1* expression levels on Rad52 [33], the effect of *NUD1* over-expression on the rate of interchromosomal recombination [31], the fact that *NUD1* disruption has a suppiesser effect on the DSB-induced lethality in *rad52* mutants and increases the rate of NHEJ at *MAT* locus [34]— all suggest that Nud1p is involved in the recombination-dependent DSB repair in yeast. It is reasonable to expect that any effect *NUD1* expression might have in mammalian cells would be reminiscent of its functions in yeast. Most importantly, however, our latest work showed that *NUD1* over-expression in murine Ltk- cell line results in an increased rate of the extrachromosomal homologous recombination (manuscript in preparation).

Our working model explaining the increase in homologous recombination by EE over-expression is as follows. EEs are required to process DSBs into suitable substrates for the search of homology, i.e. the 3' ssDNA overhangs. This step is rate limiting for the whole process of initiation of the homologous recombination at the DSB. Hence, the over-

expression of EE might generate 3' ssDNA tails at higher rate, thus increasing the rate of the initiation, and consequently, the rate of homologous recombination overall.

Ionizing radiation is known to produce DNA DSBs [45]. The expression of an active EE may stimulate the repair of these DSBs via homologous recombination pathway of the cell, perhaps by catalyzing the rate-limiting initiation step of the reaction. The drug cis-platin is a potent DNA cross-linking agent. Although the major type of DNA damage resulting from cis-platin are intrastrand cross-links, which are efficiently repaired by the excision pathway, it also produces lethal interstrand cross-links. The excision of the latter results in generation of DSBs [46]. As with ionizing radiation, the repair of these DSBs is probably enhanced by the presence of Nud1p which potentially stimulates the homologous recombination repair.

For cis-platin concentration of 0.75μM (13% mean survival of the control) no statistically significant difference in cell survival frequencies was observed. It is possible that at such high doses of the drug, the DNA damage to the cells is so extensive that the advantage provided by the recombinant EE is no longer of significance. Similarly, for radiation doses above 8 Gy (data not shown) no statistically significant difference in cell survival was observed.

The most interesting result obtained in our study was that *NUD1* expression has greatly sensitized *NUD1*-transfected cells to UV. The reduction in cell survival of the *NUD1*-transfectants as compared to the cell survival of the controls increases with an increasing UV dose. At 2.0 kJ/m^2, the cell survival of the *NUD1*-transfectants was 56% lower than that of the controls. The major type of DNA damage caused by the UV light is the dimerization of pyrimidines [47]. The major pathway for the repair of this type of lesion in placental mammals is the NER [48]. Hence, to explain our results we advance the hypothesis that Nud1p may interfere with normal functioning of the NER.

No difference in cell survival upon treatment with chlorambucil and mechlorethamine has been observed. Probably, the type of lesion resulting from these agents is not recognized

by the recombinant EE and, on the other hand, there is no interference with the endogenous repair pathways involved in this case. Hence, the observed results suggest that repair of the corresponding alkylated DNA adducts may proceed through a pathway different from recombination-dependent DNA repair and the NER pathways, and is neither stimulated nor hampered by the *NUD1* over-expression.

NUD1 expression resulted, however, in 11% higher resistance of the transfected cells to 1.0 mM EMS, another alkylating agent. EMS has been shown to cause DSBs in DNA [49], however, and the increased cell survival of *NUD1*-transfectants, we believe, once again, reflects their increased proficiency in the recombination-dependent repair. When the concentration of EMS is increased, the difference in cell survival between *NUD1*-transfected and the control cells first disappears and, at even higher EMS concentrations, *NUD1*-transfected cells feature reduced cell survival — 17% decrease at 2.5 mM EMS. The different effects of *NUD1* expression on the survival of cells at different concentrations of the drug probably reflect the nature and the relative abundance of the underlying DNA lesions. Whenever DSBs is the predominant type of damage, the presence of recombinant EE improves the cell survival, possibly by stimulating the recombination-dependent repair pathway. On the other hand, when other types of DNA lesions, that are better handled by the excision repair, are predominant, the presence of the exogenous EE becomes deleterious as it may interfere with the endogenous DNA repair pathways.

Given the high degree of conservation of the repair and recombination enzymes through the evolution, together with the fact that yeast and human endo-exonucleases are immunochemically cross-reactive, and taking into account our results with the transient expression of the yeast EE in HeLa cells, our observations suggest that human EE does potentially play a role in recombination-dependent DSBs repair of the DNA. In this light, the isolation of the mammalian homologue of the *NUD1* gene, as well as further investigations of the mechanisms of action of EEs in homologous recombination and DNA repair appear to be of great importance. These experiments are currently in progress.

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Legends

Figure 1. Northern blot analysis of total RNA from *NUD1*-transfected and pCI-transfected HeLa cells. **A.** MOPS 1% agarose gel; **B.** Autoradiogram of the Northern blot hybridization to the *NUD1*-specific probe. **Lane 1**: DNaseI treated total RNA from pCI-transfected control HeLa cells; **Lane 2**: DNaseI treated total RNA from pCI-*NUD1* transfected HeLa cells. A total of 20 µg of RNA was loaded onto each lane.

Figure 2. Nuclease activity assay. One representative experiment is shown. Nuclease activity is reported as the cpm values determined at different time points. The latter correspond to the amount of acid-soluble radioactivity released from ^{32}P-labeled ssDNA linear substrate by the nucleolytic activities of the immune complexes precipitated by rabbit antibody raised against the *N. crassa* endo-exonuclease, from the lysates of HeLa cells transfected with (o)pCI-*NUD1* vector and (•) pCI control vector.

Table 1 Survival following gamma-irradiation of *NUD1*-transfected and pCI-transfected (control) HeLa cells

Dosage	Exp.	*NUD1* - transfectants surv. freq.[a]	pCI-transfectants surv. freq.	Absolute Δ% survival	Statistical analysis[b]	P-value	Average relative Δ survival
2 Gy	1.	82.6%	69.8%	+12.8%	m_{NUD}= 76.3%;		
	2.	86.3%	70.4%	+15.9%	m_{pCI} = 66.0%;		
	3.	65.4%	64.3%	+1.1%	n= 5; m_D= +10.3; s_D=	P=0.0208	+15.6%
	4.	75.0%	71.9%	+3.1%	7.8;		
	5.	72.2%	53.7%	+18.5%	**t = 2.959 >**		
					$v_4(t_{0.025})$ = 2.776		
					97.5 % confidence int.		
4 Gy	1.	49.1%	41.8%	+7.3%	m_{NUD}= 42.3%;		
	2.	41.7%	37.7%	+4.0%	m_{pCI} = 36.1%;		
	3.	39.8%	32.8%	+7.0%	n= 5; m_D= +6.1; s_D= 2.5;	P=0.0026	+16.9%
	4.	45.8%	42.6%	+3.2%	**t = 5.528 >**		
	5.	35.0%	25.8%	+9.2%	$v_4(t_{0.005})$ = 4.604		
					99.5 % confidence int.		
8 Gy	1.	11.0%	9.64%	+1.36%	m_{NUD}= 7.53%;		
	2.	8.09%	8.13%	-0.04%	m_{pCI} = 6.67%;		
	3.	7.16%	7.02%	+0.14%	n= 5; m_D= +0.85 ;	P=0.0340	+12.7%
	4.	6.53%	4.82%	+1.71%	s_D=0.77;		
	5.	4.85%	3.76%	+1.09%	**t = 2.484 >**		
					$v_4(t_{0.050})$ = 2.1326		
					95% confidence int.		

[a] The percent survival is determined as (sum of the surviving colonies from three parallel experiments) ÷ (sum of colonies formed in three untreated control flasks) * 100.

[b] Statistical significance of the observed difference has been calculated using the paired t-test method; $v_{n-1}(t_a)$ is the critical t-value for (n-1) degrees of freedom, where a is the rejection region of the H_0 hypothesis : m_D=0 (or m_{NUD}-m_{pCI}=0). The difference is considered statistically significant with (1-a) confidence interval if the calculated t-value > $v_{n-1}(t_a)$.

[c] The relative difference in percent survival is determined as (m_{NUD} - m_{pCI}) ÷ m_{pCI} * 100.

Table 2 Survival following UV irradiation of *NUD1*-transfected and pCI-transfected HeLa cells

UVB	Exp.	*NUD1* - transfectants surv. freq.	pCI- transfectants surv. freq.	Absolute Δ% survival	Statistical analysis	P-value	Averag relativ Δ% surviv
0.25 kJ/m^2	1.	84.3%	79.7%	+4.6%	m_{NUD}= 81.6%; m_{pCI} = 90.6%; n= 6; m_D= -9.00; s_D= 13.3; **t = 1.6454 <** $v_5(t_{0.050})$ = 2.015 statistically insignificant	P=0.3140	-9.9%
	2.	56.2%	46.8%	+9.4%			
	3.	95.3%	107 %	-11.7%			
	4.	90.7%	106%	-15.3%			
	5.	74.1%	100%	-25.9%			
	6.	89.1%	104%	-14.9%			
0.50 kJ/m^2	1.	68.7%	90.9%	-22.2%	m_{NUD}= 71.4%; m_{pCI} = 84.3%; n= 6; m_D= -15.1; s_D= 10.6; **t = 3.4787 >** $v_5(t_{0.010})$ = 3.365 99 % confidence int.	P=0.0088	-18.0%
	2.	56.4%	57.6%	-1.2%			
	3.	76.4%	99.1%	-22.7%			
	4.	84.2%	89.5%	-5.3%			
	5.	46.4%	74.1%	-27.7%			
	6.	78.0%	89.7%	-11.7%			
1.00 kJ/m^2	1.	33.6%	56.6%	-23.0%	m_{NUD}= 43.1%; m_{pCI} = 65.3%; n= 6; m_D= -22.2; s_D= 5.10; **t = 10.694 >** $v_5(t_{0.0005})$ = 6.869 99.95 % confidence int.	P<0.0001	-34.0%
	2.	35.9%	52.3%	-16.4%			
	3.	50.2%	80.6%	-30.4%			
	4.	62.9%	80.5%	-17.6%			
	5.	17.8%	42.6%	-24.8%			
	6.	58.0%	79.4%	-21.4%			
2.00 kJ/m^2	1.	12.0%	50.3%	-38.3%	m_{NUD}= 18.2%; m_{pCI} = 41.8%; n= 5; m_D= -23.6 ; s_D=12.4; **t = 4.2543 >** $v_4(t_{0.010})$ = 3.747 99 % confidence int.	P=0.0066	-56.4%
	2.	15.4%	22.4%	-7.0%			
	3.	16.7%	31.9%	-15.2%			
	4.	29.4%	57.8%	-28.4%			
	5.	17.8%	46.8%	-29.0%			

Table 3 Survival in cis-platin of *NUD1*-transfected and pCI-transfected HeLa cells

[cis-Pt]	Exp.	*NUD1* - transfectants surv. freq.	pCI- transfectants surv. freq.	Absolute Δ% survival	Statistical analysis	P-value	Averag relativ Δ% surviv
0.25 μM	1.	84.1%	68.7%	+15.4%	m_{NUD}= 57.3%;		
	2.	67.8%	47.0%	+20.8%	m_{pCI} = 46.4%;		
	3.	36.1%	32.0%	+4.10%	n= 4; m_D= +10.9; s_D=8.64;	P=0.0432	+23.5%
	4.	41.2%	38.0%	+3.20%	**t = 2.518 >**		
					$v_3(t_{0.050})$ = 2.353		
					95 % confidence int.		
0.50 μM	1.	38.3%	18.8%	+19.5%	m_{NUD}= 47.4%;		
	2.	51.0%	40.8%	+10.2%	m_{pCI} = 30.0%;		
	3.	43.2%	35.8%	+7.40%	n= 4; m_D= +17.4; s_D=11.4;	P=0.0273	+58.0%
	4.	57.3%	24.7%	+32.6%	**t = 3.0672 >**		
					$v_3(t_{0.050})$ = 2.353		
					95 % confidence int.		
0.75 μM	1.	26.0%	26.7%	-0.70%	m_{NUD}= 15.2%;		
	2.	5.31%	5.65%	-0.334%	m_{pCI} = 14.8%;		
	3.	4.58%	7.95%	-3.37%	n= 4; m_D= +0.5; s_D= 4.11;	P=0.4165	-3.4%
	4.	25.1%	18.8%	+6.30%	**t = 0.2297 <**		
					$v_3(t_{0.050})$ = 2.353		
					statistically insignificant		

Table 4 Survival in EMS of *NUD1*-transfected and pCI-transfected HeLa cells

[EMS]	Exp.	*NUD1* - transfectants surv. freq.	pCI-transfectants surv. freq.	Absolute Δ% survival	Statistical analysis	P-value	Averag relativ Δ% surviva
0.5 mM	1. 2. 3.	93.1% 107% 89.1%	81.4% 102% 97.3%	+11.7% +5.0% -8.2%	m_{NUD}= 96.4%; m_{pCI} = 93.6%; n= 3; m_D= +2.8; s_D= 10.1; **t = 0.4846 <** $\nu_2(t_{0.050})$ = 2.920 statistically insignificant	P=0.3379	+2.99%
1.0 mM	1. 2. 3.	97.3% 80.3% 98.7%	89.2% 68.7% 90.4%	+8.1% +11.6% +8.3%	m_{NUD}= 92.1%; m_{pCI} = 82.8%; n= 3; m_D= +9.3; s_D= 1.96; **t = 8.224 >** $\nu_2(t_{0.010})$ = 6.965 99 % confidence int.	P=0.0072	+11.2%
2.0 mM	1. 2. 3.	77.6% 86.1% 34.6%	78.0% 86.0% 38.4%	-0.4% +0.1% -3.8%	m_{NUD}= 66.1%; m_{pCI} = 67.5%; n= 3; m_D= -1.37; s_D= 2.12; **t = 1.115 <** $\nu_2(t_{0.050})$ = 2.920 statistically insignificant	P=0.1904	-2.03%
2.5 mM	1. 2. 3.	56.9% 10.3% 2.60%	62.1% 17.2% 4.64%	-5.20% -6.90% -2.05%	m_{NUD}= 23.3%; m_{pCI} = 28.0%; n= 3; m_D= -4.71; s_D= 2.46; **t = 3.315 >** $\nu_2(t_{0.050})$ = 2.920 95 % confidence int.	P=0.0402	-16.8%
3.0 mM	1. 2. 3.	2.20% 2.32% 5.17%	2.80% 2.11% 6.97%	-0.60% +0.212% -1.80%	m_{NUD}= 3.23%; m_{pCI} = 3.96%; n= 3; m_D= -0.729; s_D=1.01; **t = 1.2503 >** $\nu_2(t_{0.050})$ = 2.920 statistically insignificant	P=0.1688	-18.4%

Table 5 Survival in mechlorethamine of *NUD1*-transfected and pCI-transfected HeLa cells

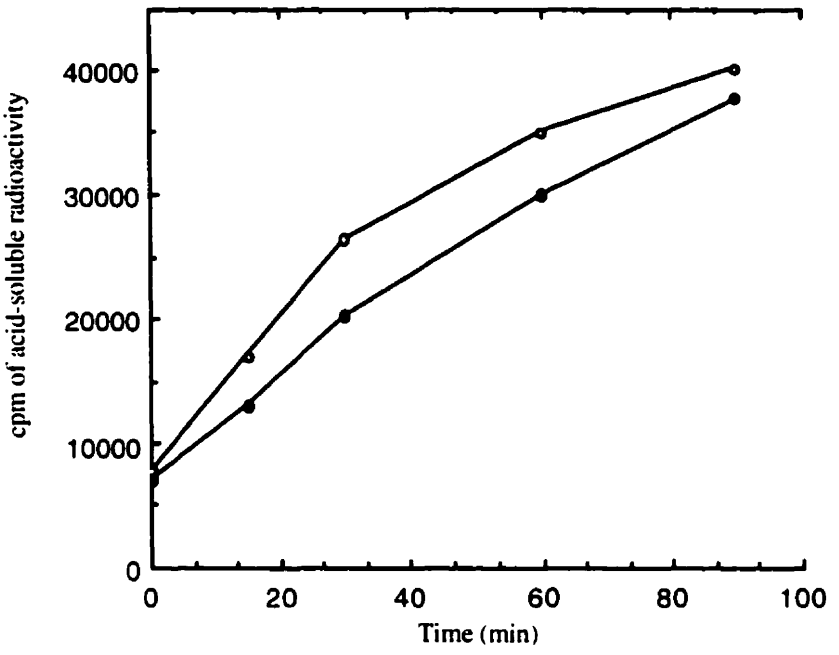
[mechloreth amine]	Exp.	*NUD1* - transfectants surv. freq.	pCI- transfectants surv. freq.	Absolute Δ% survival	Statistical analysis	P-value	Averag relativ Δ% surviv
0.25 μM	1.	85.8%	89.7%	-3.9%	m_{NUD}= 78.8%; m_{pCI} = 75.5%; n= 4; m_D= +3.27; s_D=5.97; **t = 1.580 <** $v_3(t_{0.050})$ = 2.353 statistically insignificant	P=0.1763	+4.33%
	2.	91.5%	87.0%	+4.5%			
	3.	75.9%	65.4%	+10.5%			
	4.	62.0%	60.0%	+2.0%			
0.50 μM	1.	79.0%	71.6%	+7.4%	m_{NUD}= 56.3%; m_{pCI} = 59.9%; n= 4; m_D= -3.6; s_D= 7.48; **t = 0.9628 <** $v_3(t_{0.050})$ = 2.353 statistically insignificant	P=0.2033	-6.01%
	2.	72.7%	82.0%	-9.3%			
	3.	41.9%	47.8%	-5.9%			
	4.	31.7%	38.3%	-6.6%			
0.75 μM	1.	74.5%	65.3%	+9.2%	m_{NUD}= 44.1%; m_{pCI} = 45.2%; n= 4; m_D= -1.15; s_D= 8.94; **t = 0.2572 <** $v_3(t_{0.050})$ = 2.353 statistically insignificant	P=0.4068	-2.54%
	2.	57.2%	54.6%	+2.6%			
	3.	25.3%	30.4%	-5.1%			
	4.	19.4%	30.7%	-11.3%			

Table 6 Survival in chlorambucil of *NUD1*-transfected and pCI-transfected HeLa cells

[chlorambucil]	Exp.	*NUD1* - transfectants surv. freq.	pCI- transfectants surv. freq.	Absolute Δ% survival	Statistical analysis	P-value	Averag relativ Δ% surviva
10 μM	1.	74.0%	79.4%	-5.4%	m_{NUD}= 66.8%;		
	2.	79.0%	74.7%	+4.3%	m_{pCI} = 67.4%;		
	3.	73.1%	76.0%	-2.9%	n= 5; m_D= -0.64; s_D=	P=0.3603	-0.95%
	4.	41.1%	39.9%	+1.2%	3.73;		
	5.	66.6%	67.0%	-0.4%	**t = 0.3838 <**		
					$v_4(t_{0.050})$ = 2.132		
					statistically insignificant		
20 μM	1.	37.9%	40.1%	-2.2%	m_{NUD}=35.4%;		
	2.	56.9%	50.3%	+6.6%	m_{pCI} = 32.8%;		
	3.	34.2%	24.7%	+9.5%	n= 6; m_D= +2.63;	P=0.1166	+8.03%
	4.	44.9%	42.4%	+2.5%	s_D=4.76;		
	5.	12.4%	14.9%	-2.5%	**t = 1.336 <**		
	6.	26.1%	24.2%	+1.9%	$v_4(t_{0.050})$ = 2.132		
					statistically insignificant		
30 μM	1.	15.7%	19.2%	-3.5%	m_{NUD}= 16.2%;		
	2.	39.1%	28.9%	+10.2%	m_{pCI} = 15.6%;		
	3.	8.09%	7.56%	+0.53%	n= 6; m_D= +0.607;	P=0.3891	+3.89%
	4.	18.7%	21.9%	-3.2%	s_D=5.0;		
	5.	4.06%	3.85%	+0.21%	**t = 0.2972 <**		
	6.	11.7%	12.3%	-0.6%	$v_4(t_{0.050})$ = 2.132		
					statistically insignificant		

Figure 1

Figure 2


cpm of acid-soluble radioactivity
40000
30000
20000
10000
0
0
20
40
60
80
100
Time (min)

2.2 Transient expression of *Saccharomyces cerevisiae* endo-exonuclease *NUD1* gene increases the frequency of extrachromosomal homologous recombination in mouse Ltk- fibroblasts (article published in Mutation Research, 1999; 435: 129-139. Reproduced with permission from Elsevier Science.)

In the preceding section we have presented evidence that *NUD1* gene expression specifically increases the survival of cells treated with agents that cause DNA DSBs. This type of DNA lesions are thought to be repaired, at least in part, via the recombinational repair pathway. Hence, our hypothetical conclusion is that the observed effect of *NUD1* expression is due to the fact that Nud1p increases the frequency of homologous recombination.

In this section we present more direct evidence of *NUD1* expression being stimulatory to homologous recombination. We demonstrate that transient expression of *NUD1* gene increases the frequency of extrachromosomal homologous recombination in mouse fibroblasts.

Transient expression of *Saccharomyces cerevisiae* endo-exonuclease *NUD1* gene increases the frequency of extrachromosomal homologous recombination in mouse Ltk- fibroblasts.

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Key words : endo-exonuclease, *NUD1*, DNA repair, homologous recombination

Abstract

Endo-exonucleases are nucleolytic enzymes which have been shown to participate in the processes of DNA repair and recombination in eukaryotes. Recently we have demonstrated that transient expression of *S. cerevisiae* endo-exonuclease *NUD1* gene in HeLa cells increased the resistance of the latter to ionizing radiation and cis-platin, suggesting the involvement of the *NUD1* gene product in the recombination repair of double strand breaks. Here, we report that transient expression of *NUD1* results in up to 62% increase in the frequency of homologous recombination between two co-transfected linear plasmids in mouse Ltk- cells.

1. Introduction

Homologous recombination is the process central to a number of important biological events such as accurate chromosomal segregation at meiosis, DNA damage repair, genome rearrangements and evolution of multigene families[1-5]. The precise mechanism of homologous recombination is so far unknown [6]. The proposed models, known as double-strand break (gap) repair model [7-9], single-strand annealing (SSA) model [10], one-sided invasion model [11] and synthesis-dependent strand-annealing model [12], were devised to accommodate the existing data from recombination studies done mainly in lower eukaryotes. Although all of these models differ from each other in several aspects, all postulate that the homologous recombination is initiated by a double-strand break (DSB) in one of the recombining molecules which is then processed enzymatically to a gap with long 3' single-stranded (ss) DNA tails. The 3' tails are postulated to invade the homologous duplex and prime new DNA synthesis. In *E. coli*, the generation of the ssDNA tails is catalyzed by the RecBCD complex (reviewed in [13]). In eukaryotes, the nuclease(s) required to generate the 3'-single stranded DNA tails have not been identified. Nevertheless, a group of nucleases with activities similar to RecBCD, collectively referred to as endo-exonucleases (EEs), have been isolated both from lower eukaryotes and from vertebrates, including mammals (for

review see [14]). Endo-exonucleases are likely candidates for the enzymes responsible for the generation of 3' ss DNA intermediates, and consequently, for the initial steps of the homologous recombination.

Another potential candidate for the initial processing of the DSBs is the enzymatic complex formed by the Rad50, Mre11, and Xrs2 proteins, which shares structural and functional similarities with the SbcCD protein complex of *E. coli* [15]. All three proteins were shown to be essential for DSB repair by nonhomologous end joining [16]. The yeast null mutants of either *mre11*, *rad50* or *xrs2* feature poor mitotic growth, a delay in mating type switching, elevated rates of spontaneous mitotic recombination, short telomeres, and a defect in the formation of meiosis-specific DSBs [17-19]. However, the exonuclease activity of human Mre11 alone or in association with human Rad50 has 3'—>5' orientation *in vitro* [20]. Moreover, a recent report by Moreau et al. shows that the nuclease activity of Mre11 is required only for meiotic but not for mitotic homologous recombination [21].

The *Saccharomyces cerevisiae* EE coded by the *NUD1* gene is a 72 kDa nuclear protein. It possesses ssDNA- and RNA- specific endonuclease and dsDNA 5'—>3' processive exonuclease activities [22, 23]. Immunochemically related endo-exonucleases have been isolated from *Neurospora crassa*, *Aspergillus nidulans*, *Coprinus cinereus*, monkey cells, the mitochondria of *S. cerevisiae*, and mice [24-29].

Previously, it has been shown that increased *NUD1* expression in *S. cerevisiae* is associated with increased intrachromosomal recombination and increased cell survival after irradiation with gamma-rays [30]. Conversely, reduced EE activity in *Neurospora crassa* was shown to be associated with meiotic sterility and increased sensitivity to UV, X-rays, and alkylating agents [31]. Other lines of evidence for the role of *NUD1* in homologous recombination processes include a 90% reduction in Nud1p levels of *rad52* yeast mutants [32] and Nud1p interaction with HO-endonuclease in a yeast two-hybrid assay (T.Y.-K. Chow unpublished data). Moreover, in a recent publication Asefa et al. have reported that the *nud1* yeast strain is highly sensitive to the HO-endonuclease induced DSBs at the MAT

locus, with cell survival being inversely proportional to the duration of HO-endonuclease expression [33]. Analysis of the surviving colonies from the isogenic *nud1* strain indicated that many of the survivors were sterile and the proportion of these sterile survivors increased with the time of HO-endonuclease expression. The same paper also reports that the *nud1* mutation has a suppresser effect on DSB-induced lethality in *rad52* mutants.

We also have demonstrated recently that transiently expressed Nud1p is active in HeLa cells. It increased the resistance of the latter to ionizing radiation and cis-platin [34].

Here, we report the effect of *NUD1* transient expression on the frequency of homologous recombination between two linearized plasmids co-transfected into mouse Ltk- fibroblasts.

2. Materials and Methods

pCI-*NUD1* expression vector.

The *Hind*III- *Xba*I 2.224 kb fragment of the *NUD1* cDNA [35] was subcloned into the *Sal*I site of the pCI mammalian expression vector (Promega, WI USA) under the control of the constitutive CMV enhancer/ promoter. The expression from the vector was confirmed by Northern blot analysis of the RNA extracted from the transfected Ltk- cells and by the measurement of the nuclease activity of the transfected cell lysates.

The recombination system

The recombination system used is similar to the one described by Shapira et al. [36]. It is based on two plasmids carrying the herpes simplex virus (HSV) thymidine kinase (TK) gene with non-overlapping insertion and deletion mutations (cf. Fig.1 for details). Both mutated TK genes are non-functional. Ltk- fibroblasts are deficient in TK activity and, hence, can not survive in hypoxanthine-aminopterin-thymidine (HAT) medium. The appearance of HAT-resistant (HAT^R) colonies, subsequent to co-transfection of the two plasmids, is diagnostic of an interplasmid homologous recombination event.

The Thx26 vector

The Thx26 vector contains an HSV TK gene with an 8 bp *Xho*I insertion mutation at position 738 of the TK coding sequence, subcloned into pBR322 [36].

The pAd-Thx76Δ vector

The HSV TK gene with a *Xho*I insertion mutation at position 1611 of the TK coding sequence (Thx76) was deleted from the sequence 3' to the *Xho*I linker and subcloned into the commercial pAdBM1 vector (Quantum, Canada) . The CMV-*neo* cassette was inserted 5' to the HSV TK.

Cell culture

Ltk- cells were grown and maintained in DMEM (Bio Media, Canada) supplemented with 10% FBS (GibcoBRL, NY USA) and Antimyotic-Antibiotic (GibcoBRL) at 37°C in 5% CO_2/ 95% air.

Transfections and selection

About $3x10^6$ cells, plated on 100-mm dishes (Costar, MA USA) 20 hours prior to transfection, were transiently transfected with equimolar quantities of Thx26 (4 µg) and pAd-Thx76Δ (6 µg) linearized plasmids, and 10 µg of pCI-*NUD1* or pCI circular supercoiled plasmids using Superfect poly-cationic reagent from QIAGEN Canada, according to the specifications provided by the manufacturer.

At 20 hours post-transfection, the cells were trypsinized. 1/100 of all cells was put in an 80 cm^2 TC flask (NUNC, IL USA) containing DMEM supplemented with 250 µg/ml G418. The rest of the cells were plated in four 80 cm^2 TC flasks containing DMEM supplemented with HAT.

The cells were then incubated in a CO_2 incubator for 11 days with the selection media being changed every 4 days. Surviving cells were finally fixed with 10% formaldehyde

phosphate buffered saline, stained with methylene blue. The colonies, formed by at least 20 cells, were counted.

Northern analysis

Total RNA was extracted from $6x10^6$ Ltk- cells transfected with either pCI-*NUD1* or pCI (control) plasmids, using TRIzol reagent from GibcoBRL. 20 µg of RNA was then treated for 30 min with DNAse I (Pharmacia) and run on MOPS 1% agarose gel. The RNA was then transferred to a Hybond-N membrane (Amersham, UK) and probed with the ^{32}P-dCTP labeled 973 bp *Cla*I restriction fragment of the *NUD1* cDNA.

Nuclease activity determination

The nuclease activity assay was performed as previously described [34]. The transfected cells were trypsinized and resuspended in lysis buffer (0.02 M Tris-HCl, 1 mM EDTA, and 1 mM phenylmethysulfonyl fluoride). The cells were lysed by sonication. The lysate was microcentrifuged for 10 min at maximum speed. The protein concentration of the supernatant was determined by Bradford assay [37]. The crude cell extracts from *NUD1*-transfected and control cells, containing the same amount of protein, were incubated with anti-EE antibody at 4°C overnight. Following the incubation, protein A-Sepharose was added to the solutions and the mixtures were incubated for 30 min on ice. The immune-precipitate complex was isolated by centrifugation, washed with TE and resuspended in nuclease buffer [22]. The deoxyribonuclease activities were then determined by measuring the release of acid-soluble radioactivity from ^{32}P-labeled, heat-denatured, single-stranded plasmid DNA, according to the method described by Chow and Resnick [22].

Southern Analysis of the HAT^R clones

HAT^R colonies were randomly selected and expanded. The genomic DNA (gDNA) from HAT^R clones was extracted by standard Proteinase K/Phenol/Chloroform method. 20 µg of the gDNA were then digested with *Eco*RI, *Hind*III, and *Xho*I enzymes (Pharmacia)

and subjected to Southern analysis by standard method. The *Eco*RI-*Hind*III fragment of the HSV TK labeled with ^{32}P-dCTP by random primer oligolabeling kit (Pharmacia) was used as the probe.

3. Results

NUD1 transient expression

The expression of *NUD1* from the pCI-*NUD1* mammalian expression vector upon its transfection into Ltk- cells was confirmed by Northern blot analysis. The Northern blot (Fig. 2) features a hybridization signal with the RNA extracted from *NUD1*-transfected Ltk- cells, whereas none is seen with the RNA extracted from the control Ltk- cells transfected with the pCI plasmid.

The enzymatic activity of the yeast *NUD1* gene product expressed from the transfected pCI-*NUD1* plasmid was also confirmed by the nuclease activity assay. This assay measures the rate of generation of acid-soluble radioactivity from the denatured linear ^{32}P-dCTP labeled DNA substrate resulting from the nucleolytic activities of the fraction being assayed. Figure 3 shows the results of one representative experiment. The nucleolytic activity of the fraction purified from the pCI-transfected control cell lysate is due to the fact that the antibody to the yeast EE cross-reacts with the endogenous human enzyme. From the initial part of the curves the rate of the nucleolytic reaction for the transfected and the control lysates can be determined as following:

$$v(t) = dP/dt,$$

where v(t) is the rate of the reaction at time t, and P is the amount of the reaction product represented here by cpm of the acid-soluble radioactivity. Hence,

$$v_{NUD} = [P_{NUD}(t=30') - P_{NUD}(t=15')] \div 15 \text{ min} = [21\,677 - 12\,012] \div 15 = 644 ;$$

$$v_{pCI} = [P_{pCI}(t=30') - P_{pCI}(t=15')] \div 15 \text{ min} = [19\,530 - 10\,941] \div 15 = 572 .$$

Assuming that the reaction is first order in EE, $v(t) = k[S][E]$, where [S] is the concentration of the substrate and [E] the concentration of the nuclease. The concentration of the substrate [S] was initially the same in both reaction mixes, hence

$$[E]_{NUD} / [E]_{pCI} = (k[S][E]_{NUD}) / (k[S][E]_{pCI}) = v_{NUD} / v_{pCI} = 644 / 572 = 1.13 .$$

Hence, in the experiment shown, *NUD1* transient expression led to approximately 13% increase in the nuclease activity of the EE-specific-antibody precipitable fraction at t = 30 min.

Three additional assays of the kind were performed, each featuring for t = 30 min an increase in nuclease activity of the *NUD1*-transfectants ranging from 10% to 55%. The average increase in the nuclease activity of the *NUD1*-transfectants as compared to the controls at t = 30 min was 36%. The observed increase is statistically significant with a 97.5% confidence interval, as determined using the paired t-test method (n = 4, P = 0.0189).

The effect of *NUD1* expression on interplasmid recombination

The appearance of HAT^R colonies subsequent to co-transfection of the Thx26 and pAd-Thx76Δ plasmids is diagnostic of an interplasmid homologous recombination event followed by random integration of the corrected plasmid into the genome. Hence, the frequency of appearance of HAT^R colonies is the product of the frequency of interplasmid homologous recombination and of the frequency of their random integration. It is possible, however, that functional TK gene can be produced by inter or intra-chromosomal recombination between two randomly integrated complementary plasmids, but the frequency of this event should be many fold lower than that of the event described above, $2x10^{-9}$ ($2x10^{-6}$ x 10^{-3}) *vs* 10^{-4} events/cell/generation. According to Waldman and Waldman [38], the frequency of interchomosomal recombination between HSV TK sequences in Ltk- cells is $2x10^{-6}$ events/cell/generation; the average frequency of integration of the pBR322-HSV TK vector is 10^{-3}. The frequency of interplasmid recombination foliowed by random integration into the Ltk- cell genome, as reported by Shapira et al. [36], is 10^{-4}. Hence, it is reasonable to assume that the vast majority of HAT^R colonies detected arose by interplasmid recombination

followed by random integration. The CMV-*neo* cassette incorporated into the pAd-Thx76Δ vector allows us to monitor the frequency of random integration of the plasmid by scoring the number of G418-resistant colonies produced from an aliquot of the transfected cells. The frequency of interplasmid homologous recombination is then calculated as a ratio of the frequency of HAT^R colony appearance to the frequency of appearance of $G418^R$ colonies.

To demonstrate that HAT^R clones arose as a result of a homologous recombination event that gave rise to the wild type HSV TK, the genomic DNA of randomly selected HAT^R clones was subjected to triple digestion with *Eco*RI, *Hind*III and *Xho*I restriction enzymes and analyzed by Southern blotting for the presence of the intact *Xho*I-resistant HSV TK gene (Figure 3). *Eco*RI cuts both plasmids once 187 bp 5' to the initiation codon of the TK gene; *Hind*III cuts the Thx26 plasmid 0.6 kb 3' to the stop codon of the TK gene, and pAdThx76Δ — within the CMV-*neo* cassette; *Xho*I cuts both plasmids once at the position of their respective *Xho*I linker insertions (cf.Figure 1). The presence of the 1.9 kb *Eco*RI - *Hind*III restriction fragment resistant to *Xho*I digestion is characteristic of the reversion of one of the mutated TK genes to the wild type, either by SSA (the dominant type of extrachromosomal homologous recombination), by double crossing-over, or by gene conversion at the position of the *Xho*I linker insertion. Every HAT^R clone analyzed in that manner featured the wild type HSV TK gene.

The results from all recombination experiments are summarized in Tables 1-3. The statistical significance for the differences observed was determined by the paired t-test.

No statistically significant difference in the rate of illegitimate recombination could be seen in the *NUD1*-transfected cells as compared to the controls.

On the other hand, *NUD1* expression gave rise to a consistent ($P = 0.004$) increase in the rate of homologous recombination in the case where both recombining plasmids were linearized within the region of homology at the *Xho*I restriction site (cf. Figure 1). The average increase in homologous recombination frequency is 35.5% and ranges from 9.88% to 54.5%.

No consistent difference in homologous recombination frequency was seen in the case where both plasmids were linearized outside of the homology region at the *Cla*I restriction site (two-tailed t-test, P = 0.6705).

The recombination experiments in which one of the plasmids was linearized within the region of homology and the other outside of it, gave the following results. When Thx26, linearized with *Xho*I, was co-transfected with pAd-Thx76Δ linearized with *Cla*I, the *NUD1* co-transfected cells featured an average of 23.8% increase in the frequency of homologous recombination between the plasmids (P = 0.0275).

4. Discussion

Although the minute details of the homologous recombination process in eukaryotes are lacking to date, the available data from various recombination experiments allows one to postulate that the initial steps of the process involve the generation of DSBs which are processed into 3' ssDNA tails. The dominant paradigms for the homologous recombination mechanism, namely the DSB repair model and the SSA model, both feature 3' ssDNA tails as an early intermediate of the process. According to these models, ssDNA may play a role in the formation of heteroduplex structures, either by strand invasion, or by annealing of the single-stranded regions of homology. Evidence for ssDNA involvement in recombination is provided by the study of meiotic recombination at *ARG4* and during mating type switching in *S. cerevisiae* [39-41]. ssDNA overhangs were detected as recombination intermediates when DNA was injected into *Xenopus laevis* oocytes [42, 43].

Over the past decade evidence for the role of endo-exonucleases in DNA recombination and repair has been continuously accumulating (see Introduction). This data, together with consideration of their biochemical properties, notably the dsDNA-specific processive 5'—>3' exonuclease activity, make EEs primary candidates for the role of the enzymes generating 3' ssDNA tailed intermediates during homologous recombination.

Although *NUD1* gene product homologues have been demonstrated in vertebrates and even in primates [27, 44], the corresponding genes have not yet been cloned. This shortcoming brought us to use the available yeast *NUD1* gene in order to investigate the effect of EE overexpression on recombination in mammalian cells. Previously we were able to demonstrate that *NUD1* could be successfully expressed and exhibited biological activity in human HeLa cells [34].

The transient expression of *NUD1* from a mammalian expression vector resulted in a modest but consistent increase in the frequency of extrachromosomal homologous recombination in mouse fibroblasts (35.5% average increase, P = 0.004). The most consistent results were seen in case when both recombining plasmids had been linearized within the region of homology, i.e. at the *Xho*I site of both plasmids (cf. Figure 1 and Table 1). When both recombining plasmids were linearized outside the region of homology (at the *Cla*I site of both plasmids), there was no significant difference in the frequency of interplasmid recombination between *NUD1*-transfected cells and the controls (cf. Figure 1 and Table 2). In the case where only one of the plasmids was linearized within the region of homology, while the other was linearized outside of the region of homology (i.e. the Thx26 was linearized with *Xho*I and the pAd-Thx76Δ was linearized with *Cla*I), an increase in homologous recombination was observed. The magnitude of the increase, however, was less than in the case where both plasmids were linearized with *Xho*I (23.8% *vs* 35.5% average increase, respectively).

The observed increase in the homologous recombination frequency appears to be proportional to the number of 3' ssDNA tails that can be generated by 5'—> 3' exonuclease activity from the site of linearization within the region of homology. Thx26 features stretches of homologous sequence on both sides of the *Xho*I linker insertion, whereas pAd-Thx76Δ features homologous sequence only 5' to its *Xho*I linker insertion. When both plasmids are linearized with *Xho*I, the number of potential 3' ssDNA "homologous" tails is 3 (two for Thx26 and one for pAd-Thx76Δ); when Thx26 is linearized with *Xho*I and pAd-Thx76Δ

with *Cla*I there are only 2 "homologous" 3' ssDNA tails (assuming that the ssDNA tails are shorter than 3.4 kb); only one "homologous" 3' ssDNA tail can be generated when both plasmids are linearized with *Cla*I, if the 5'—> 3' exonuclease processing extends beyond 0.65kb from the *Cla*I site, and none if the latter assumption is false.

The expression of Nud1p appears to specifically affect the homolgous recombination, since it had no effect on the frequency of random integration events. The frequency of random integration (illegitimate recombination), as reflected by the rate of appearance of G418-resistant colonies, remained the same regardless of the presence or absence of *NUD1*.

The reported differences in homologous recombination frequencies between the *NUD1*-transfectants and the controls, although consistent and significant, are relatively small. We believe that the magnitude of the effect of *NUD1* expression is undermined by at least two phenomena. First, the experiments were performed in transiently transfected cells. Most probably the use of cell lines stably transfected with *NUD1* would resolve this problem. Unfortunately, we have not succeeded so far in producing such cell lines. We believe that the failure to obtain stable transfectants of *NUD1* stems from the fact that the constitutive expression of *NUD1*, resulting from the "leaky" inducible promoter, is deleterious to the cells. Second, mammalian cells are extremely proficient in extrachromosomal homologous recombination per se, thus rendering so much more difficult the detection of an increase in the frequency of the latter. Recombination frequencies of 1 to 20% have been reported for extrachromosomal homologous recombination using assays similar to the one we used in the present study [45, 46].

Our working model explaining the increase in homologous recombination by EE overexpression is as follows: EEs are required to process DSBs into suitable substrates for the search of homology (i.e. the 3' ssDNA overhangs). If this step is rate limiting for the whole process of initiation of homologous recombination at the DSB, then the overexpression of EE should increase the rate of initiation, and consequently, the rate of homologous recombination overall. It is possible, however, that the role of EE in the process

of homologous recombination is not limited to simple exonucleolytic activity, but involves more complex interactions with the substrate DNA molecules and possibly with other proteins of the recombination machinery.

It is generally believed that extrachromosomal homologous recombination in mammalian cells proceeds via SSA [10] which involves extensive deletions of the DNA free ends and the generation of long (>1 kb) ssDNA tails. In yeast, Nud1p is believed to function in the homologous recombination pathway which relies upon conservative DSB repair recombination [31-33]. It is possible then that Nud1p interacts with the ends of the plasmids and directs the latter to undergo recombination via DSB repair rather than via the endogenous default SSA pathway. It is also possible that the former pathway is more efficient than the latter in the generation of wild type HSV TK by gene conversion at the sites of the insertion/ deletion mutations. Hence, the observed increase in homologous recombination of the *NUD1*-transfectants. Supporting this hypothesis are the report of yeast *RAD52* overexpression in human cells resulting in an increase in extrachromosomal recombination [47] and extensive evidence indicating that the *NUD1* and *RAD52* gene products function in the same DNA repair / recombination pathway [14, 30-34].

Recent studies by Henderson and Simons [48] show that exogenous DNA integration by illegitimate recombination is frequently preceded by extensive 5'—>3' and by less extensive 3'—>5' exonuclease digestion. Although, *NUD1* expression potentially affects the 5'—>3' exonuclease processing of the transfected DNA, no difference in the rate of illegitimate recombination frequency between *NUD1*-transfectants and the controls could be detected in our study. This observation strengthens the hypothesis formulated above, that the role of EEs in homologous recombination involves more than just the generation of 3' ssDNA tails required for the search of homology and heteroduplex formation.

Overall, our results provide additional evidence for the involvement of EEs in homologous recombination in mammalian cells. They also demonstrate that the overexpression of EEs such as Nud1p can potentially be used as a means of selectively

increasing the frequency of homologous recombination in mammalian cells without a concomitant increase in the illegitimate recombination frequency. This might be of value for such applications as gene targeting, which in turn is potentially useful with regard to a number of applications in molecular biology and somatic gene therapy.

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Legends

Figure 1. Recombination detection systems. The HSV *tk* gene of the Thx26 vector is inactivated by the *Xho*I linker insertion at position 738. The pAd-Thx76Δ vector contains the HSV *tk* gene of the original Thx76 vector (with the *Xho*I linker insertion at position 1611) deleted 3' to the *Xho*I site. The homologous sequences between both vectors, surrounding the *Xho*I linker of the Thx26 are 1.12 kb 5' to the linker and 0.9 kb 3' to the linker. The pAd-Thx76Δ vector contains the CMV-*neo* cassette which allows the detection of illegitimate recombination events. Recombination between the target and the targeting vector would yield functional *tk*, resulting in HAT-resistant clones. Prior to transfections both plasmids were linearized at the site of their respective *Xho*I linkers (open triangles). The DNA probe used for the Southern analysis is shown as an open rectangle with thick borders.

Figure 2. Northern blot analysis of total RNA from *NUD1*-transfected and pCI-transfected Ltk- cells. **A.** MOPS 1% agarose gel; **B.** Autoradiogram of the Northern blot hybridization to the *NUD1*-specific probe. **Lane 1**: DNaseI treated total RNA from pCI-transfected control Ltk- cells; **Lane 2**: DNaseI treated total RNA from pCI-*NUD1*-transfected Ltk- cells. A total of 20 μg of RNA was loaded onto each lane.

Figure 3. Nuclease activity assay. One representative experiment is shown. Nuclease activity is represented by the cpm values determined at different time points. The cpm values correspond to the amount of acid-soluble radioactivity released from the ^{32}P-labeled ssDNA linear substrate by the nucleolytic activities immunoprecipitated from the lysates of HeLa cells transfected with (°)pCI-*NUD1* vector and (•) pCI control vector. The immunoprecipitation was done using the rabbit polycloncal antibodies raised against the *N. crassa* endo-exonuclease.

Figure 4. Southern blot. Lanes 1-4 feature, respectively, pAdThx76Δ digested with *Hind*III and *Eco*RI; pAdThx76Δ digested with *Hind*III, *Eco*RI, and *Xho*I; Thx26 digested with *Hind*III and *Eco*RI; Thx26 digested with *Hind*III, *Eco*RI, and *Xho*I. Lanes 5-15: 10μg of gDNA isolated from randomly selected HAT-resistant colonies, digested with *Hind*III, *Eco*RI, and *Xho*I. The 1918 bp band corresponds to the *Hind*III/*Eco*RI fragment of HSV TK which is resistant to *Xho*I digestion and can be seen for every HAT-resistant clone analyzed.

Table 1 Effect of *NUD1*-expression on recombination between Thx26 / *Xho*I and pAD / *Xho*I

Exp.	*NUD1* expression	HAT-resistent colonies per $6x10^6$ cells transfected	G418-resistent colonies per $6x10^4$ cells transfected	$HAT^R/G418^R$ ratio	% difference
1	+	40	40	1.00	+34.6%
	-	26	35	0.743	
2	+	176	39	4.51	+39.2%
	-	120	37	3.24	
3	+	178	19	9.37	+9.88%
	-	162	19	8.53	
4	+	68	27	2.52	+26.0%
	-	66	33	2.00	
5	+	51	20	2.55	+54.5%
	-	33	20	1.65	
6	+	32	3	10.7	+47.6%
	-	29	4	7.25	
7	+	33	3	11.0	+37.5%
	-	40	5	8.00	
8	+	36	5	7.20	+35.0%
	-	48	9	5.33	
35.5% average increase in $HAT^R/G418^R$ ratio, n = 8, P = 0.004					

Table 2 Effect of *NUD1*-expression on recombination between Thx26/*Cla*I and pAD/*Cla*I

Exp.	*NUD1* expression	HAT-resistent colonies per $6x10^6$ cells transfected	G418-resistent colonies per $6x10^4$ cells transfected	$HAT^R/G418^R$ ratio	% difference
1	+	31	28	1.11	+39.3%
	-	31	39	0.795	
2	+	7	29	0.241	-16.8%
	-	9	31	0.290	
3	+	6	12	0.50	-16.7%
	-	9	15	0.60	
4	+	5	6	0.833	+94.5%
	-	3	7	0.428	
5	+	1	8	0.125	-68.7%
	-	2	5	0.400	
The difference in $HAT^R/G418^R$ ratios is statistically insignificant, n = 5, P = 0.6705					

Table 3 Effect of *NUD1*-expression on recombination between Thx26/*Xho*I and pAD /*Cla*I

Exp.	*NUD1* express	HAT-resistent colonies per $6x10^6$ cells transfected	G418-resistent colonies per $6x10^4$ cells transfected	$HAT^R/G418^R$ ratio	% difference
1	+	23	3	7.67	+15.0%
	-	20	3	6.67	
2	+	30	11	2.73	+27.3%
	-	30	14	2.14	
3	+	13	4	3.25	+62.5%
	-	14	7	2.00	
4	+	6	4	1.50	+50.0%
	-	2	2	1.00	
5	+	16	11	1.45	-18.5%
	-	16	9	1.78	
6	+	12	6	2.00	+38.5%
	-	13	9	1.44	
7	+	26	30	0.867	-8.45%
	-	18	19	0.947	
23.8% average increase in $HAT^R/G418^R$ ratio, n = 7, P = 0.0275					

Figure 1

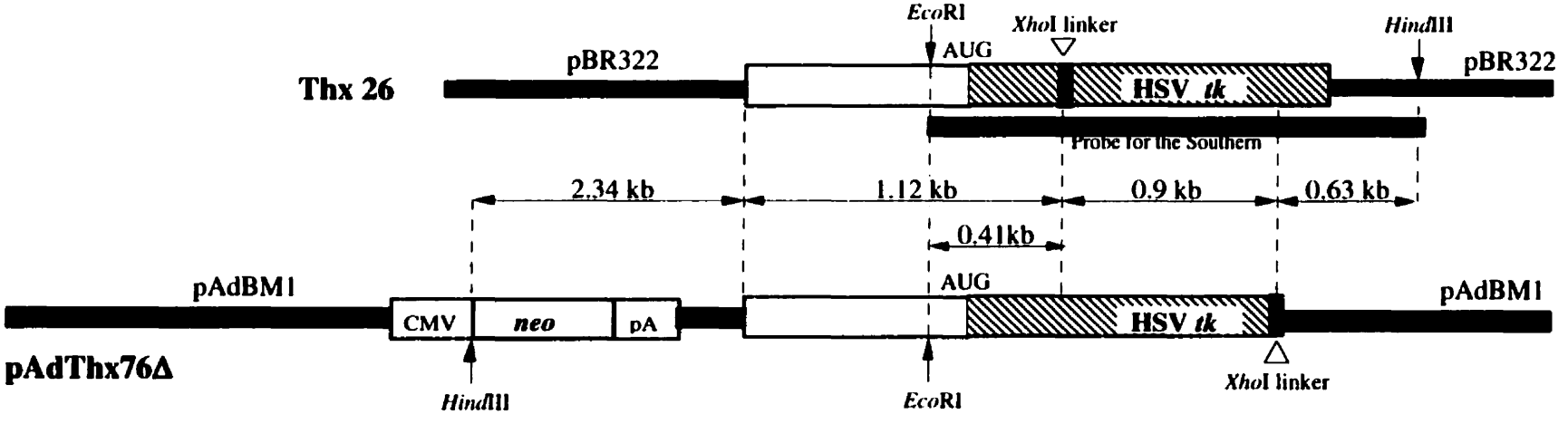

EcoRI
XhoI linker
HindIII
AUG
pBR322
Thx 26
pBR322
HSV tk
Probe for the Southern
2.34 kb
1.12 kb
0.9 kb
0.63 kb
0.41kb
pAdBM1
AUG
pAdBM1
CMV
neo
pA
HSV tk
pAdThx76Δ
HindIII
EcoRI
XhoI linker

Figure 2

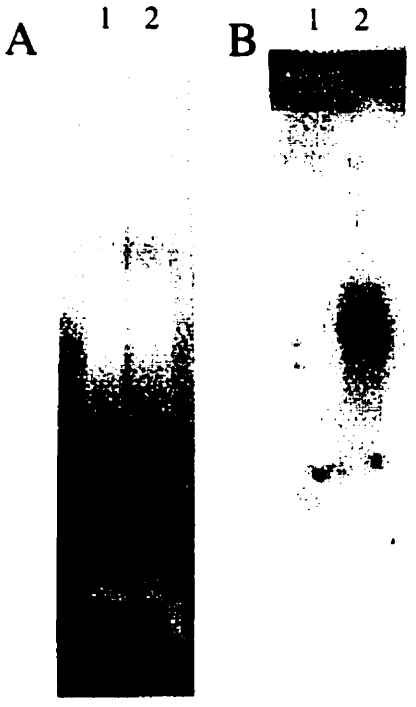


Figure 3

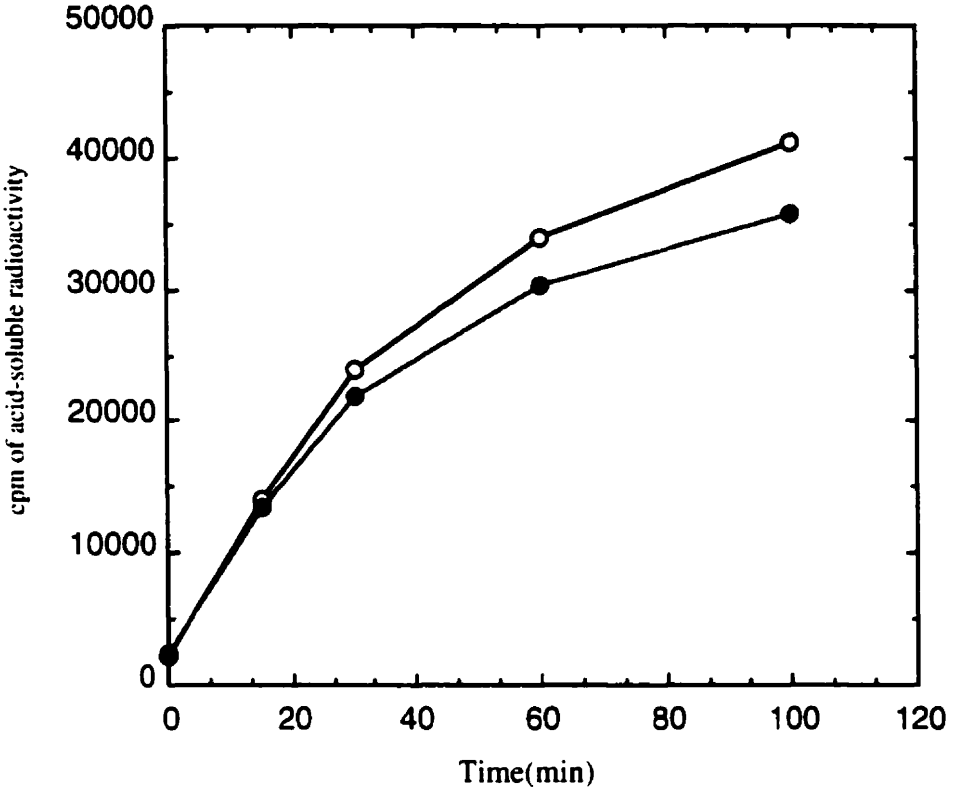
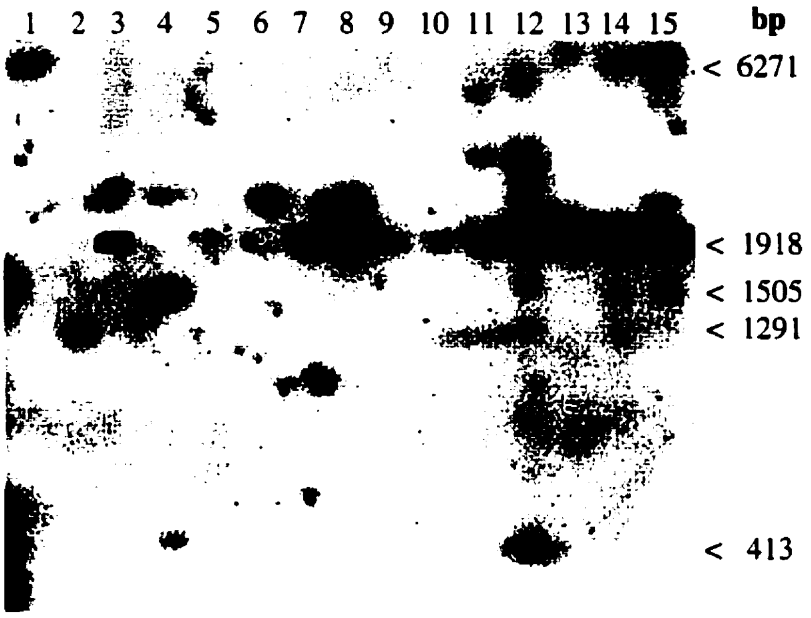

50000
40000
30000
20000
10000
0
cpm of acid-soluble radioactivity
0
20
40
60
80
100
120
Time(min)

Figure 4



CHAPTER 3 Inhibition of poly(ADP-ribose) polymerase with 1,5-isoquinolinediol stimulates homologous recombination in Ltk- cells.

Chemical inhibition of PARP is known to increase the frequency of sister chromatid exchange, of intrachromosomal homologous recombination, and gene amplification in mammalian cells. These results suggest that PARP inhibition generally stimulates homologous recombination processes. However, two previously published studies of the effect of PARP inhibition on the rate of extrachromosomal homologous recombination and gene targeting showed that the rate of the former remained unchanged and the frequency of the latter decreased in response to PARP inhibition with 3-methoxybenzamide.

In this chapter we present our findings that treatment of cells with an inhibitor of PARP 1,5-isoquinolinediol (ISQ) results in increase in the frequency of extrachromosomal homologous recombination in mouse Ltk- fibroblasts. We have also demonstrated that treatment of mouse Ltk- cells with ISQ leads to an increase of up to 8-fold in the frequency of gene targeting of the stably integrated HSV *tk* gene. We believe that our results concerning the effect of ISQ on gene targeting may have potential application for the improvement of such technologies as the generation of genetic knock-outs and the *ex vivo* gene therapy.

3.1 Inhibition of poly(ADP-ribose) polymerase stimulates extrachromosomal homologous recombination in mouse Ltk- fibroblasts (article published in Nucleic Acids Research, 1999; 27: 4526-4531. Reproduced with permission from Oxford University Press.)

Inhibition of poly(ADP-ribose)polymerase stimulates extrachromosomal homologous recombination in mouse Ltk- fibroblasts.

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Key words : PARP inhibition, 1,5-isoquinolinediol, homologous recombination

Abstract

Poly(ADP-ribose)polymerase (PARP) is an abundant nuclear enzyme activated by DNA breaks. PARP is generally believed to play a role in maintaining the integrity of the genome in eukaryote cells via anti-recombinogenic activity by preventing inappropriate homologous recombination reactions at DNA double-strand breaks. While inhibition of PARP reduces non-homologous recombination, at the same time it stimulates sister chromatid exchange and intrachromosomal homologous recombination. Here we report that the inhibition of PARP with 100 μg/ml (0.622 mM) 1,5-isoquinolinediol results in an average 4.6-fold increase in the frequency of extrachromosomal homologous recombination between two linearized plasmids carrying Herpes Simplex Virus thymidine kinase (HSV *tk*) genes inactivated by non-overlapping mutations, in mouse Ltk- fibroblasts. These results are in disagreement with the previously reported observation that PARP inhibition had no effect on extrachromosomal homologous recombination in Ltk- cells.

Introduction

Homologous recombination is the process by which two DNA molecules exchange homologous sequences. In eukaryotes this process is the basis of a number of important biological events such as recombination between homologous chromosomes during mitosis and meiosis, generation of the antigen recognition molecule repertoire of the immune system, gene amplification, and DNA repair [1-5]. The complexity of the cellular machinery involved in DNA repair and recombination has now become apparent. In yeast alone, the number of genes whose products are believed to participate in recombination/ repair is estimated at over 100 [6].

PARP is an abundant nuclear protein found in all higher eukaryotes [7, 8]. It recognizes, binds to, and becomes activated by DNA breaks. As its name implies, the enzyme catalyzes the synthesis of polyADP-ribose from NAD+ on a number of different

protein targets. Its substrates include ligases, topoisomerases, histones and PARP itself. Although the biological role of PARP is as yet unclear, it has been recently suggested by Satoh and Lindahl, and others that one of PARP's overall functions might be the preservation of genomic integrity by decreasing spontaneous homologous recombination at sites of DNA breaks [9-11].

There have been a number of reports indicating that PARP inhibition results in a decrease of random integration of exogenous DNA into the mammalian genome [12, 13]. PARP inhibition was also reported to increase the frequency of sister chromatid exchange (SCE) in mammalian cells, a process that proceeds via homologous recombination [14]. Finally, Waldman and Waldman have reported that PARP inhibition increased the frequency of intrachromosomal homologous recombination in mouse Ltk- fibroblasts [15]. Recent studies with PARP knock-out mice have confirmed the anti-recombinogenic function of PARP at DNA strand breaks [16, 17].

The above mentioned results suggest that PARP inhibition generally stimulates homologous recombination processes. However, studies of the effect of PARP inhibition on the rate of extrachromosomal homologous recombination in mouse Ltk- fibroblasts and gene targeting in Chinese hamster ovary cells showed that the rate of the former remained unchanged and the frequency of the latter decreased 2-fold in response to PARP inhibition with 3-methoxybenzamide (3-MB) [13, 18].

Here, we report the effect of PARP inhibition with 1,5-isoquinolinediol (ISQ) on extrachromosomal recombination in mouse Ltk- fibroblasts. Conversely to the previously published reports, we have observed that PARP inhibition results in a consistent dose-dependent increase in the frequency of extrachromosomal homologous recombination.

Materials and Methods

The recombination system

The recombination system used is similar to the one described by Shapira et al. [19]. It is based on two plasmids carrying the Herpes Simplex Virus (HSV) thymidine kinase (*tk*) gene with non-overlapping insertion and deletion mutations (cf. Figure 1 for details). Both mutated *tk* genes are non-functional. Ltk- fibroblasts are deficient in *tk* activity, and hence, can not survive in hypoxanthine-aminopterin-thymidine (HAT) medium. The appearance of HAT-resistant (HAT^R) colonies, after co-transfection with the two plasmids, is diagnostic of an interplasmid homologous recombination event.

The Thx26 vector

The Thx26 vector contains an HSV*tk* gene with an 8 bp *Xho*I insertion mutation at position 738 of the *tk* coding sequence, subcloned into pBR322 [19].

The pAd-Thx76Δ vector

The HSV*tk* gene with a *Xho*I insertion mutation at position 1611 of the *tk* coding sequence (Thx76) was deleted from the sequence 3' to the *Xho*I linker and subcloned into the commercial pAdBM1 vector (Quantum, Canada) . The CMV-*neo* cassette was inserted 5' to the mutated HSV*tk*.

Cell culture

Ltk- cells were grown and maintained in DMEM (Bio Media, Canada) supplemented with 10% FBS (GibcoBRL, NY USA) and Antimyotic-Antibiotic (GibcoBRL) at 37°C in 5% CO_2/ 95% air.

Transfections and selection

$3x10^6$ cells were plated on 100-mm dishes (Costar, MA USA) and allowed to divide once. The cells were transfected with equimolar quantities of Thx26 (4μg) and of pAd-Thx76Δ (6 μg) plasmids linearized with *Xho*I, using Superfect poly-cationic reagent from QIAGEN Canada, according to the specifications provided by the manufacturer.
At 20 hours post-transfection, the cells were trypsinized. 1/100 of all cells were seeded in an 80 cm^2 TC flask (NUNC, IL USA) containing complete DMEM supplemented with 250

μg/ml G418. The rest of the cells were plated in four 80 cm^2 TC flasks containing complete DMEM supplemented with HAT.

The cells were then incubated in a CO_2 incubator for 11 days with the selection media being changed every 4 days. Surviving cells were finally fixed with 10% formaldehyde phosphate buffered saline, stained with methylene blue. The colonies, formed by at least 20 cells, were counted.

The statistical significance for the differences observed for recombination frequencies was determined by the paired t-test. The difference was considered significant for P-values lower than 0.05.

PARP inhibition

The competitive inhibitor of PARP, 1,5-isoquinolinediol (Sigma) was dissolved in DMSO at 100 mg/ml (622 mM). The inhibition was performed by direct addition of appropriate amounts of the above solution to the cell culture medium immediately after the completion of the transfection procedure. The PARP inhibition was maintained for 20h.

Southern Analysis of the HAT^R clones

HAT^R colonies were randomly selected and expanded. The genomic DNA (gDNA) from HAT^R clones was extracted by standard Proteinase K/Phenol/Chloroform method. 10μg of the gDNA were then digested with *Eco*RI, *Hind*III and *Xho*I enzymes (Pharmacia) and subjected to Southern analysis by standard methods. The *Eco*RI-*Hind*III fragment of the HSV*tk* from the Thx26 plasmid labeled with ^{32}P-dCTP by a random primer oligolabeling kit (Pharmacia), was used as the probe.

Results

The effect of 1,5-isoquinolinediol on cell survival and illegitimate recombination frequency

To assess the toxicity of the ISQ to the Ltk- fibroblasts we have performed a number of standard cell survival clonogenic assays. The results are summarized in Figure 2A. The exposure of cells to the inhibitor for 20h resulted in a dose-dependent drop in cell survival. At

100 µg/ml (0.622 mM) — the highest drug concentration used — the cell survival was on average only 29% (±2%) of the control.

The effect of ISQ inhibition of PARP on the frequency of random integration (illegitimate recombination) was determined by transfecting the pAd-Thx76Δ plasmid linearized with *Xho*I into Ltk- cells, followed by treatment with different concentrations of ISQ for 20h and selection of the G418-resistant colonies. The ISQ treatment decreased the frequency of random integration. The maximum decrease of 46.5% (±4.28%) was observed at 100 µg/ml ISQ (Figure 2B). Given the large variation in the random integration frequencies observed for the lower doses of the drug, it is unclear whether there is an early dose dependence followed by a plateau or if there is a biphasic response, perhaps indicating two different actions of the drug at different dosages.

The effect of 1,5-isoquinolinediol on interplasmid homologous recombination

The appearance of HAT^R colonies subsequent to co-transfection of the Thx26 and pAd-Thx76Δ plasmids is diagnostic of an interplasmid homologous recombination event followed by random integration of the corrected plasmid into the genome. Hence, the frequency of appearance of HAT^R colonies is the product of the frequency of interplasmid homologous recombination and of the frequency of their random integration. It is possible, however, that a functional *tk* gene can be produced by inter or intra-chromosomal recombination between two randomly integrated complementary plasmids, but the frequency of this event should be many fold lower than that of the event described above — $2x10^{-9}$ ($2x10^{-6}$ x 10^{-3})*vs* 10^{-4} events/cell/generation. According to Waldman and Waldman [15], the frequency of intrachromosomal recombination between HSV*tk* sequences in Ltk- cells is $2x10^{-6}$ events/cell/generation; the average frequency of integration of the pBR322-HSV *tk* vector is approximately 10^{-3}. The frequency of interplasmid recombination followed by random integration into the Ltk- cell genome, as reported by Shapira *et al.* [19], is around 10^{-}

^{4}. Hence, it is reasonable to assume that the vast majority of the HAT^{R} colonies detected arose by interplasmid homologous recombination followed by random integration.

The CMV-*neo* cassette incorporated into the pAd-Thx76Δ vector allows us to monitor the frequency of random integration of the plasmid by scoring the number of G418-resistant (G418^{R}) colonies produced from an aliquot of the transfected cells. The frequency of interplasmid homologous recombination is then calculated as a ratio of the number of HAT^{R} colonies to the number of G418^{R} colonies.

To demonstrate that HAT^{R} clones arose as a result of a homologous recombination event that gave rise to the wild type HSV*tk*, the genomic DNA of randomly selected HAT^{R} clones was subjected to triple digestion with *Eco*RI, *Hind*III and *Xho*I restriction enzymes and analyzed by Southern blotting for the presence of the intact *Xho*I-resistant HSV *tk* gene (Figure 3). *Eco*RI cuts both plasmids once 187 bp 5' to the initiation codon of the *tk* gene; *Hind*III cuts the Thx26 plasmid 0.6 kb 3' to the stop codon of the *tk* gene, and pAdThx76Δ within the CMV-*neo* cassette; *Xho*I cuts both plasmids once at the position of their respective *Xho*I linker insertions (cf.Figure 1). The presence of the 1.9 kb *Eco*RI - *Hind*III restriction fragment resistant to the *Xho*I digestion is characteristic of the reversion of one of the mutated *tk* genes to the wild type, either by SSA (the dominant type of extrachromosomal homologous recombination), by double crossing-over, or by gene conversion of Thx26 at the position of the *Xho*I linker insertion. Although a simple loss of the *Xho*I linker from Thx26 would restore the wild type *tk* gene, no HAT^{R} colonies could be detected in the control experiments where the Ltk- cells were transfected with the *Xho*I-linearized Thx26 plasmid alone. The diagnostic 1918 bp *Hind*III/*Eco*RI fragment resistant to *Xho*I digestion was detected for every HAT^{R} clone analyzed with the exception of one clone (Figure 3, lane 7). In the case of the latter, the band corresponds to a fragment of higher molecular weight. It has probably arisen as a result of a double crossing-over with the resolution of the Holiday junction 5' to the *Hind*III restriction site of Thx26.

The results from all recombination experiments are summarized in Table 1. The statistical significance for the differences observed was determined by the paired t-test. Although no significant difference in the rate of extrachromosomal homologous recombination, as compared to the untreated cells, was observed at the lowest concentration of the drug used (30 μg/ml = 0.186 mM), for the ISQ concentrations of 50, 75 and 100 μg/ml (0.310, 0.466 and 0.622 mM), the average increase in homologous recombination was, respectively, 85.6% (P=0.0066; ranging from 34.4% to 182%), 191% (P=0.0370; ranging from 150% to 261%) and 463% (P=0.0030; ranging from 294% to 709%).

Discussion

The precise mechanism of homologous recombination in eukaryotes remains unknown [20]. The proposed models, known as the double-strand break (gap) repair model [21-23], the single-strand annealing (SSA) model [24], the one-sided invasion model [25] and the synthesis-dependent strand-annealing model [26], were devised to accommodate the existing data from recombination studies done mainly in lower eukaryotes. Although all of these models differ from each other in several aspects, all postulate that homologous recombination is initiated by a double-strand break (DSB) in one of the recombining molecules.

Two of the major proteins known to recognize, interact with, and become activated by free DNA ends are DNA-dependent protein kinase (DNA-PK) and PARP [27]. Whereas DNA-PK activation by free DNA ends most probably leads to the activation of the non-homologous end-joining DSB repair pathway [28], the role of the PARP in DNA repair/recombination remains unclear.

PARP is an abundant nuclear enzyme present in all higher eukaryotes. Although its structure and enzymatic activities have been extensively studied and are fairly well understood, its biological function remains unclear [7, 8, 11]. It is known that PARP is activated by breaks in chromosomal DNA. It can bind to both single strand and double strand breaks in the DNA and initiate the polymerization of polyADP-ribose on itself and on a

number of its protein substrates. The resulting negatively charged branched ADP-ribose polymer is thought to protect the DNA nicks from inappropriate recombination with homologous genomic sequences allowing the former to undergo repair via end-joining reactions. Lindhal *et al* and others [9-11] have proposed that the role of PARP could be the negative regulation of recombination which ensures the stability of the genome. This hypothesis predicts that PARP inhibition or deactivation should result in an increase of homologous recombination processes. Indeed, PARP inhibition stimulates SCE and increases the frequency of intrachromosomal homologous recombination in mouse cells [14, 15]. Similarly, knock-out mice that lack PARP feature increased levels of SCE [16]. Moreover, increased recombination activity after the loss of PARP anti-recombinogenic function is demonstrated by the fact that the PARP knock-out can rescue V(D)J recombination in SCID mice lacking the DNA-PK [17].

It is generally believed that extrachromosomal recombination is, to a certain extent, similar to meiotic and mitotic recombination and most probably makes use of the same enzymes as the latter. It would be reasonable then to expect, that PARP inhibition would be stimulatory to extrachromosomal homologous recombination the same way it is for SCE, intrachromosomal and V(D)J recombination.

Using plasmids carrying HSV*tk* genes inactivated by complementary insertion and deletion mutations, we were able to show that treatment of the transfected cells with a PARP inhibitor, ISQ, resulted in a dose-dependent increase in the rate of interplasmid homologous recombination ranging, for the highest ISQ concentration tested, from 2.8 to 7.0 -fold.

Given that there is no clear understanding of PARP effects on the cellular processes, the only explanations for the observed increase of extrachromosomal homologous recombination are purely speculative. One possible explanation is that extrachromosomal recombination, similarly to inter- and intrachromosomal recombination, requires a protein whose activity is repressed by PARP. It is also possible that PARP inhibition makes the free ends of the linearized plasmids more readily accessible to the homologous recombination

machinery. It might also be that ISQ acts on a protein or proteins other than PARP and that the former affect(s) recombination directly or indirectly. However, in spite of the fact that over the past six years ISQ has been widely used in the studies involving PARP, there have been no reports of ISQ's affecting any enzyme other than PARP. Similarly, the suggestion that the observed increase in homologous recombination might be due to ISQ's promoting DNA damage and thus stimulating recombination, although plausible is not supported by any reports of ISQ causing any DNA damage directly or indirectly [Guy Poirier, personal communication].

Yet another possible explanation involves the well established fact that PARP inhibition decreases the frequency of illegitimate recombination [12, 13, Figure 2B and Table 1, this paper]. If illegitimate and extrachromosomal recombination represent two competing pathways, then the repression of the former would result in stimulation of the latter.

In light of the recent finding by Ruscetti et al. that the protein kinase activity of DNA-PK can be stimulated by PARP in the presence of NAD+ in a reaction that is blocked by ISQ [27], the following alternative explanation of our results can be offered. PARP might actively promote the functioning of a DNA repair pathway other than the recombination repair pathway, for example the non-homologous end-joining (NHEJ) repair pathway. If NHEJ competes with recombination repair, and if the former is stimulated by PARP, then PARP's inhibition may result in increased homologous recombination.

As mentioned in the introduction, a study similar to ours was conducted by Waldman and Waldman in 1990 [13]. They investigated the effect of 3-methoxybenzamide (3-MB), a competitive inhibitor of PARP, on illegitimate and extrachromosomal recombination in Ltk- cells. In their study, cells were transfected with a wild type HSV*tk* gene or with two defective *tk* gene sequences, circular or linearized, followed by selection for *tk* -positive colonies. While treatment of the cells with 2 mM 3-MB was found to reduce the number of colonies recovered with a wild type *tk* 10 to 20-fold (numbers non-corrected for the cell survival rates), it reduced the number of colonies recovered with defective *tk* genes only to the same

extent as in transfection with a wild type gene. Hence, the authors have concluded that PARP played a role in illegitimate recombination, but did not play an important role in extrachromosomal homologous recombination.

The two major factors that distinguish our study of the effect of PARP inhibition on the extrachromosomal recombination from the one described above, and which might be responsible for the diverging results of these studies, are the nature of the PARP inhibitor and the method of transfection used.

Our predecessors made use of 3-MB, whereas we used ISQ. ISQ is a more potent and more specific inhibitor of PARP. ISQ was first identified as a potent inhibitor of PARP by Banasik and co-workers during a large scale survey using an *in vitro* assay systems [29]. They showed that its 50% inhibitory concentration was two orders of magnitude lower than that of 3-aminobenzamide. Subsequently, Shah *et al* reported that ISQ was also a potent inhibitor of PARP *in vivo* and showed that it could completely abolish oxidant-induced activation of PARP in C3H10T1/2 cells [30].

As far as the method of transfection of the plasmids is concerned, we used lipofectin in our study, because in our case, it gives more consistent transfection efficiency than the calcium phosphate/DNA coprecipitation or electroporation methods used by Waldman and Waldman in their study. The method of transfection can be of importance, since the same authors have reported that an identical concentration of 3-MB resulted in different degrees of inhibition of random integration depending on whether the plasmid DNA had been electroporated or transfected via calcium phosphate/DNA coprecipitation [18].

Also, since the plasmids containing the mutated HSV *tk* genes used by Waldman and Waldman did not have a marker allowing the direct determination of the rate of illegitimate recombination, the determination of the frequency of homologous recombination in their experiments required two separate transfections: one to determine the rate of random integration, and the second to determine the rate of appearance of HAT^R colonies due to homologous recombination followed by random integration. The frequency of homologous

recombination was then calculated using the results from the two transfections without taking into account the variability in transfection efficiency from one experiment to another. We believe that our determinations of the frequency of homologous recombination are more accurate, since they derive from the data generated by a single transfection experiment and, hence, are not affected by the variability in the transfection efficiency.

Overall, we believe our finding that PARP inhibition stimulates extrachromosomal homologous recombination while decreasing non-homologous recombination is important. Firstly, it appears to solve the conflict in the previously reported results that PARP inhibition stimulates chromosomal but not extrachromosomal homologous recombination. Secondly, it suggests that PARP inhibition with ISQ may be used to increase gene targeting frequency. We are now investigating this possibility.

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Legends

Figure 1. Cf. Legend for Figure 1 on page 170.

Figure 2. The effect of 1,5-isoquinolinediol on cell survival and on illegitimate recombination in Ltk- cells. **A.** 300 Ltk- cells were plated in 25 cm^2 TC flasks and treated 5h after plating with different concentrations of ISQ for 20h. The cells were then washed twice with PBS and incubated in DMEM/10% FBS/Antibiotic-Antimyotic in a CO_2 incubator for 11 days. The cells were finally fixed with 10% formaldehyde phosphate buffered saline, stained with methylene blue and the colonies, formed by at least 20 cells, were counted. For every drug concentration the experiment was carried out in triplicate. An average of 175 colonies per flask was scored in the absence of the inhibitor. The percent survival is calculated as the number of colonies scored for the given concentration of the drug divided by the number of colonies scored in the absence of the drug multiplied by 100. Each point represents the mean of 5 separate experiments, and each error bar one standard deviation of that mean. **B.** $3x10^5$ Ltk- cells, plated on 60-mm dishes 20 hours prior to transfection, were transfected with the *Xho*I-linearized pAd-Thx76Δ plasmid using Superfect poly-cationic reagent from QIAGEN Canada, and treated with different concentrations of ISQ for 20h. At 20 hours post-transfection, the cells were trypsinized, plated onto 80 cm^2 TC flasks in complete DMEM supplemented with 250 μg/ml G418 and incubated in a CO_2 incubator for 11 days. At which point the cells were fixed, stained and the colonies were counted. An average of 61 $G418^R$ colonies was scored in the absence of the drug. The percentage of illegitimate recombination is calculated as the number of $G418^R$ colonies scored for the given concentration of the drug divided by the number of $G418^R$ colonies scored in the absence of the drug multiplied by 100. The percentage of illegitimate recombination has been adjusted for the cell death due to the drug toxicity by dividing the observed percentage of illegitimate recombination by the average cell survival rate determined for this drug concentration. The standard deviation of the mean for the percentage of illegitimate recombination has been similarly adjusted by

multiplying it by the standard deviation of the mean for the percentage of cell survival for the same drug concentration. Each point represents the mean of 5 separate experiments.

Figure 3. Representative Southern analysis of HAT-resistant colonies arising after co-transfection of Ltk- cells with the *Xho*I-linearized Thx26 and pAdThx76Δ plasmids. The ^{32}P-dCTP-labeled *Eco*RI-*Hind*III fragment of the HSV *tk* was used as a probe. Lanes 1-4 feature, respectively, pAd-Thx76Δ digested with *Hind*III and *Eco*RI; Thx26 digested with *Hind*III and *Eco*RI; pAd-Thx76Δ digested with *Hind*III, *Eco*RI and *Xho*I; Thx26 digested with *Hind*III, *Eco*RI and *Xho*I. Lanes 5 - 21: 10μg of gDNA isolated from randomly selected HAT-resistant colonies, digested with *Hind*III, *Eco*RI and *Xho*I. The 1918 bp band corresponds to the *Hind*III/*Eco*RI fragment of HSV*tk* which is resistant to *Xho*I digestion and can be seen for every HAT-resistant clone analyzed, with only one exception (lane 7).

Table 1 Effect of 1,5-isoquinolinediol on recombination between Thx26/*Xho*I and pAd-Thx76Δ/ *Xho*I

Exp.	[Inhib.] (μg/ml)	HAT-resistant colonies per $6x10^6$ cells transfected	G418-resistant colonies per $6x10^6$ cells transfected	$HAT^R/G418^R$ ratio	% difference
1	0	80	15×10^2	0.0533	—
	30	63	16×10^2	0.0394	-26.0%
	50	73	9×10^2	0.0811	+52.2%
	75	80	6×10^2	0.133	+150%
	100	63	3×10^2	0.210	+294%
2	0	78	57×10^2	0.0137	—
	30	62	26×10^2	0.0238	+74%
	50	54	14×10^2	0.0386	+182%
	75	49	12×10^2	0.0408	+198%
	100	73	8×10^2	0.0912	+567%
3	0	33	7×10^2	0.0471	—
	30	40	6×10^2	0.0667	+41.6%
	50	19	3×10^2	0.0633	+34.4%
	75	17	1×10^2	0.170	+261%
	100	18	1×10^2	0.180	+282%
4	0	92	60×10^2	0.0153	—
	30	58	36×10^2	0.0161	+5.10%
	50	72	27×10^2	0.0267	+73.9%
	75	107	27×10^2	0.0396	+158%
	100	124	10×10^2	0.124	+709%

Figure 1 Cf. page 174.

Figure 2

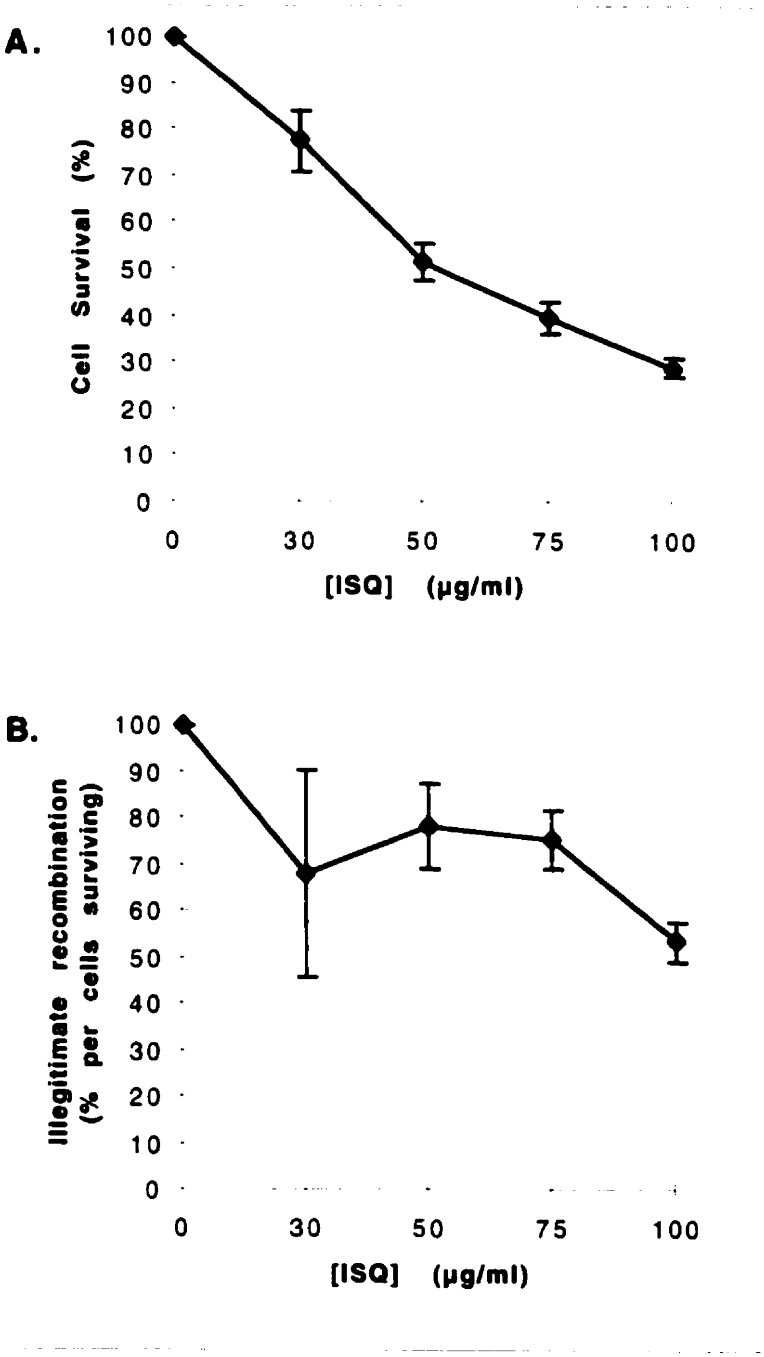
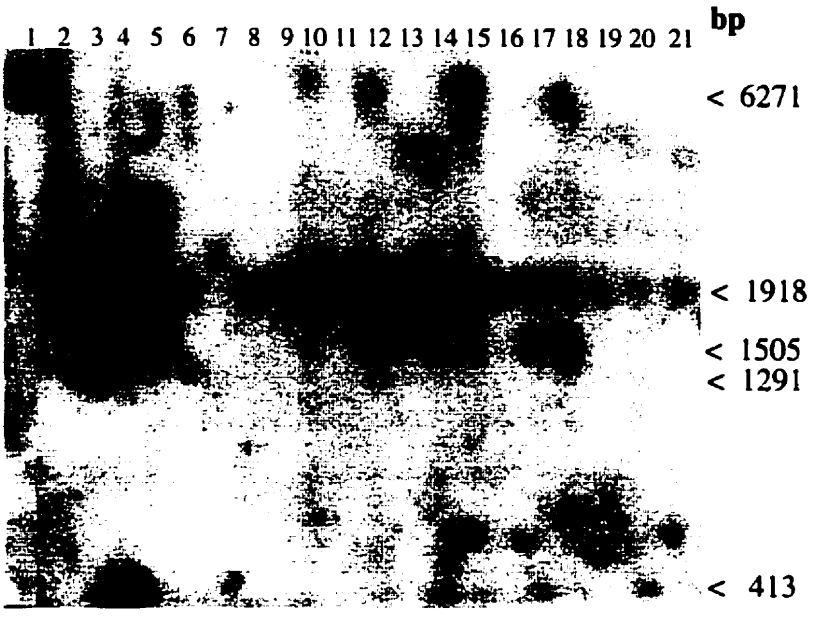

A.
Cell Survival (%)
100
90
80
70
60
50
40
30
20
10
0
0
30
50
75
100
[ISQ] (µg/ml)
B.
Illegitimate recombination (% per cells surviving)
100
90
80
70
60
50
40
30
20
10
0
0
30
50
75
100
[ISQ] (µg/ml)

Figure 3


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
bp
< 6271
< 1918
< 1505
< 1291
< 413

3.2 1,5-isoquinolinediol increases the frequency of gene targeting by homologous recombination in mouse fibroblasts (article submitted to Gene Therapy)

In the section above we have demonstrated that, conversely to the previously reported results, treatment of cells with a potent specific inhibitor of PARP, such as ISQ, leads to increased extrachromosomal homologous recombination.

In the present section we extend our study of the effects of ISQ on homologous recombination to gene targeting. Using mutated HSV *tk* gene stably integrated into Ltk- cells as a genetic target, we were able to demonstrate that PARP inhibition with ISQ leads to a significant increase in the frequency of gene targeting.

1,5-isoquinolinediol increases the frequency of gene targeting by homologous recombination in mouse fibroblasts

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Summary

Gene targeting is a technique which allows the introduction of predefined alterations into chromosomal DNA. It involves a homologous recombination reaction between the targeted genomic sequence and an exogenous targeting vector. In theory, gene targeting constitutes the ideal method of gene therapy for single gene disorders. In practice, gene targeting remains extremely inefficient for at least two reasons : very low frequency of homologous recombination in mammalian cells and high proficiency of the mammalian cells to randomly integrate the targeting vector by illegitimate recombination. One known method to improve the efficiency of gene targeting is inhibition of poly(ADP-ribose) polymerase (PARP). It has been shown that PARP inhibitors, such as 3-methoxybenzamide, could lower illegitimate recombination, thus increasing the ratio of gene targeting to random integration. However, the above inhibitors were reported to decrease the absolute frequency of gene targeting. Here we show that treatment of mouse Ltk- cells with 1,5-isoquinolinediol, a new generation PARP inhibitor, leads to an increase up to 8-fold in the absolute frequency of gene targeting of the stably integrated HSV*tk* gene.

Key words : gene targeting, PARP inhibition, 1,5-isoquinolinediol

Introduction

Gene targeting, or a homologous recombination reaction between a transfected DNA sequence and a homologous genomic target sequence, is an experimental application of a cell's ability to perform homologous recombination in order to bring about a desired alteration in an endogenous sequence. This technique can be exploited for the precise disruption of the targeted genes (known as genetic knock-outs) or the correction of genetic mutations. The former application is already widely used in research to study gene structure

and function, embryonic development, as well as to generate animal models of human diseases; the latter will undoubtedly become one day the basis for gene therapy of genetic disorders and cancer.

Although appreciable progress has been made over the years in developing gene therapy procedures based on gene supplementation strategies, the latter approach presents a number of serious limitations. [1] First of all, only recessive genetic disorders are amenable to gene therapy by gene supplementation. Secondly, gene supplementation does not allow the precise control of the expression of the supplemented gene. The latter might be of importance in situations where the expression of the gene must be tightly regulated. For example, in the case of β-thalassemia, expression of the supplemented β-globin gene must be coordinated with that of the endogenous α-globin gene. Thirdly, since gene supplementation relies on the transfection of the entire cDNA, the size of the defective gene can become a limiting factor, for example, as is the case for the dystrophine gene. Finally, there exists the risk of insertional mutagenesis if the supplemented gene randomly integrates into the genome of the transfected cell.

All of the above problems associated with the gene supplementation approach can be circumvented by gene targeting of the defective gene.[2] Since gene targeting involves *in situ* repair of the endogenous gene, both recessive and dominant disorders can be treated and gene expression remains under endogenous control. The size of the deleterious gene is not a limiting factor, since only the portion surrounding the mutation is required for gene targeting. And, since the targeting vector does not have to be maintained in the transfected cells, the risk of insertional mutagenesis is reduced as well.

The main obstacle in the application of gene targeting to gene therapy is the low frequency of homologous recombination in mammalian cells. The ability to specifically increase the frequency of homologous recombination in mammalian cells could make gene targeting applicable to gene therapy.

Previously, we were able to show that treatment of cells with 100 µg/mL (0.622 mM) 1,5-isoquinolinediol (ISQ) resulted in an average 4.6-fold increase in the frequency of extrachromosomal homologous recombination in mouse Ltk- fibroblasts.[3]

ISQ is a very potent inhibitor of poly(ADP-ribose) polymerase, an abundant nuclear protein found in all higher eukaryotes.[4] PARP has been shown to recognize, bind to, and become activated by DNA breaks. Once activated, the enzyme catalyzes the synthesis of polyADP-ribose from NAD+ on itself and on a number of protein substrates. Although the biological role of PARP is as yet uncertain, it has been suggested that one of PARP's overall functions might be the preservation of genomic integrity by decreasing spontaneous homologous recombination at sites of DNA breaks.[5-7]

PARP inhibition is known to decrease the frequency of random integration of exogenous DNA into the mammalian genome.[8, 9] PARP inhibition was also reported to increase the frequency of sister chromatid exchange (SCE) in mammalian cells, a process that proceeds via homologous recombination.[10] Finally, it has been shown that PARP inhibition increased the frequency of intrachromosomal homologous recombination in mouse Ltk- fibroblasts.[11] Recent studies with PARP knock-out mice have confirmed the anti-recombinogenic function of PARP at DNA strand breaks.[12, 13]

The above results suggest that PARP inhibition generally stimulates homologous recombination processes. However, early studies of the effect of PARP inhibition on the rate of extrachromosomal homologous recombination in mouse Ltk- fibroblasts showed that the rate of the latter remained unchanged in response to PARP inhibition with 3-methoxybenzamide (3-MB).[9] Similarly, it has been reported that treatment with 3-MB decreased the frequency of gene targeting 2 to 13-fold in Chinese hamster ovary cells.[14]

We were able to demonstrate that by using an inhibitor of PARP more specific than benzamide derivatives, one could substantially increase the rate of extrachromosomal homologous recombination in murine cells. We thus investigated the effect of ISQ on gene targeting.

Here we report that treatment of the Ltk- cells with 100 µg/mL (0.622 mM) ISQ results in an average 4.3-fold increase in the absolute frequency of gene targeting of a stably integrated Herpes Simplex Virus thymidine kinase (HSV *tk*) gene.

Materials and Methods

Cell culture

Cells were grown and maintained in DMEM (Bio Media, Canada) supplemented with 10% FBS (GibcoBRL, NY USA) and Antibiotic-Antimyotic (GibcoBRL) at 37°C in 5% CO_2/ 95% air. Continuous G418 selection (300 µg/mL) was applied to the target cell lines which were stably transfected with *neo*-containing pAd-Thx76Δ vector.

Plasmid DNA

Plasmids pAd-Thx76Δ and Thx26 are described elsewhere[3] (also cf. Fig. 1A). Briefly, pAd-Thx76Δ contains the HSV *tk* gene which has been inactivated by a deletion of the sequence 3' to nucleotide 1611 according to the nomenclature of Wagner et al.[15] It also contains a CMV-*neo* cassette inserted 5' to the mutated HSV *tk*.

The Thx26 vector contains an HSV *tk* gene with an 8 bp *Xho*I linker insertion mutation at position 738 of the *tk* coding sequence.

Transfections and selection

For the generation of target cell lines, approximately $3x10^5$ Ltk- cells were transfected with the pAd-Thx76Δ vector linearized at the *Cla*I recognition site, using Superfect poly-cationic reagent from QIAGEN Canada, according to the specifications provided by the manufacturer. Twenty-four hours after transfection, cells were subjected to selection with 300 µg/mL G418 for 12 days.

For gene targeting experiments, a total of $12x10^6$ target cells were plated on 100-mm dishes ($3x10^6$ cells per dish) (Costar, MA USA) and allowed to divide once. The cells were transfected with Thx26 linearized with *Xho*I (10 µg of plasmid per dish), again using

Superfect poly-cationic reagent (QIAGEN Canada). At 24 hours post-transfection, the cells were trypsinized and transferred into sixteen 80 cm^2 TC flasks (NUNC, IL USA) containing complete DMEM supplemented with hypoxanthine-aminopterin-thymidine (HAT) (GibcoBRL). The cells were then incubated in a CO_2 incubator for 11 days with the selection medium being changed every 4 days. Surviving cells were either isolated for further analyses, or fixed with 10% formaldehyde phosphate buffered saline and stained with methylene blue.

PARP inhibition

The inhibitor of PARP, 1,5-isoquinolinediol (Sigma) was dissolved in DMSO at 100 mg/mL (0.622 mM). The inhibition was performed by direct addition of appropriate amounts of the above solution to the cell culture medium immediately after the completion of the transfection procedure. The PARP inhibition was maintained for 24h.

Determination of ISQ toxicity

Three hundred target cells were plated in 25 cm^2 TC flasks and, 5h later, were treated with ISQ for 24h. The cells were then washed twice with PBS and incubated in DMEM/10% FBS/Antibiotic-Antimyotic in a CO_2 incubator for 11 days. The cells were finally fixed with 10% formaldehyde phosphate buffered saline, stained with methylene blue, and colonies of at least 20 cells were counted. For every target cell line, this experiment was repeated ten times. Cell survival was calculated as the number of colonies scored in the presence of the drug divided by the number of colonies scored in the absence of the drug multiplied by 100.

Southern Analysis of the HAT^R clones

HAT^R colonies were randomly selected and expanded. The genomic DNA (gDNA) from HAT^R clones was extracted by standard methods. Ten micrograms of the gDNA were then digested with *Eco*RI, *Hind*III and *Xho*I enzymes (Pharmacia) and subjected to Southern analysis by standard methods. The *Eco*RI-*Hind*III fragment of HSV *tk* from the Thx26 plasmid labeled with ^{32}P-dCTP by a random primer oligolabeling kit

(Pharmacia), was used as the probe for the *tk*. Similarly labeled pBR322 backbone of Thx26 was used as a probe for the integrated targeting vector.

Results

Target cell lines

Ten different pAd-Thx76Δ stably transfected clones were expanded and characterized with respect to their ability to undergo gene targeting with a consistent baseline frequency of at least 10^{-6}. When electroporation was used as a means of transfection of the targeting vector, no consistent frequency of targeting was observed with any of the target cell lines assayed. Lipofection with Superfect gave reproducible baseline targeting frequencies for all of the target cell lines assayed. Three target cell lines pAd4, pAd7 and pAd9, which featured consistent gene targeting frequency in the range of 10^{-5}, were chosen for further investigation.

The effect of ISQ on cell survival and illegitimate recombination

The toxicity of ISQ on Ltk- has been previously determined by clonogenic assays.[3] To take into account the clonal variability of the pAd-Thx76Δ stable transfectants, we tested the effect of ISQ on cell survival of the selected target cell lines. We found that 24 hour exposure of the cells to 100 μg/mL ISQ resulted in 53.5%, 21% and 56.6% cell survival for pAd4, pAd7 and pAd9, respectively.

We have previously established that 100 μg/mL ISQ resulted in an average 46.6% (±4.28%) (corrected for cell survival) reduction in the rate of illegitimate recombination in Ltk- cells.[3]

The effect of ISQ on gene targeting

Three separate experiments were performed for each target cell line. The results are summarized in Table 1. In every case, treatment of the cells with 100 μg/mL ISQ increased the absolute frequency of gene targeting. The magnitude of the increase varied with the target cell line. Thus, the highest increase has been observed with pAd7 — 7.3 to

9.5-fold. Targeting of pAd9 was increased on average 2.8-fold, and targeting of pAd4 — 2-fold.

To demonstrate that HAT^R colonies arose as a result of a homologous recombination, we performed a Southern blot analysis of the genomic DNA of randomly selected HAT^R clones. The gDNA was subjected to triple digestion with *Eco*RI, *Hind*III and *Xho*I restriction enzymes, and assayed for the presence of the intact *Xho*I-resistant HSV *tk* gene (Figure 1B). The presence of a 1.94 kb *Eco*RI - *Hind*III restriction fragment resistant to *Xho*I digestion is characteristic of the reversion of the 3'-deleted *tk* of the pAd-Thx76Δ to the wild type. The diagnostic band was detected for every HAT^R clone analyzed.

No HAT^R colonies were detected in the control experiments where the parent Ltk- cells were transfected with the *Xho*I-linearized Thx26 plasmid.

In order to establish whether gene targeting was accompanied by the integration of the targeting vector, we have also performed a Southern blot analysis on the gDNA of the randomly selected HAT^R clones using the targeting vector-specific sequences as a probe. None of the HAT^R clones analyzed in this manner featured any concomitant integration of the targeting vector (data not shown).

Discussion

Most of the current strategies in gene therapy have focused on the random delivery of therapeutic genes using viral or plasmid vectors.[1] However, most of the expression vector based gene therapy protocols fail to achieve the level of transgene expression required for treating genetic diseases.[1, 2] Besides, the gene supplementation approach presents a number of limitations. With respect to the latter, direct *in situ* repair of the affected genes by targeted homologous recombination appears as the ideal solution.

Unfortunately, homologous recombination in mammalian cells is a highly inefficient process. The average frequency for gene targeting in mammalian cells is estimated at 1 for

every 10^6 transfected cells .[16,17] Another major obstacle that stands in the way of applying gene targeting to such useful ends as gene therapy is the extreme proficiency of the mammalian cells in illegitimate recombination. [18,19] The ratio of random integration to gene targeting in transfected cells is typically in the order of 1000 to 1.[20] Hence, ways must be found to selectively stimulate homologous recombination in the target cells without, however, increasing the frequency of illegitimate recombination.

One known way to lower the rate of illegitimate recombination in mammalian cells is to inhibit PARP. Thus, Waldman et al. have reported that inhibition of PARP with 3 mM 3-MB reduced the rate of illegitimate recombination 150-fold in Chinese hamster ovary cells. Unfortunately, at the same time, 3 mM 3-MB lowered the absolute frequency of gene targeting 2 to 11-fold.[14]

PARP is an abundant nuclear enzyme present in all higher eukaryotes. Although its structure and enzymatic activities have been extensively studied for more than thirty years, its biological function remains unclear.[4,7] It has been proposed that the role of PARP could be the negative regulation of recombination to ensure the stability of the genome.[5-7] This hypothesis predicts that PARP inhibition or deactivation should result in an increase of homologous recombination processes. Indeed, PARP inhibition stimulates SCE and increases the frequency of intrachromosomal homologous recombination in mouse cells.[10,11] Similarly, knock-out mice that lack PARP feature increased levels of SCE.[12] Moreover, increased recombination activity after the loss of PARP anti-recombinogenic function is demonstrated by the fact that the PARP knock-out can rescue V(D)J recombination in SCID mice lacking DNA-PK.[13]

Thus, it appears that PARP inhibition or deactivation correlates with an increase in homologous recombination. Hence, it was surprising that PARP inhibition with 3-methoxybenzamide (3-MB) was reported to lower the frequency of gene targeting.[14] The same authors have also reported that PARP inhibition with 3-MB had no effect on the rate of extrachromosomal homologous recombination.[9]

Recently, using plasmids carrying HSV *tk* genes inactivated by complementary mutations, we were able to show that treatment of the transfected Ltk- cells with another PARP inhibitor, 1,5-isoquinolinediol (ISQ), resulted in a dose-dependent increase in the rate of interplasmid homologous recombination ranging, for the highest ISQ concentration tested (0.622 mM), from 2.8 to 7.0 -fold.[3] Hence, we decided to verify whether ISQ would have a similar effect on gene targeting.

By treating cells with 0.622 mM ISQ we were able to achieve a consistent increase in gene targeting of the stably transfected HSV *tk* gene in three different target cell lines derived from mouse Ltk- cells. The magnitude of the stimulation of gene targeting varied with the cell line from an average increase of 108% to 712%. The cell line-specific differences in the effect of ISQ are probably due to the difference in chromosomal location of the target sequence. Concomitantly, 0.622 mM ISQ lowered the rate of illegitimate recombination 2-fold. Thus the increase in the gene targeting: random integration ratio observed in our study ranged from 2.2 to 16-fold.

ISQ was first identified as potent inhibitor of PARP by Banasik and co-workers during a large scale survey using an *in vitro* assay system.[21] Among the wide panoply of PARP inhibitors, isoquinoline derivatives are the most potent and the most specific.[21, 22] The most potent of the latter are 4-amino-1,8-naphalimide, 6(5H)- and 2-nitro-6(5H)-phenanthridinones and ISQ. ISQ is the least toxic of the four. The 50% inhibitory concentrations of the above compounds for PARP, 0.18 - 0.37 μM, are 30 to 100-fold lower than that of 3-methoxybenzamide and other benzamide derivatives. [22] Shah *et al* reported that ISQ was also a potent inhibitor of PARP *in vivo* and showed that it could completely abolish oxidant-induced activation of PARP in C3H10T1/2 cells.[23]

We believe that the apparently contradictory results previously reported on the effect of PARP inhibitors on different homologous recombination processes were probably due to the low specificity and potency of the inhibitors used. Benzamide and its derivatives, preferentially used in these studies have been found to have several additional side-effects

in vivo. There have been reports of their effects on nicotinamide N-methyltransferase[24], on the *de novo* synthesis of purines[25], glucose metabolism, and DNA synthesis.[26] However, as our work shows, if a highly specific and potent inhibitor of PARP is used, such as ISQ, the resulting stimulatory effect on homologous recombination is seen not only for SCE and intrachromosomal recombination, but for extrachromosmal recombination and gene targeting as well.

Given that there is no clear understanding of PARP effects on cellular processes, the explanations for the observed increase of gene targeting remain speculative. One possible explanation is that gene targeting requires a protein whose activity is repressed by PARP. It is also possible that PARP inhibition makes the free ends of the linearized targeting plasmid more readily accessible to the homologous recombination machinery. It might also be that ISQ acts on a protein or proteins other than PARP and that the former affect(s) recombination directly or indirectly. However, there have been no reports of ISQ's affecting any enzyme other than PARP. Similarly, the suggestion that the observed increase in homologous recombination might be due to ISQ's promoting DNA damage and thus stimulating recombination, although plausible, is not supported by any reports.

Yet another possible explanation involves the fact that PARP inhibition decreases the frequency of illegitimate recombination.[8, 9] If illegitimate recombination and gene targeting represent two competing pathways, then the repression of the former might result in stimulation of the latter.

We believe that our finding that ISQ can stimulate gene targeting is important. The fact that a simple addition of a chemical agent can increase the absolute frequency of gene targeting up to 8-fold, while at the same time decreasing the rate of random integration 2-fold, makes it a very attractive method for the improvement of such applications as the generation of genetic knock-outs and the *ex vivo* gene therapy. However, several issues must be addressed before ISQ can be safely used for such applications. Will ISQ have the same effect on gene targeting in different cell types and in different species? Will it have the

same effect on the targeting of endogenous chromosomal sequences? And finally, does ISQ have any adverse effect on the genetic integrity of the treated cells? If these issues can be addressed adequately, ISQ inhibition of PARP may very well become a valuable tool for genomic manipulations by gene targeting.

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Table 1 Effect of 1,5-isoquinolinediol on gene targeting of pAd-Thx76Δ- target cell lines

Target cell line	Exp	HAT^R colonies $12x10^6$ cells treated		Percent increase	Average percent increase; *P* value[2]
		No inhibitor	0.66 μM ISQ [1]		
pAd4	1	82	112	+36.9%	+108% *P* = 0.0369
	2	90	131	+45.4%	
	3	60	206	+243%	
pAd7	1	43	324	+653%	+ 712% *P* = 0.0285
	2	20	190	+852%	
	3	13	95	+633%	
pAd9	1	39	87	+122%	+176% *P* = 0.0023
	2	20	71	+253%	
	3	37	94	+153%	

[1] The numbers are adjusted for the survival rate.

[2] The statistical significance for the observed difference in gene targeting frequencies was determined by the paired t-test. The difference was considered significant for P-values lower than 0.05.

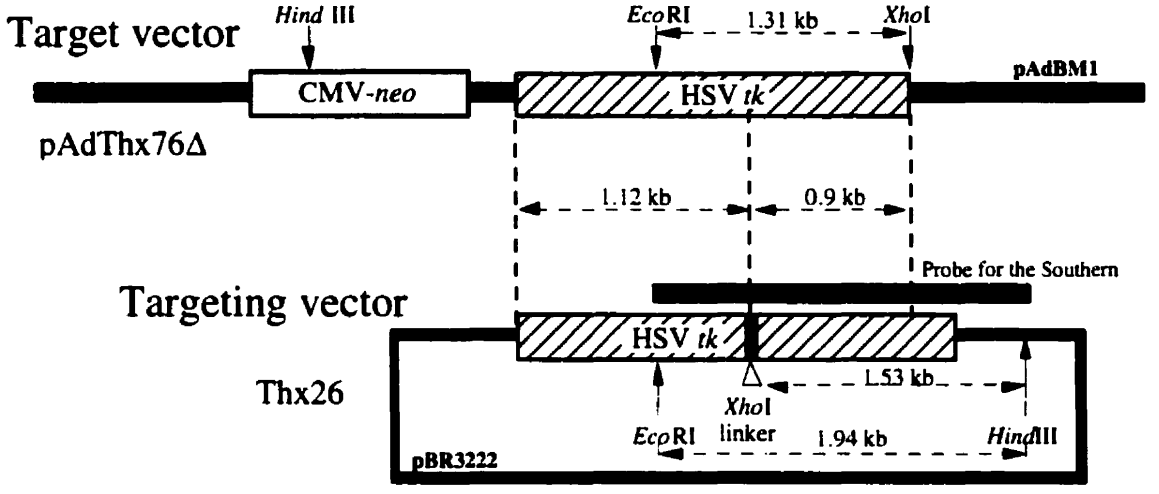
Legends

Figure 1. A. Gene targeting system. The target vector pAd-Thx76Δ contains the HSV *tk* gene deleted 3' to position 1611. The HSV *tk* gene of the targeting vector Thx26 is inactivated by the *Xho*I linker insertion at position 738. The homologous sequences between both vectors, surrounding the *Xho*I linker of the Thx26 are 1.12 kb 5' to the linker and 0.9 kb 3' to the linker. The pAd-Thx76Δ vector contains the CMV-*neo* cassette which allows the continuos selection with G418 to ensure the maintenance of the target within the genome of the target cell-lines. Recombination between the target and the targeting vector would yield functional *tk*, resulting in HAT-resistant clones. Prior to transfections the Thx26 vector was linearized at the site of its *Xho*I linker (open triangle). The DNA probe used for the Southern analysis is shown as an open rectangle with thick borders.

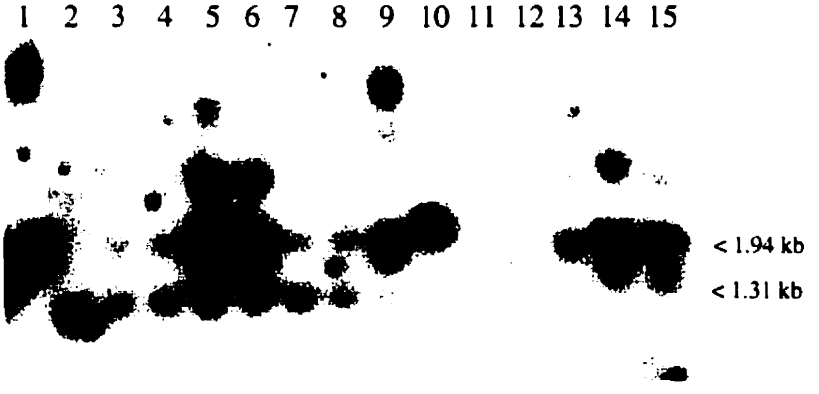
B. Representative Southern analysis of HAT-resistant colonies arising after targeting of Ltk- derived target cells with the *Xho*I-linearized Thx26 plasmid. The ^{32}P-dCTP-labeled *Eco*RI-*Hind*III fragment of HSV *tk* from Thx26 was used as a probe. Lanes 1and 2 feature 10μg of gDNA of untransfected pAd4 digested, respectively, with *Hind*III and *Eco*RI, and with *Hind*III, *Eco*RI and *Xho*I. Lanes 3 - 9: 10μg of gDNA isolated from randomly selected HAT-resistant colonies resulting from targeting pAd4 with Thx26, digested with *Hind*III, *Eco*RI and *Xho*I. Lane 10 contains 120 pg of the Thx26 plasmid digested with *Hind*III and *Eco*RI. Lanes 11 and 12 are empty. Lanes 13-15: 10μg of gDNA isolated from randomly selected HAT-resistant colonies resulting from targeting pAd7 with Thx26, digested with *Hind*III, *Eco*RI and *Xho*I. The 1940 bp band corresponds to the *Hind*III/*Eco*RI fragment of HSV *tk* which is resistant to *Xho*I digestion and can be seen for every HAT-resistant clone analyzed.

Figure 1

A.


Target vector
Hind III
EcoRI
1.31 kb
XhoI
CMV-neo
HSV tk
pAdBM1
pAdThx76Δ
1.12 kb
0.9 kb
Probe for the Southern
Targeting vector
HSV tk
Thx26
1.53 kb
XhoI
linker
EcoRI
1.94 kb
HindIII
pBR3222

B.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
< 1.94 kb
< 1.31 kb

Chapter 4: Discussion

Gene targeting *vs* gene supplementation

Most of the current strategies in gene therapy have focused on the delivery of therapeutic genes using viral or plasmid vectors, the so-called "gene supplementation" approach. However, most of the expression vector based gene therapy protocols fail to achieve the level of transgene expression required for treating genetic diseases. Besides, the gene supplementation approach presents a number of intrinsic limitations : requirement of full length cDNA for the construction of the therapeutic vector, lack of the complex transcriptional regulation of the therapeutic gene, inability to achieve long term expression with non-integrating vectors and risk of insertional mutagenesis in the case of integrating vectors.

With respect to the problems enumerated above, direct *in situ* repair of the affected genes by targeted homologous recombination appears as the ideal solution. Successful gene targeting would give rise to the permanent expression and normal regulation of corrected genes in appropriate cells or organs. It is not limited by the size of the gene to be corrected and can be used for treating dominant genetic diseases.

Although conceptually the *in situ* repair of the genetic mutations by gene targeting is fairly straightforward, the practical application of gene targeting to gene therapy, given the current status of gene targeting technology, is not feasible. There are at least two problems that must be solved to make gene therapy by gene targeting realistic : the extremely low rates of homologous recombination in mitotic cells must be overcome, and ways of specifically and reversibly inhibiting illegitimate recombination must be found.

In our studies, we have concentrated on two approaches to achieve the above goals. The first consisted in overexpressing a protein, Nud1p, which, we believe, acts early on in homologous recombination. The second involved chemical inhibition of PARP, an enzyme known to be involved in regulation of illegitimate recombination in mammalian cells.

Overexpression of recombinational proteins

Homologous recombination is not at its highest in mammalian mitotic cells. For example, homologous recombination is 1500-fold less frequent in mitotic cells than in spermatids, meiotic cells (Murti et al., 1994). Another example of the fact that higher homologous recombination rates can be achieved by virtue of a shift in enzymology of the cells is the observation that a chicken transformed B-cell lines exhibit up to two orders of magnitude greater rates of gene targeting than the non B-cell lines derived from the same animal (Buerstedde and Takeda, 1991). It is then reasonable to expect that homologous recombination could be stimulated in somatic cells by overexpression of some of the relevant genes, such as the genes of the *RAD52* epistasis group.

For instance, it has been reported that overexpression of human *RAD52* stimulates recombination between two *lacZ* direct repeats about 3-fold in monkey FSH2 cells, and confers resistance to ionizing radiation (Park, 1995). Expression of the yeast *RAD52* gene in human HT1080 cells was reported to stimulate extrachromosomal recombination and to confer resistance to ionizing radiation (Johnson et al., 1997).

It has also been reported that *RAD51* overexpression confers resistance to ionizing radiation and stimulates homologous recombination between direct repeats in CHO cells (Vispé et al., 1998).

All currently available models for homologous recombination postulate the generation of the ss DNA overhangs as early intermediates. It is presently unclear what step is rate limiting in homologous recombination. One of the possibilities is that the production of these ssDNA intermediates is the rate limiting step. These ss overhangs are produced enzymatically from DSBs. However, the nature of the nuclease(s) involved remains unknown. Endo-exonucleases present in all eukaryotes appear as reasonable candidates, given the similarities they show with bacterial recBCD.

In Chapter 2 above, the results of our study of the effects of yeast endo-exonuclease *NUD1* gene overexpression on homologous recombination in mammalian cells are presented.

We have found that *NUD1* overexpression slightly stimulates extrachromosomal recombination in mouse cells and confers some resistance to ionizing radiation and cis-platin, but not UV or alkylating agents, in human HeLa cells. These results are interesting, for they provide additional evidence that endo-exonucleases might be involved in the initiation of homologous recombination in mammalian cells.

The observed effects of *NUD1* overexpression on extrachromosomal recombination and cell resistance to DNA DSB-inducing agents, although consistent and significant, were nevertheless small. If the effect of *NUD1* expression on gene targeting is of similar magnitude, it will not be of any practical usefulness for gene targeting applications. It should be noted however, that the effects of *NUD1* in our experiments were observed against a background of extremely efficient DSB repair by NHEJ prevailing in mammals and of high proficiency of mammalian cells to catalyze extrachromosomal homologous recombination. It is reasonable to expect that the stimulatory effect of *NUD1* on gene targeting would be greater than it was for DSB repair or on the extrachromosmal recombination. Secondly, it is highly probable that Nud1p needs to interact with other proteins to function efficiently. For instance, *NUD1* expression is tightly regulated by Rad52. Also Nud1p seems to interact with HO-endonuclease in the case of mating-type switching in yeast. Consequently, it is possible that the effect of *NUD1* expression on homologous recombination can be increased if combined with the concomitant expression of Nud1p partner enzymes. Evidently, work must be done to identify those. Finally, *NUD1* is an yeast gene, and it is not known if its gene product is sufficiently similar to its mammalian homologue to permit its complete functional substitution. It is possible that the overexpression of the mammalian endo-exonuclease would result in greater stimulation of homologous recombination. The corresponding mammalian gene has remained elusive so far, but the attempts at its cloning are presently pursued.

Inhibition of illegitimate recombination

The controversy of whether illegitimate and homologous recombination represent two competing or independent pathways has not been definitely settled. What ever is the case, inhibition of illegitimate recombination appears as a promising approach for improving gene targeting in mammalian cells. If the two pathways compete for incoming targeting molecules, then the mere inhibition of illegitimate recombination should stimulate gene targeting. If the two pathways are independent, then inhibition of illegitimate recombination can be used in combination with some other procedure known to stimulate recombination (e.g. introduction of DSB within the targeted homology) in order to achieve an increase in gene targeting.

It is generally believed that illegitimate recombination occurs via the NHEJ repair pathway catalyzed by Ku80 protein of the DNA-PK. For instance, it has been observed that Ku80-deficient CHO cells have reduced stable transfection frequencies under some conditions (Liang et al., 1996). Hence, inhibition of Ku80 could be in theory a potential strategy for lowering the rate of illegitimate recombination.

Rad50, *XRS2* and *MRE11* gene products are also involved in NHEJ. Deletion of *Rad50*, or *XRS2* or *MRE11* in yeast reduces illegitimate recombination by up to 70-fold (Moore and Haber, 1996). So, at least in theory the proteins encoded by these gene could be potential targets for inhibition of random integration.

Modification of DNA ends in the transfected molecules might also be a way to reduce illegitimate recombination. Dideoxynucleotides added to the 3' ends of a linear plasmid transfected into monkey COS-1 cells increase the intramolecular homologous recombination/ random integration ratio about 5-fold (Chang and Wilson, 1987).

To date the only method known to effectively inhibit illegitimate recombination is the inhibition of the poly(ADP-ribose) polymerase (PARP). The biological function of PARP remains so far unclear, but it appears that the enzyme has anti-recombinogenic properties. PARP inhibition has been known for years to decrease illegitimate recombination in

mammalian cells. The idea to use it in gene targeting in order to achieve better homologous recombination : NHR ratios has been formulated early on.

In a previously reported attempt to improve gene targeting by inhibiting PARP, it has been found that PARP inhibition with 3-methoxybenzamide (3-MB) reduced both illegitimate recombination and gene targeting in CHO cells. But since the latter was reduced less than the former, a 100-fold increase in the homologous recombination : NHR was reported (Waldman and Waldman, 1991). The same group previously reported that 3-MB treatment did not affect extrachromosomal homologous recombination in mouse Ltk- cells (Waldman et al., 1996).

In Chapter 3 our own work with ISQ inhibition of PARP is presented. It shows that the inhibition of PARP by this competitive inhibitor, not only improves the ratios of homologous recombination : NHR by selectively inhibiting illegitimate recombination, but improves the absolute frequency of extrachromosomal homologous recombination, as well as that of gene targeting in mouse Ltk- cells.

Although the mechanism accounting for these observations remains unclear, the results in themselves are of great practical interest for gene targeting applications. They also appear to solve the conflict in the previously reported results that PARP inhibition stimulates chromosomal but not extrachromosomal homologous recombination or gene targeting. We believe that these apparently contradictory results were probably due to the low specificity and potency of the inhibitors used. Benzamide and its derivatives, preferentially used in these studies have been found to have several additional side-effects *in vivo*. However, as our work shows, if a highly specific and potent inhibitor of PARP, such as ISQ, is used, the resulting stimulatory effect on homologous recombination is seen not only for the SCE and the intrachromosomal recombination, but for the extrachromosmal recombination and gene targeting as well.

Future work

With respect to the study of the role of Nud1p in homologous recombination the most obvious and pressing step is the isolation of the corresponding mammalian gene, followed by thorough biochemical characterization of the mammalian recombinant protein.

As mentioned above, the isolation of proteins interacting with Nud1p and the unraveling of the mechanisms of its regulation in yeast, and eventually in mammals, is important as well.

Generation of cell lines stably transfected with *NUD1* under the control of a "tight" inducible promoter should be considered, if a more precise evaluation of the effect of the yeast enzyme on DSB repair and homologous recombination in mammalian cells is to be done. These cell lines could also be used in experiments targeted on isolation and characterization of early intermediates of homologous recombination. The latter can provide some additional insight about the mechanism of Nud1p action in recombination.

As far as future work on PARP inhibition is concerned, it would be interesting to see whether disruption of PARP's function by means other than chemical inhibition (e.g. utilization of PARP gene knock-outs, antisense approach) would have similar effects on extrachromosomal recombination and gene targeting as we observe with ISQ.

An alternative explanation of the effect of ISQ observed in our experiments was proposed by one of the reviewers of this thesis. In our experiments, homologous recombination events were scored as Ltk- cells reverting to TK-positive phenotype. The calculated frequency of homologous recombination (referred to as "absolute frequency of homologous recombination/ gene targeting") of the ISQ-treated cells takes into account the average frequency of cell death associated with ISQ treatment. Hence, the reviewer argued that the increase in the number of TK-positive colonies per number of surviving cells might be due to an increased killing of TK-negative cells by ISQ. In that case ISQ does not increase the frequency of homologous recombination, but simply enriches the population of surviving

cells for TK-positive clones. However, as our experiments show, ISQ-related cytotoxicity in TK-negative Ltk- cells is of the magnitude comparable to that reported for different TK-positive cell lines. Thus, ISQ does not appear to be more toxic to TK-negative cells than to TK-positive cells. We agree, nevertheless, that in order to settle this controversy definitively, the relative sensitivity of Ltk- cells and of the Ltk- cells that have reverted to TK-positive phenotype, as a result of integration of a plasmid containing wild type HSV *tk* , or as a result of gene targeting, must be determined.

Although ISQ is a much more specific and potent inhibitor of PARP than benzamide derivatives, it remains nevertheless relatively toxic. Hence, identification and characterization of less toxic competitive inhibitors of PARP is needed.

Though utilization of ISQ appears as a very promising method for improvement of gene targeting techniques, several issues must be addressed before ISQ can be safely used for such applications. Will ISQ have the same effect on gene targeting in different cell types and in different species? For example, can ISQ improve gene targeting in mouse ES cells? Will it have the same effect on the targeting of endogenous chromosomal sequences? And finally, does ISQ have any adverse effect on the genetic integrity of the treated cells? If these issues can be addressed adequately, ISQ inhibition of PARP may very well become a valuable tool for genomic manipulations by gene targeting.

List of Frequently Used Abbreviations

3-AB	—	3-aminobenzamide
3-MB	—	3-methoxybenzamide
APRT	—	adenine phosphoribosyltransferase
bp	—	base pair
CHO	—	Chinese hamster ovary
DHFR	—	dihydrofolate reductase
ds	—	double strand(ed)
DSB	—	double strand break
DSBR	—	double strand break repair
DSG	—	double strand gap
EE	—	endo-exonuclease
EMS	—	ethyl methanesulfonate
Gy	—	gray
HAT	—	hypoxanthine-amethopterin-thymine
hDNA	—	heterologous DNA
HPRT	—	hypoxanthine-guanine phosphoribosyltransferase
HSV	—	Herpes Simplex Virus
ISQ	—	1,5-isoquinolinediol
kb	—	kilobase
MMS	—	methyl methanesulfonate

NAD+	—	nicotinamide adenine dinucleotide
NER	—	nucleotide excision repair
NHEJ	—	non-homologous end joining
ORF	—	open reading frame
pADPR	—	polyADP-ribose
PARP	—	poly(ADP-ribose)polymerase
PK	—	protein kinase
RPA	—	replication particle A
SCE	—	sister chromatid exchange
SCID	—	severe combined immunodeficiency
ss	—	single strand(ed)
SSA	—	single strand annealing
TK	—	thymidine kinase