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Reverse genetic studies of homologous DNA recombination using the chicken B-lymphocyte line, DT40

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DT40 is an avian leucosis virus-transformed chicken B-lymphocyte line which exhibits high ratios of targeted to random integration of transfected DNA constructs. This efficient targeted integration may be related to the ongoing diversification of the variable segment of the immunoglobulin gene through homologous DNA recombination-controlled gene conversion. DT40s are a convenient model system for making gene-targeted mutants. Another advantage is the relative tractability of these cells, which makes it possible to disrupt multiple genes in a single cell and to generate conditionally gene-targeted mutants including temperature-sensitive mutants. There are strong phenotypic similarities between murine and DT40 mutants of various genes involved in DNA recombination. These similarities confirm that the DT40 cell line is a reasonable model for the analysis of vertebrate DNA recombination, despite obvious concerns associated with the use of a transformed cell line, which may have certain cell-line-specific characteristics. Here we describe our studies of homologous DNA recombination in vertebrate somatic cells using reverse genetics in DT40 cells.

Keywords: homologous recombination; DT40; reverse genetic study, double-strand break repair

1. TARGETED INTEGRATION OF DT40 CELLS

Modification of genetic loci by homologous recombination (HR) is a powerful way to study gene function and regulation. Many gene-targeting experiments have been performed in murine embryonic stem (ES) cells as well as mammalian somatic cell lines. However, this approach has been limited by the low efficiencies (10^{-2} to 10^{-5}) with which mammalian cells integrate exogenous DNA into their chromosomes through HR. Most transfected DNA integrates into the genome at random chromosomal positions in not only mammalian cells but also in other higher eukaryotic cells, including insect and plant cells. Unique exceptions to this rule among the higher eukaryotic cells are chicken B-lymphocyte lines, where targeted integration occurs at frequencies similar to those of random integration (Buerstedde & Takeda 1991). These targeting efficiencies are orders of magnitude higher than those observed in mammalian cells. The expression state of the targeted loci has little, if any, effect on the frequency of HR in DT40 cells.

The molecular basis for the high targeting efficiencies of chicken B-lymphocyte lines is not clear. High targeting efficiencies were observed in three chicken B-lymphocyte lines examined, including the two avian leucosis virus (ALV)-transformed cell lines DT40 and RP9 (Buerstedde *et al*. 1990) and a *v-rel*-transformed cell line 27L2, while no targeting events were detected in the chicken non-B-lymphocyte lines used as controls (Buerstedde & Takeda 1991). These observations support the notion that efficient HR is an intrinsic characteristic of primary chicken B lymphocytes. B-lymphocyte precursors in the bursa of Fabricius and in the splenic germinal centres diversify the variable segment of their immunoglobulin (Ig) light chain locus by intrachromosomal HR called Ig gene conversion (Reynaud *et al*. 1985, 1987). There are a number of V region pseudogenes at the upstream area of a V(D)J segment in the chicken Ig loci. In Ig gene conversion, nucleotide sequence blocks derived from V region pseudogenes are transferred into the functional rearranged V gene. The Ig gene conversion process exhibits an unusual characteristic in the HR reactions in DT40 cells. Although the presence of mismatches between the homologous substrate DNAs strongly suppresses HR reactions (De Wind *et al*. 1999), divergence of sequences between the V region pseudogenes and the rearranged V gene does not appear to interfere with intragenic HR. DT40 cells also carry out Ig gene conversion. The increased ratios of gene targeting in DT40 cells may be related to this Ig gene conversion activity, since both processes are mediated by HR and are observed only in chicken B lymphocytes but not in chicken non-B cells or in any mammalian cell lines. Thus, both processes appear to share the same enzymatic activities.

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Besides efficient gene targeting, another advantage of DT40 cells is their relative tractability. The phenotype, as well as the karyotype of wild-type DT40 cells, is highly stable. The DT40 karyotype comprises a modal chromosome number of 80, which comprises 11 autosomal macrochromosomes, the ZW sex chromosomes and 67 microchromosomes (Sonoda *et al*. 1998). The karyotype does not show obvious abnormalities except for a trisomy of chromosome two and one additional microchromosome, both of which were consistently observed in DT40 cells. Thus, any abnormal phenotype of gene-targeted clones derived from DT40 cells is attributable solely to the mutation of the genes rather than to clonal variation of the parental DT40 cells. Furthermore, the invariable character of DT40 cells during extended periods of *in vitro* cell culture allows us to perform sequential gene targeting of up to three genes in a single cell employing seven different selection marker genes. Since some DNA repair pathways are complementary to each other, cells deficient in two such repair pathways often exhibit an extremely severe phenotype when compared with cells deficient in either pathway alone. The availability of seven different selection markers in DT40 cells, as well as the relatively invariant character of wild-type DT40 cells, allows us to investigate the distinct and overlapping roles of independent repair pathways.

2. CONDITIONAL GENETIC MUTATIONS IN DT40 CELLS

Since a large number of DNA lesions are caused by endogenous sources, such as radicals generated during metabolic processes, defects in multiple DNA repair pathways may result in the accumulation of DNA lesions during the cell cycle and eventually cause cell death. Thus, the disruption of multiple DNA repair pathways often must be done conditionally. To date, three different methods have been developed to disrupt essential genes conditionally in DT40 cells. We used a tetracycline (tet) repressible promoter to express the human *RAD51* cDNA in DT40 cells and subsequently disrupted the endogenous *RAD51* alleles (Sonoda *et al*. 1998). A problem with the tet system is that leaky expression of the transgene cannot be fully excluded.

One way to overcome this disadvantage has been to employ a chimeric Cre recombinase (Fukagawa & Brown 1997). The Cre recombinase recognizes *loxP* sequences, and deletes or inverts sequences between two *loxP* sites depending on the relative orientation of the two *loxP* sequences. The chimeric Cre recombinase is flanked on both sides by a mutated hormone-binding domain of the murine oestrogen receptor (Zhang *et al*. 1998), which no longer binds oestrogen but does bind the antagonist 4-hydroxytamoxifen (OH-TAM). In the absence of OH-TAM, the chimeric Cre recombinase is retained in the cytoplasm by associating with the heat-shock protein Hsp90. In the presence of OH-TAM, the chimeric Cre recombinase is translocated into the nucleus where it recognizes *loxP* sites and recombines the DNA. Cre-mediated recombination works efficiently in DT40 cells. While this system allows us to completely inhibit the expression of a gene of interest, Cre-mediated recombination does not occur in a synchronous manner in a population of cells, as does repression by the tet repressible promoter.

T. Fukagawa (Mishima, Japan) has developed a third method to disrupt conditionally essential genes in DT40 cells, i.e. the generation of temperature-sensitive (ts) mutants. Since the physiological temperature of chickens is higher than that of mammals, ts mutant clones can be generated more easily with DT40 cells than with mammalian cells. DT40 cells can be maintained, without compromising their viability, at temperatures from 34 °C to as high as 42.5 °C. Ts mutant cDNAs of a gene of interest can be designed based on information from yeast ts mutants, if the base sequences of ts mutations of the homologue yeast gene are available. Following the standard protocol to generate ts mutant cells, each mutated cDNA is introduced by targeted integration into the intact endogenous locus of the gene in heterozygous mutant (+/−) DT40 cells. The resulting cells should be homozygous mutant (−/−) cells and express only the mutated protein. The temperature-dependent character of each clone can be evaluated by comparing their phenotype (e.g. viability) between 34 °C and 42.5 °C. However, ts mutant cells would allow us to examine precisely the role of the ts mutant protein by only transiently inactivating the protein at a particular phase of the cell cycle in a synchronized population.

3. THE REPAIR SYSTEMS WHICH ACT ON DNA DAMAGE

A wide range of potential insults to the genomic DNA is caused not only by the environment, such as ionizing radiation, but also by cellular activities *per se*. Estimates of the number of lesions produced daily per human genome range from rather few to several thousand, depending on the lesion and the detection technology (Kunkel 1999; Lindahl 1993). Uninduced damage comes in many forms and is efficiently repaired by a variety of repair processes. If damage is not repaired before the cell progresses to the next stage of the cell cycle, the nature of the damage may alter, resulting in the formation of the secondary lesions. For example, if a G1 cell carrying single-strand breaks in its genomic DNA progresses through S phase, the single strand lesions will be converted to secondary lesions, i.e. double-strand breaks (DSBs) in sister chromatids (reviewed in Haber 1999). Similarly, some types of covalently modified base residues are known to arrest DNA replication, causing a daughter strand gap that encompasses the damage. In addition, bacterial studies have indicated that stalled replication folks are actively converted to DSBs as part of the replication fork restart process (Kogoma 1997) and although not yet demonstrated, it seems possible that a similar mechanism of replication restart might occur in higher eukaryotes (reviewed in Flores-Rozas & Kolodner 2000). Thus, DNA replication at primary lesions in the template strand could result in more severe secondary DNA lesions such as gaps and chromatid breaks.

A chromosomal break is a lethal event, if left unrepaired. Two major repair pathways exist to deal with DSBs in metazoans: non-homologous end-joining (NHEJ) and HR (reviewed in Kanaar *et al*. 1998; Paques & Haber 1999). NHEJ repairs adjacent broken DNA ends with little

or no requirement for extensive sequence homology, while the more accurate HR requires an intact homologous sequence (in a homologous chromosome or a sister chromatid) to effect repair. Our molecular knowledge of the eukaryotic systems of HR-mediated repair dates from the definition of yeast mutants that are hypersensitive to DSBs induced by ionizing radiation (IR). Genetic studies revealed that some of the radiosensitive mutants are also defective in HR during meiosis and belong to the same epistasis group, called the *RAD52* epistasis group. Key proteins in the *RAD52* epistasis group are *RAD51*, *RAD52* and *RAD54*. Subsequent work defined the metazoan counterparts of the yeast HR genes and showed that these proteins are required for DSB repair following IR (reviewed in Shinohara & Ogawa 1995). In contrast, our understanding of NHEJ began with the characterization of certain radiosensitive mammalian mutants, which proved to be defective in NHEJ genes rather than in those responsible for HR (reviewed in Jeggo 1998; Lieber 1999; Smith & Jackson 1999). Genes involved in end-joining include *LIGASE IV*, *XRCC4*, *KU70*, *KU80* and *DNA-PKcs*. The yeast homologues of mammalian NHEJ genes were subsequently defined and shown to act as a back-up for HR in DSB repair. These observations indicate that both the NHEJ and HR pathways are shared by single-celled and multicellular eukaryotes as DSB repair mechanisms.

4. HOMOLOGOUS DNA RECOMBINATION IS ESSENTIAL FOR THE VIABILITY OF VERTEBRATE CELLS

HR-deficient yeast cells can proliferate, though murine cells deficient in Rad51 or in Mre11 are not viable. To investigate the essential roles of Rad51 and Mre11 in vertebrate cells, we generated conditionally Rad51- and Mre11-deficient cells from DT40 cells. The depletion of Rad51 or Mre11 caused both the appearance of randomly distributed chromosomal break ups to a few breaks per mitotic cell and subsequent cell death (Sonoda *et al*. 1998; Yamaguchi-Iwai *et al*. 1999). Furthermore, chromosomal breaks also occur in cells deficient in other genes of the *RAD52* epistasis group, including *RAD51B* and *RAD54* in DT40 cells (Takata *et al*. 2000, 1998) and *XRCC2* and *XRCC3* in Chinese hamster cell lines (Liu *et al*. 1998; reviewed in Thompson & Schild 1999). These observations indicate that a defect in HR-mediated DSB repair accounts for the appearance of chromosomal breaks during the cell cycle. Thus, DSBs may occur frequently during the cell cycle in vertebrate cells. Conceivably, HR has to play a more important role in maintaining chromosomal DNA in vertebrate cells than HR in yeast, probably due to the several hundredfold difference in genome size between vertebrates and lower eukaryotes, to which we attribute the lethality of HR defects in vertebrate cells.

It is likely that HR-mediated DNA repair occurs during DNA replication in vertebrate cells. This idea is supported by the presence of Rad51 foci in the S phase (Haaf *et al*. 1995; Tashiro *et al*. 1996), which may reflect an active, polymeric form of Rad51 (Raderschall *et al*. 1999), the structural and functional homologue of *Escherichia coli* RecA. Furthermore, some types of DNA lesions on a template strand are converted to chromatid breaks and daughter strand gaps by DNA replication. These secondary lesions could stimulate HR with the other intact chromatids and could be then repaired by gene conversion using homologous sequences from the intact sister chromatid. Such gene conversion events might be associated with crossover of sister chromatids, whereas gene conversion events associated with crossover occur frequently during meiosis but only occasionally during mitosis in yeast. To assess the presence of HR between sister chromatids, we evaluated the involvement of HR in sister chromatid exchange (SCE) by measuring the level of SCE in HR-deficient cells. SCE was known to be an S-phase-associated repair process and induced by treating cells prior to DNA replication with various environmental mutagens, including cross-linking agents and ultra violet (Carrano *et al*. 1978). Furthermore, cycling mammalian as well as chicken cells exhibit up to five spontaneous SCEs per mitosis. We showed that HR is indeed responsible for mediating both spontaneous SCE and SCE induced by a cross-linking agent, suggesting the presence of HR-mediated repair during the cell cycle (Sonoda *et al*. 1999). Since crossing over is known to be a relatively infrequent event during mitosis, these visible crossing-over events (SCEs, up to five per cell cycle; Zwanenburg *et al*. 1985), suggest that the level of HR-mediated replication-associated repair may be quite high in vertebrate cells.

5. DNA REPLICATION-ASSOCIATED HOMOLOGOUS DNA RECOMBINATION MAY BE RESPONSIBLE FOR TARGETED INTEGRATION

The ability to manipulate HR would remove a major bottleneck in various approaches of gene therapy, as well as facilitating further biological research. The recent findings linking HR to DNA replication may have important ramifications for those interested in genome manipulation by HR. Gene-targeting efficiency is highly dependent on the length of homology of the targeting sequence. Indeed, when this length of homology is increased from 6 to 12 kb, the efficiency of targeted integration increases by as much as tenfold in murine ES cells (Deng & Capecchi 1992). This observation is in marked contrast with gene targeting in yeast, where fewer than one hundred bases of homology are enough for efficient gene targeting. Thus, the mechanism of gene targeting in yeast is not necessarily shared by vertebrate cells. Assuming that HR is initiated by DNA damage such as DSBs in one of homologous DNAs, an interesting question is whether such DNA damage is at the ends of the linearized gene-targeting construct or DSBs in the genomic DNA. In yeast, homologous sequences at the end of linearized targeting construct appear to invade intact duplex DNA in the genome to initiate HR (Leung *et al*. 1997). Thus, DNA damage in a gene-targeting construct might be repaired by interacting with intact homologous sequences in the genome, resulting in the targeted integration of the selection marker gene, depending on the manner of resolution of HR intermediates (figure 1). By contrast, we have assumed that a gene-targeting construct participates in HR-mediated repair as an intact template DNA, similar to an intact sister chromatid when damaged sister chromatids are repaired by HR. This model is supported by the fact that the induction of DSBs in the genome stimulates gene targeting by more than two orders of

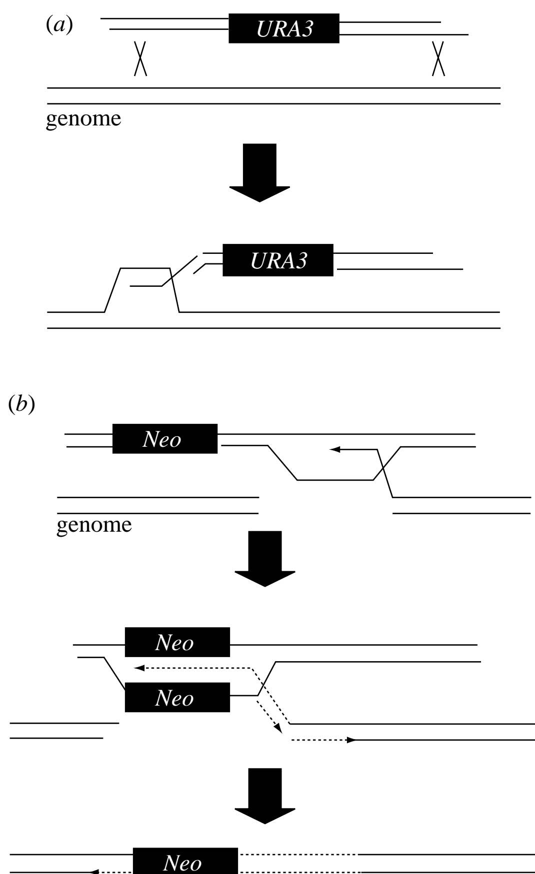

Figure 1. Models in gene targeting. (*a*) In yeast, homologous sequences at the end of linearized targeting construct appears to invade intact duplex DNA in the genome to initiate HR. (*b*) In vertebrates, a gene-targeting construct may participate in HR-mediated repair as an intact template DNA. *URA3* and *Neo* represent selection marker genes for yeast and vertebrate cells, respectively. Arrows indicate DNA synthesis associated with DNA recombination.

magnitude in rodent cells (Richardson *et al.* 1998). Furthermore, it is in agreement with the known targeting efficiencies in murine ES cells: if five HR repair events occur per mammalian genome per cell cycle (a conservative estimate based on SCE frequency), i.e. one HR site per 10^9 base pairs (bp), a linear DNA molecule of 10^4 bp should encounter an HR site with a frequency of 10^{-5} per cell. ES cells perform targeted integration with a frequency of 10^{-6} to 10^{-7} per transfected cell and with the frequency of 10^{-5} to 10^{-6} per cell that incorporated the transfected DNA in the nucleus. These calculated frequencies of cells that undergo HR-mediated repair at the genomic homologue of the targeting construct are in rough agreement with the observed targeting frequencies.

6. FUNCTIONAL INTERACTION BETWEEN HOMOLOGOUS RECOMBINATION AND NON-HOMOLOGOUS END-JOINING

Multiple pathways have evolved to deal with chemical damage to individual bases, sequence alterations during replication and DNA strand breaks. Eukaryotic cells have acquired larger genomes during their evolution and, accordingly, their DNA repair and damage-induced checkpoint regulation pathways have to play a more important role in maintaining the integrity of the genome. Furthermore, the relative role of each DNA repair pathway appears to differ between yeast and vertebrate cells. For example, in yeast, HR-mediated repair is functional at any stage of the cell cycle and genes involved in HR are induced following genotoxic treatments. On the other hand, genes involved in HR, such as *Rad51* or *Rad54*, are not expressed in resting vertebrate cells even after various genotoxic treatments (Tan *et al.* 1999). Additionally, we previously demonstrated that the HR pathway in DT40 cells does not repair DSBs in the G1–early S phase of the cell cycle but it is preferentially employed in late S–G2, indicating that HR may repair a DSB in a chromatid by using the intact sister chromatid (Takata *et al.* 1998). Moreover, it has been suggested that HR may be suppressed in G1, since *Rad51* foci are not observed in this phase of the cell cycle (Bishop *et al.* 1998). Presumably, HR-mediated repair in G1 phase requires a more intensive homology search between homologous chromosomes in vertebrate cells than in yeast, whereas the close proximity of a pair of sister chromatids may account for the efficient HR-mediated repair during late S–G2 phase. Thus NHEJ, the other DSB repair pathway, should play a major role in the G0–G1 phase in vertebrate cells. Furthermore, the HR pathway may even be suppressed in these phases in order to avoid the possible interference of HR with NHEJ, as has been suggested by the competition for a DSB end noted between Ku and Rad52 (Van Dyck *et al.* 1999).

The roles for the HR and end-joining pathways appear to differ depending on the cause of DSBs. While HR plays a major role in repairing spontaneously arising DNA lesions during DNA replication, NHEJ is essential for repairing IR-induced DNA lesions especially in the G0–G1 phases. This conclusion is supported by the following observations. Rad51-deficient cells display a few chromosomal breaks that may cause cell death without having most cells enter the next round of the cell cycle (Sonoda *et al.* 1998). In marked contrast, cell lines deficient in the end-joining pathway are capable of proliferating, indicating a minor role, if any, for the NHEJ pathway in the maintenance of chromosomal integrity. However, a role for the NHEJ pathway in some tissues during mouse development has been suggested recently by the following observations in knockout mutants. *XRCC4*- or *ligase IV*-deficiency causes embryonic lethality, and embryonic fibroblasts derived from these embryos exhibit slow growth and marked genomic instability, including chromosomal translocations. Chromosomal translocation is presumably initiated by unrepaired or misrepaired DSBs, which may occur during DNA replication or the other phases of the cell cycle. Since chromosomal translocation is maintained for extended periods, the subtle chromosomal instability of NHEJ-deficient cells can be assessed by measuring the level of accumulated chromosomal translocation events. While the NHEJ pathway plays a less important role in repairing spontaneously-arising DNA lesions than the HR pathway, NHEJ plays a more important role than HR in repairing IR-induced DSBs in adult mice. In fact, end-joining-deficient *DNA-PKcs*$^{-/-}$ mice are hypersensitive to IR at the adult stage, although HR-deficient *RAD54*$^{-/-}$ mice are not (Essers *et al.* 2000). Additionally, Rad54 deficiency

Figure 2. A model of functional interaction between DSB repair and DNA damage-induced checkpoint in vertebrate cells. The manner of DSB repair following ionizing radiation (IR) may be different at each lesion. Some lesions are recognized initially by proteins involved in DSB repair while other lesions are initially recognized by proteins involved in checkpoint regulation. Subsequently, molecules involved in DSB repair (Rad52 and Ku) may activate a checkpoint pathway while a molecule involved in checkpoint (Atm) can also facilitate DSB repair.

increases IR sensitivity only in the absence of DNA-PKcs, suggesting that HR repairs IR-induced DSBs as a back-up for end-joining at the adult stage, when most cells are in G0 or G1. In summary, these observations indicated that the relative roles for HR and NHEJ in vertebrate cells are not necessarily the same as those in yeast.

7. CELL-CYCLE CHECKPOINT REGULATION OF DNA REPAIR PATHWAYS IN VERTEBRATE CELLS

Cell-cycle checkpoint regulation also appears to play a more important role in the maintenance of chromosomal DNA in vertebrate cells than it does in yeast. Indeed, while defective checkpoint regulation does not interfere with the cell cycle in yeast, defects in Atr, a homologue of yeast Mec1, causes lethality-associated massive chromosomal breaks in murine ES cells (De Klein *et al*. 2000). Second, the human genetic disorder ataxia telangiectasia (AT), caused by mutation in the *ATM* gene, is characterized by chromosomal instability in addition to defective cell-cycle checkpoint activation (reviewed in Lavin & Shiloh 1997; Meyn 1999). Since the length of a single cell cycle in AT cells is elongated, rather than decreased, the observed chromosomal instability in AT cells is not fully explained by the lack of a damage-induced cell-cycle arrest. Thus, checkpoint regulation involving Atm may rather promote DNA repair of spontaneously occurring DNA lesions in cycling vertebrate cells. However, in yeast, there is no evidence of direct activation of DSB repair pathways by cell-cycle checkpoint. The major evidence for a DNA DSB repair defect, additional to the cell-cycle checkpoint deficiencies, caused by ATM deficiency was reviewed recently (Jeggo *et al*. 1998). This evidence consists of (i) the inability of experimentally imposed cell-cycle delay to compensate for the absence of the ATM-imposed arrest (since time for DNA repair is considered to be a major reason for checkpoint arrest), (ii) high levels of radiation-induced chromosomal damage in AT cells without passage through the cell cycle, and (iii) the ability to distinguish separate ATM domains responsible for the radiosensitivity and the cell-cycle defects. Furthermore, we recently presented genetic evidence that both ATM and HR-mediated DSB repair following IR act in the same pathway in DT40 cells (Morrison *et al*. 2000).

If cell-cycle checkpoint promotes DSB repair, how do these two pathways interact with one another? Presumably, the manner of DSB repair following IR is quite different at each lesion (figure 2); some lesions are recognized initially by proteins involved in DSB repair while other lesions are initially recognized by proteins involved in checkpoint regulation. Consequently, proteins directly involved in DSB repair may not only initiate repair but may also be able to stimulate a cell-cycle checkpoint. Similarly, proteins involved in cell-cycle checkpoints may promote DSB repair in vertebrate cells directly, although a defect in checkpoint regulation does not affect the kinetics of DSB repair in yeast. The presence of such interactions between the DSB repair and cell-cycle checkpoint pathways agrees with the physical interaction between Mre11 involved in DSB repair and Nbs1, whose defect causes defective IR-induced cell-cycle arrest in Nijmegen syndrome (Carney *et al*. 1998; Matsuura *et al*. 1998; Varon *et al*. 1998). In contrast, there is no evidence for the involvement of the Rad50–Mre11–Nbs1 complex in cell-cycle checkpoint regulation in yeast (reviewed in Haber 1998). Furthermore, Rad51-related proteins (reviewed in Thompson & Schild 1999) and Brca1/2, which are all associated with Rad51 (reviewed in Welcsh *et al*. 2000), might also form interface, through which checkpoint pathways regulate HR. These possible functional interactions between DNA repair pathways and damage-induced checkpoint regulation in vertebrate cells need to be addressed in the future.

8. CONCLUSION AND PROSPECTS

HR-mediated repair is an essential process in vertebrate cells and appears to be intimately associated with

DNA replication. While a great deal has been learned about the actual proteins carrying out homologous recombination, how the homologous recombination systems interact with those of the cell-cycle and checkpoint regulation remains to be resolved. Furthermore, the manner of these interactions in vertebrate cells is not necessarily identical to that in yeast. We have summarized the direction of our future studies.

(i) Why is Ig gene conversion indifferent to the presence of mismatches between homologous sequences? The involvement of recently identified DNA polymerases (Woodgate 1999) in Ig gene conversion should be investigated because of their indifference to mismatches between template and primer sequences.

(ii) Assuming that HR-mediated repair of replication associated DNA lesions is responsible for targeted integration in vertebrate cells, can the efficiency of gene targeting be elevated by manipulating such DNA lesions?

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