Transcription Associated Recombination in Mammalian Cells

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Stockholm University, Sweden 2010

Cover photograph Laduviken

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ISBN 978-91-7447-079-6

Printed in Sweden by Universitetsservice US-AB, Stockholm 2010
Distributor: Department of Genetics, Microbiology and Toxicology

Abstract

There is increasing evidence that the movement of the transcription machinery through DNA has profound effects on the genomic stability. One such example is a phenomenon known as Transcription Associated Recombination (TAR). Transcription enhances recombination levels to a high degree in all organisms studied, from bacteria to mammals. The underlying causes of the high recombination levels observed are unknown, as are the rationale for the rather hazardous recombination event in this context. Recombination is not a risk-free event; there is e.g. the chance of loss of heterozygozity, which may eventually lead to tumour formation. So, why is TAR so ubiquitous? This thesis deals with the factors inducing TAR, trying to elucidate the mechanisms catalyzing this event. The proteins involved in executing TAR are unknown in mammals, and one of the aims of this thesis has been to investigate the role of well-known DNA repair proteins in TAR. In order to do so, cell lines deficient in crucial DNA repair proteins were stably transfected with a novel recombination construct. Transcription can be controlled over this recombination construct, enabling the detection of transcription associated recombination. We found that TAR is dependent on replication and that inhibition of transcription elongation had no further effect on TAR levels in our system. Further, we found that TAR employs a recombination pathway mechanistically separate from the recombination pathway induced by DNA double strand breaks. This pathway is dependent on BRCA2, a protein required for homologous recombination, but independent of the RAD51 paralog XRCC2. In subsequent studies, we found that the XPD subunit of the combined transcription and repair factor TFIIH is required for TAR, but is dispensable for DNA DSB repair by HR. We went on to investigate the connection between HR repair of UV damages and transcription and found that repair of UV damages requires transcription, but not via the transcription-coupled repair pathway. In conclusion, we found that TAR operates by a recombination pathway separate from DNA double strand break induced recombination. We found a connection with stalled replication, and revealed several of the proteins required for TAR in mammals.

List of publications

I. Transcription-Associated Recombination Is Dependent on Replication in Mammalian Cells.

Gottipati P, Cassel TN, Savolainen L, Helleday T.
Mol Cell Biol. 2008 Jan;28(1):154-64

II. Transcription-Associated Recombination is independent of XRCC2 and mechanistically separate from homology-directed DNA double-strand break repair

Savolainen L, Helleday T.
Nucleic Acids Res. 2009 Feb;37(2):405-12.

III. The XPD subunit of TFIIH is required for transcription-associated but not DNA double-strand break-induced recombination in mammalian cells

Savolainen, L., Cassel, T., Helleday, T.
Submitted

IV. Transcription-associated UV-induced DNA damage triggers futile homologous recombination repair in mammalian cells

Stoimenov, I., Gottipati, P., Savolainen, L., Schultz, N., Helleday, T.
Manuscript

Contents

cont.

Introduction

Genome Integrity and Maintenance

The genome is under constant attack from DNA damaging agents. These agents occur both exogenously in our environment in the form of e.g. UV irradiation or exhausts from cars, or endogenously within the cell, for example as a bi-product of metabolism. The DNA in each cell in our bodies is estimated to get 10^4 DNA lesions each day in the form of single strand DNA breaks and spontaneous base loss (Lindahl, 1993)**.** Together with other types of DNA damage, the number can be as high as 10^5 lesions per cell per day (Sander et al., 2004). Unrepaired DNA damages can have grave consequences for the cell and thereby, for multi-cellular organisms, also for the organism as a whole. DNA damages can cause mutations, if unrepaired before replication, leading to malfunction of the cell and even cell death. It is thus essential for the cell to have effective damage repair pathways. As a consequence, a number of intricate DNA repair pathways exist to ensure the integrity of the genome, for a recent review see e.g. (Hoeijmakers, 2009). Mutations caused by damaged DNA can lead to cancer by a variety of mechanisms. They can inactivate proto-oncogenes or inactivate tumour suppressor genes, which can lead to, for instance, uncontrolled cell division. DNA damages can also cause translocations of genetic material that may alter the transcription pattern of genes and cause loss of heterozygosity (LOH). In individuals with one normal and one mutated allele, LOH can leave the individual with the mutated allele alone, which can lead to development of cancer (Barnes et al., 1993).

There are different types of DNA damage; bases can become oxidized or alkylated, bulky adduct can be formed, the DNA can become cross-linked, or single-or double strand breaks can occur. In mammals, there are four major repair pathways (figure 1); base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and recombinational repair including non-homologous end-joining (NHEJ) and homologous recombination (HR), for a review see (Hoeijmakers, 2001). NER is involved in the repair

of lesions that distort the base pairing of the DNA, such as bulky adducts. Examples of agents that may cause this type of DNA damage are polycyclic aromatic hydrocarbons (PAHs, found in for instance grilled meat and car exhausts). During the NER process, the DNA adduct and a stretch of nucleotides surrounding it is removed and the correct bases are inserted in its place by a DNA polymerase (de Boer and Hoeijmakers, 2000). A subpathway of NER, transcription-coupled repair, specifically removes lesions that prevent transcription. If the NER machinery is unable to remove a lesion before replication, the lesion can be bypassed by translesion synthesis (TLS). In TLS, low fidelity polymerases are used to bypass the damage so that replication can continue. The lesion left in the DNA can then be repaired at a later stage (Lehmann, 2006). BER is mainly involved in the repair of base damages, which can be caused by e.g. oxidative agents or spontaneous deaminations. In BER, the damaged base is removed and replaced with an undamaged base using the intact DNA strand as a template (Lindahl and Wood, 1999). There are also repair pathways that are able to directly repair certain types of DNA damages such as O^6 –methylguanine, where a methyl group has been added to a guanine. This is a lesion that can cause mismatches as it modifies the base so that it can base pair with either C or T. The protein dedicated to removing the methyl group from the guanine residue is called O^6 –methylguanine methyltransferase (MGMT). The O^6 –methylguanine lesions are repaired by transferring the methyl from guanine in DNA to a cysteine in MGMT, which is thereby inactivated. MGMT can thus only function once (Hoeijmakers, 2001). Mismatch repair (MMR) is a repair pathway that removes mispaired nucleotides. The mispairing can arise from errors during replication either by misincorporation by the DNA polymerase or from the formation of insertions or deletions that can be the result of replication slippage (Kunz et al., 2009).

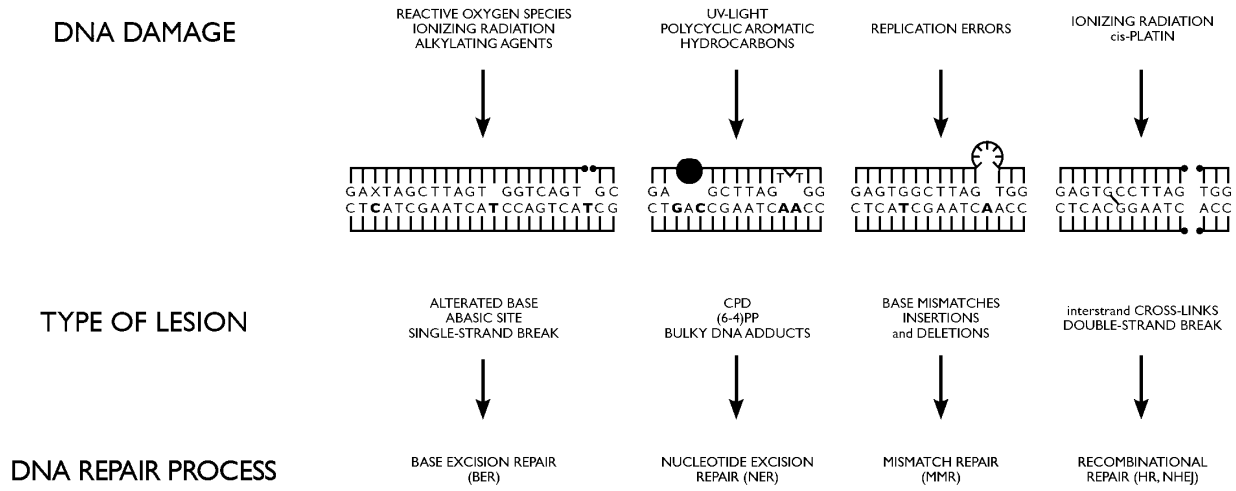


Fig. 1. Overview of DNA damages, the type of lesion they can induce and the DNA repair processes used to correct them. Adapted from (Hoeijmakers, 2001).

NHEJ and HR are responsible for the repair of DNA double strand breaks (DSBs). Two-ended DSBs of the type generated by endonucleases or ionising radiation are repaired by either NHEJ or HR; one-ended DSBs produced as a consequence of collapsed replication forks are mainly repaired by HR (Arnaudeau et al., 2001; Rothstein et al., 2000). This is not surprising, given the nature of a one-ended DSB, as there is no second DNA end for NHEJ to join. In NHEJ the two loose ends of the DNA strand are re-ligated, and in HR the broken DNA is repaired by using a homologous DNA strand, for instance the one found in the sister chromatid in replicating cells, as a template for repair (Jackson, 2001).

Mechanisms of DNA Double-Strand Break Repair

Non-Homologous End-Joining

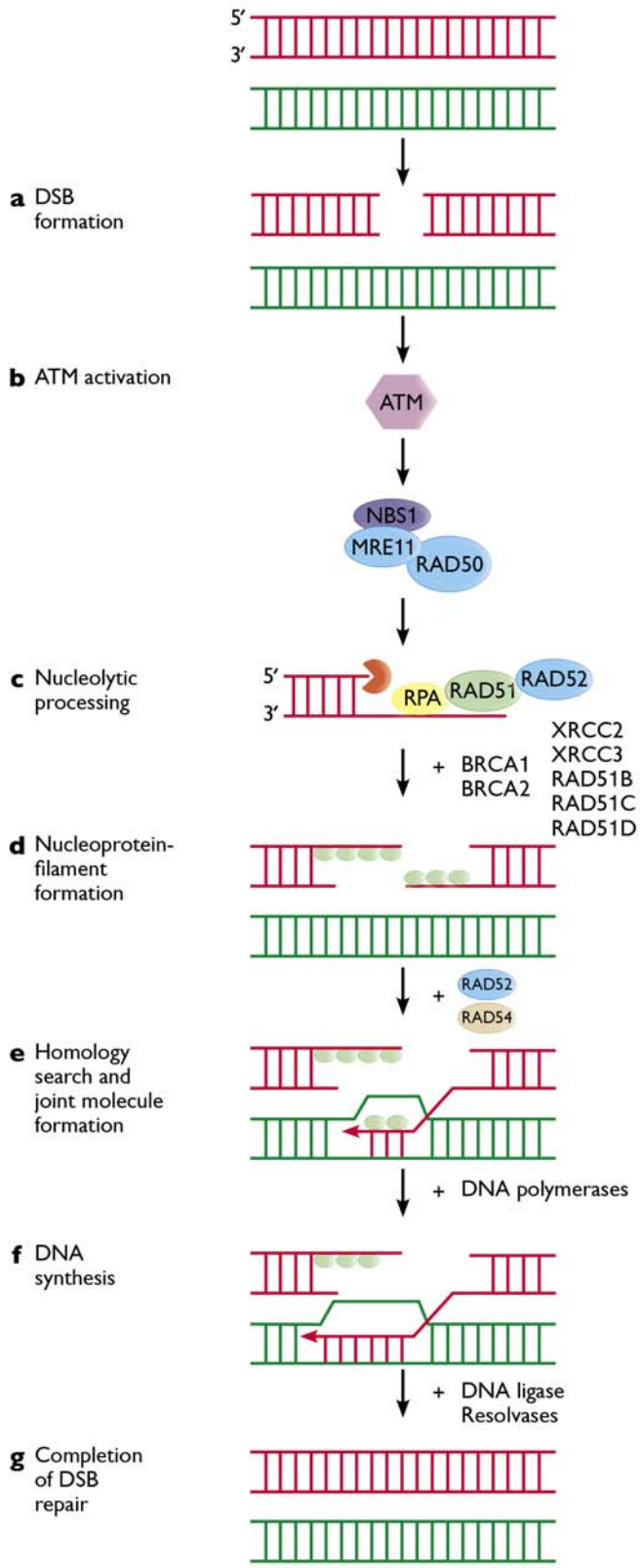
NHEJ is the faster of these two repair pathways. In NHEJ, the DNA ends are ligated without any requirement for homology, even though microhomology (1-6 bp) has been shown to facilitate the process. For ligation to take place, the DNA ends are processed, which involves the removal or, more rarely, addition of nucleotides. Consequently, this repair pathway often involves deletions (or insertions) and is therefore considered error-prone. So far, DNA-dependent protein kinase (DNA-PK) and the XRCC4, DNA-ligase IV and XLF complex have been shown to be involved in NHEJ (Lieber et al., 2003). The NHEJ process begins with end-processing by the MRE11/RAD50/NBS1 (MRN) complex (Paull and Gellert, 1998). The dsDNA ends are then bound by the Ku70-Ku80 complex, which recruits the catalytic subunit of DNA-PK, DNA-PKcs (Shrivastav et al., 2008; Yoo and Dynan, 1999). Once DNA-PKcs is bound to the DNA and the Ku complex it becomes autophosphorylated. It can then phosphorylate other targets such as Artemis which thereby gains an exonuclease activity which is thought to play a role in end-processing of the DNA ends prior to ligation by DNA-ligase IV together with XLF and XRCC4 (Ma et al., 2004)

Homology Directed Repair

Homology directed repair requires, as the name implies, longer stretches of homology than NHEJ, generally about 100 bp (Hasty et al., 1991). HR is a slow repair pathway that is considered error-free, although this statement is overly simplified as the accuracy of this repair process is dependent on the template used. Unless a sister chromatid is used, the template is not completely homologous, which can lead to loss of heterozygosity as a consequence of gene conversion. Further, it has been shown in yeast that point mutations are more frequent at DNA DSB repair sites, perhaps due to lower repair fidelity of repair

polymerases compared to replicative polymerases (Strathern et al., 1995) In HR, a homologous sequence is used as a template for repair. HR is mainly active during the S and G2 phases of the cell cycle since this is when the DNA is replicated and consequently there is a sister chromatid available to use as a template (Jackson, 2001). The homologous chromosome may also be used as a template, but this occurs less frequently; the sister chromatid is preferred with 2-3 orders of magnitude over a homologous or heterologous chromosome (Johnson and Jasin, 2001). Recently, evidence has emerged that cohesins, responsible for binding the sister chromatids together during replication also are important in DSB repair. Cohesins have been shown to migrate to DSB repair sites independently of the cell cycle, for a recent review see (Sjogren and Strom). The first step in HR is a 5' to 3' trimming of the DNA to produce a single-stranded 3' overhang. This is thought to be performed by the MRN complex, which consists of the proteins MRE11, RAD50 and NBS1 (Tauchi et al., 2002). The single-stranded 3' overhang is protected by RPA (replication protein A) and the ends are further protected by the RAD52 protein (Van Dyck et al., 1999). The next step is strand invasion. This is performed by the RAD51 protein, which has an affinity for single-stranded DNA. When bound to single-stranded DNA, RAD51 catalyses a search for homologous sequences and also has a crucial role in strand-pairing and strand exchange (Baumann et al., 1996). RPA has a greater affinity for ssDNA than RAD51, so the replacement of RPA with RAD51 needs to be catalysed. This may be performed by the RAD51 paralogues RAD51B, RAD51C, RAD51D and XRCC2 (Masson et al., 2001).

The BRCA2 protein may also have a role in replacing RPA with RAD51(Powell et al., 2002). When a homologous sequence has been found, strand invasion takes place. The invading strand anneals to the template and the 3' end of the invading strand is used as a primer for DNA synthesis. Synthesis extends beyond the break point in the damaged strand to replace the missing sequence. The process of strand invasion causes the formation of a D-loop structure and a Holliday junction (HJ), where the invading strand crosses the template strand. For a schematic figure of homologous recombination, see figure 2.


5′
3′
a DSB formation
b ATM activation
ATM
NBS1
MRE11
RAD50
c Nucleolytic processing
5′
3′
RPA
RAD51
RAD52
+ BRCA1
BRCA2
XRCC2
XRCC3
RAD51B
RAD51C
RAD51D
d Nucleoprotein-filament formation
+
RAD52
RAD54
e Homology search and joint molecule formation
+ DNA polymerases
f DNA synthesis
+ DNA ligase
Resolvases
g Completion of DSB repair

The Szostak Model

A double HJ may also form by the invasion of the other DNA end (Szostak et al., 1983). This can be followed by branch migration, which can result in either a crossover or non-cross-over event. Recently, it was shown in yeast that double HJs indeed form after a DSB (Bzymek et al.). However, it is still disputed whether this mechanism actually occurs in mammalian DSB repair. For instance, cross-over events are rarely seen when analysing products of DSB repair (Johnson and Jasin, 2000). This may indicate that HJs are preferentially resolved without crossing over, or that double HJ structures are not formed. However, the scarcity of cross-over products may be due to BLM and topoisomerase III proteins, which might function in HJ dissolution, in order to avoid cross-over products (Raynard et al., 2006; Wu and Hickson, 2003).

Synthesis-Dependent Strand-Annealing

As an alternative to the Szostak model, the synthesis-dependent strand-annealing (SDSA) model emerged; in this model the second DNA end does not perform an invasion (Paques and Haber, 1999). Instead, DNA synthesis is performed beyond the breakage point and the newly synthesised strand re-anneals with the other DNA end. When synthesis is complete, the strand is released by sliding the HJ towards the 3' end. Several proteins have been implicated in HJ migration (e.g WRN, BLM, p53)(Karow et al., 2000; Plank et al., 2006; Prabhu et al., 2002; Yang et al., 2002) but the mechanisms are poorly understood.

Fig 2. Schematic overview of HR repair of a DNA double strand break a) a DNA DSB is formed
b) the damage is sensed by ATM and cell cycle checkpoints are activated c) the dsDNA ends are trimmed by the MRN complex d) the ssDNA ends are coated with RPA and RAD51 and the RAD51 paralogues are recruited e) RAD51 performs a homology search and strand invasion f) DNA is synthesised beyond the damage g) the HJ is resolved and the nicks ligated. Figure adapted from (van Gent et al., 2001)

Once branch migration is complete, the free DNA end is protected by RPA and the DNA ends are annealed. This process is probably facilitated by RAD52 and p53 (New et al., 1998; Oberosler et al., 1993; Van Dyck et al., 1999). If necessary, the remaining flap is removed by XPF/ERCC1 (Al-Minawi et al., 2008). Finally, the gaps are filled and ligated. The outcome is a gene conversion event (Haber et al., 2004).

Single-Strand Annealing

In cases where the DSB occurs within, or in proximity to, repetitive sequences the damage can be repaired via a RAD51 independent pathway termed single-strand annealing (SSA)(Paques and Haber, 1999). This appears to be a common pathway for DSB repair in mammals (Liang et al., 1998). The mechanism of repair in SSA is poorly understood, but evidence suggests that RAD52 and RPA are involved in binding the single stranded DNA and bringing the ends together. The DNA ends are then annealed and DNA between the sequences is flipped out and removed by the XPF protein followed by ligation of the nick in the newly annealed DNA (Sargent et al., 2000). SSA is associated with loss of sequence, as deletions are inevitable.

Break-Induced Replication

When a replication fork encounters a SSB, a one-ended DSB may arise. After resection, the 3' end can perform a RAD51 dependent strand invasion, restoring the replication fork. The HJ produced as a result of the strand invasion is cleaved, and DNA replication is resumed. This pathway is called break-induced replication (BIR) (Haber, 2000; Kraus et al., 2001). However, recently it was shown that one-ended DSBs do not restart replication forks in mammalian cells. This puts into question the validity of this model for higher organisms (Petermann et al.). For a summary of HR pathways, see fig. 3.

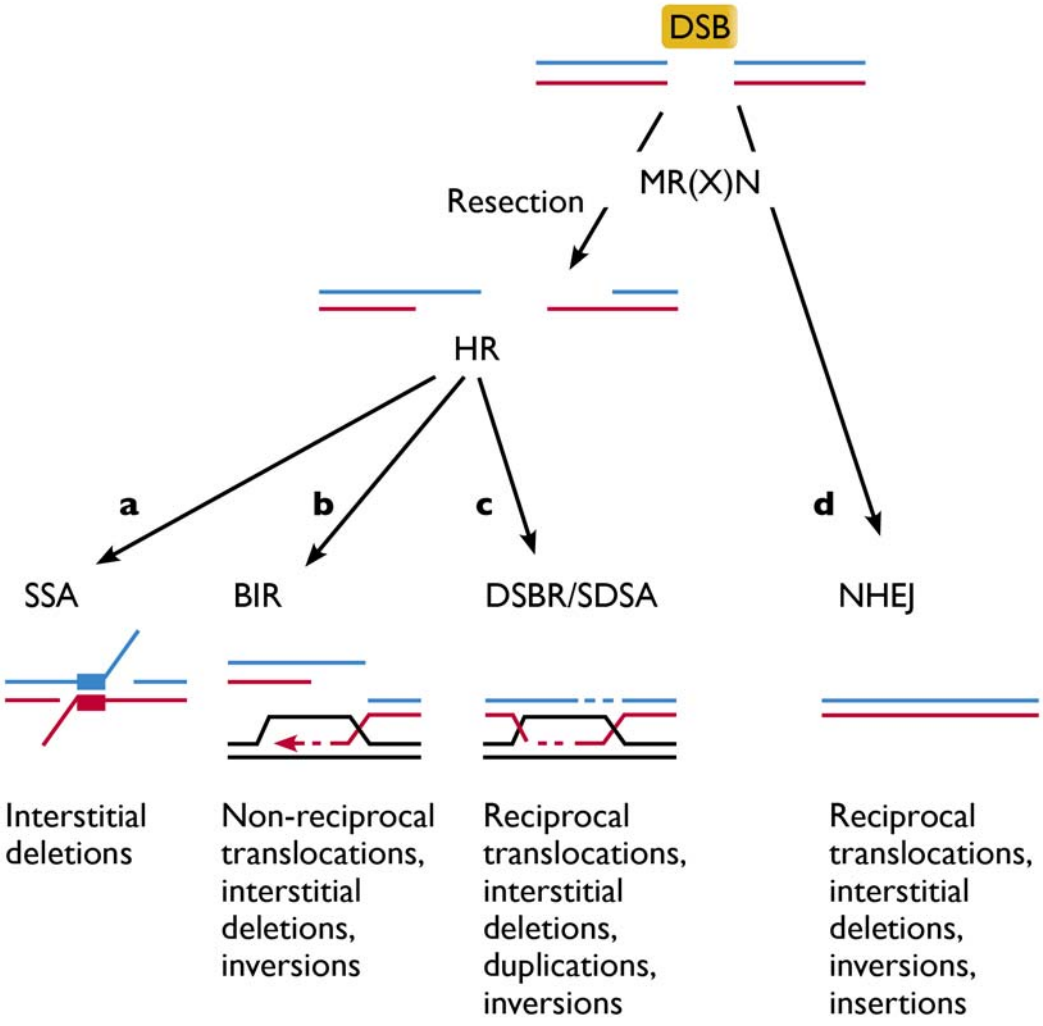


Figure 3. A DSB can be repaired by a) single-strand annealing (SSA) if the DSB is flanked by repetitive sequences b) break-induced replication (BIR) c) Double strand break repair (DSBR) involving a double Holliday junction (HJ) or Synthesis Dependent Strand Annealing (SDSA) d) a DSB can also be repaired by Non-Homologous End-Joining (NHEJ) in the absence of a homologous sequence. Adapted from (Aguilera and Gomez-Gonzalez, 2008)

The Role of XRCC2 in HR

As described previously, a vital step in HR is the replacement of the ssDNA binding protein RPA with RAD51. RPA has a higher affinity for ssDNA than RAD51 and the assembly of RAD51 onto ssDNA has to be facilitated by several proteins. It has been shown that a number of proteins are required for the formation of RAD51 foci in mammalian cells following IR (Liu, 2002). Among these are five RAD51 paralogues XRCC2, XRCC3, RAD51B, RAD51C and RAD51D (Masson et al., 2001). XRCC2 was isolated after its ability to correct IR sensitive hamster cells (Liu et al., 1998). The XRCC2 protein appears to interact with RAD51 and to be essential for the formation of RAD51 foci, as foci are unable to form in XRCC2 deficient hamster irs1 cells after subjection to DNA damage, even though RAD51 protein levels are normal (Johnson et al., 1999). XRCC2 has been shown to be essential for repair of DSBs by HR; hamster cells deficient in XRCC2 exhibit a 100-fold decrease in DSB-induced HR compared to the parental cell line. In contrast, the XRCC2 deficient cells did not exhibit any defect in repair by NHEJ. When the cells were transfected with a plasmid expressing wt XRCC2, the HR repair defect could be corrected to almost wt levels (Johnson et al., 1999). The HR deficiency due to XRCC2 deficiency has also been confirmed in mouse knock-outs (Deans et al., 2003). The precise function of XRCC2 is unknown. It associates with three other RAD51 paralogues; RAD51B, RAD51C and RAD51D; this complex is termed BCDX2 (Thacker and Zdzienicka, 2003). The BCDX2 complex has been shown to bind preferentially to Holliday junction-type structures (Yokoyama et al., 2004) and replication forks (Compton et al.). Despite the fact that the function of XRCC2 is unknown, it is essential for survival. Knocking out XRCC2 in mice leads to embryonic lethality. Thus XRCC2 may be essential for development but not for the viability of cells. Cell lines deficient in XRCC2 are viable, but exhibit chromosomal aberrations, sensitivity to cross-linking agents, some sensitivity to gamma radiation and, as mentioned previously, defective RAD51 foci formation following exposure to gamma rays (Deans et al., 2000). Recently, it has been reported that RAD51 foci do indeed form in XRCC2 deficient cells, but that the foci formation is slower than in wild type cells (Tambini et al.). Interestingly, irs1 hamster cells, deficient in XRCC2, have been shown to be deficient in replication

associated HR repair of lesions caused by hydroxyurea treatment, but proficient in repair of lesions caused by thymidine, indicating involvement of the XRCC2 protein in separate repair pathways at replication forks (Liu and Lim, 2005).

The BRCA2 Protein in DNA Repair

Another protein that is paramount for functional HR is the BRCA2 protein. BRCA2 binds directly to RAD51 via short peptide repeats called BRC repeats (Bork et al., 1996). Cells deficient in BRCA2 have been shown to be deficient in DSB repair by HR(Moynahan et al., 2001; Xia et al., 2001) but not in NHEJ (Xia et al., 2001), and are also unable to form RAD51 foci following ionising radiation (Yuan et al., 1999). Biochemical studies indicate that the function of BRCA2 can be to regulate the ability of RAD51 to form nucleoprotein filaments; incubation of a BRC repeat peptide with RAD51 and DNA has been shown to revert the RAD51 filaments to monomeric form and disrupted the interaction between RAD51 and DNA (Davies et al., 2001). However, RAD51 foci and SCE can form in the absence of BRCA2 in S-phase, indicating that BRCA2 is not required for RAD51 foci formation or SCE per se, but that replication associated RAD51 foci and ionising radiation induced foci utilise separate pathways (Saleh-Gohari and Helleday, 2004; Tarsounas et al., 2003). Apart from its role in HR, BRCA2 may act in the BER pathway (Hay and Clarke, 2005), as BRCA2 deficient cells are sensitive to MMS, an agent that causes methylated bases which are primarily repaired by BER. (Bogliolo et al., 2000; Chen et al., 1998).

Replication and Repair

The predominant source of lesions repaired by HR in mammalian cells probably arises at replication forks (Saleh-Gohari et al., 2005). In human cells, approximately ten replication associated breaks are estimated to occur at each cell division, as based on the number of sister-chromatid exchanges (Haber, 1999). RAD51 foci also form at replication forks, further showing the importance of HR at replication forks (Tashiro et al., 1996) (Lundin et al., 2003). Cells deficient in RAD51 are not viable, but a cell line with an inducible promoter controlling RAD51 expression has been established. This cell line exhibits an accumulation of chromosome breaks during replication when the promoter for the Rad51 gene is turned off, further showing the importance of the RAD51 protein in the repair of replication associated lesions (Sonoda et al., 1998). A one-ended DSB can also occur if a replication fork encounters an unrepaired DNA single-strand break (SSB). This may lead to a collapsed replication fork that can be restored by HR(Arnaudeau et al., 2001; Lundin et al., 2002; Petermann et al.). Evidence in bacteria indicates that the replication forks can regress when encountering an obstacle, forming a so-called chicken foot structure that can serve as a substrate for HR (McGlynn and Lloyd, 2002; Seigneur et al., 1998), for a review see (Helleday, 2003) An alternative pathway to resolve stalled replication forks is demonstrated by Pomerantz and O'Donnell in a recent paper where they show that, in E.Coli, the replisome stalls when colliding with a transcription complex, but instead of collapsing, the replication fork resumes replication after the displacement of the RNAP by Mfd, the bacterial homolog of CSB in humans (Pomerantz and O'Donnell). The human CSB protein is, however, unable to dislocate an RNAP stalled at a lesion (Selby and Sancar, 1997b), instead the stalled RNAP is ubiquitinylated and degraded(Woudstra et al., 2002).

DNA Damage Signalling at Stalled Replication Forks

To ensure the integrity of replicating cells, cell cycle checkpoints have evolved. The role of the cell cycle checkpoints is to detect DNA damages, so that these damages can be repaired before the next round of replication, thus avoiding mutagenesis. This is achieved by slowing or arresting cell cycle progression, making sure that there is sufficient time for DNA repair to act.

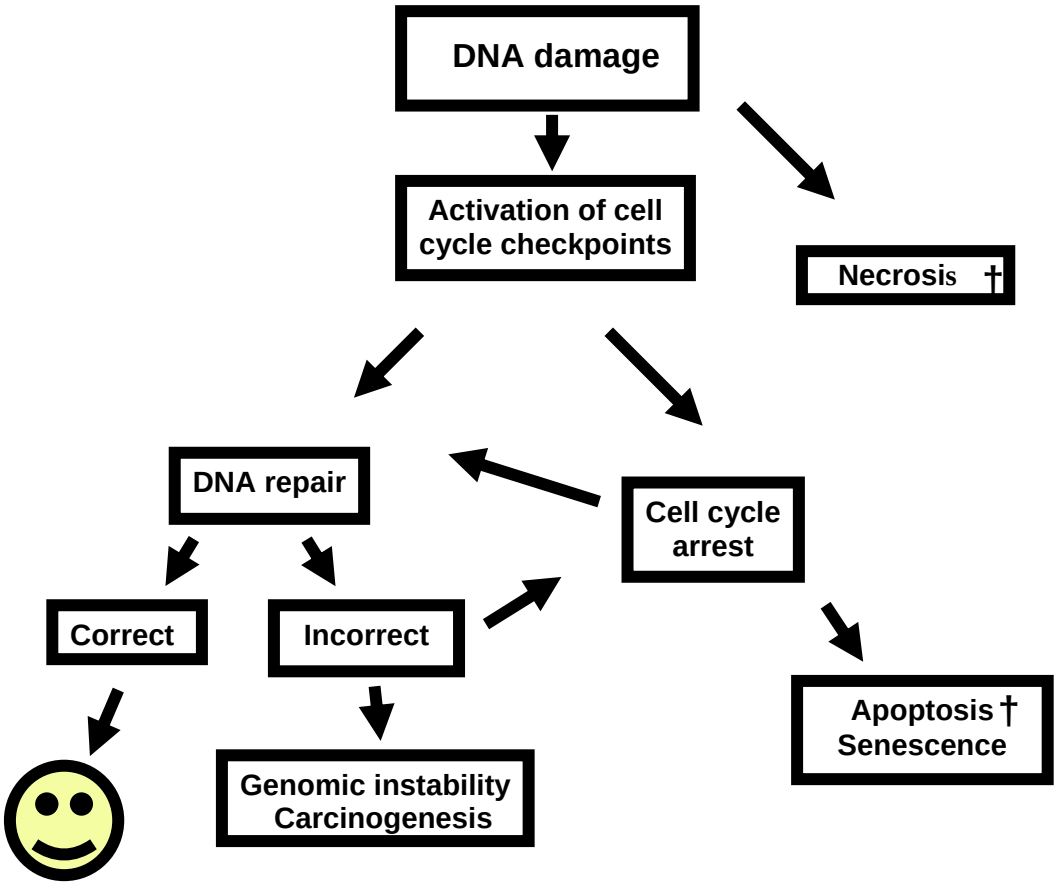


Fig 4. Overview of DNA damage response. DNA damage activates cell cycle checkpoints. DNA repair takes place, which can be either correct, in which case the cell cycle continues, or incorrect, which may lead to genomic instablility, irreversible growth arrest or apoptosis. Adapted from (Houtgraaf et al., 2006)

If the damage to DNA is too extensive for repair, an option is programmed cell death, apoptosis, for an overview see fig 4. The kinases Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia and RAD3 related (ATR) are key players in the signalling at replication stress in human cells (Abraham, 2001). Replication stress, for instance after replication fork stalling following thymidine treatment, leads to ATM and Chk1 activation and subsequent repair by HR (Bolderson et al., 2004; Sorensen et al., 2005). ATM is activated mainly by DSBs, to which it is probably recruited by the MRN complex (Su, 2006). ATR is activated by stalled replication forks and other lesions leading to the accumulation of ssDNA, such as resected DSBs; DNA-PKcs has also been shown to play a role in signalling for stalled replication forks (Yajima et al., 2006). ATM and ATR phosphorylate the effectors Chk1 and Chk2, and these in their turn trigger the checkpoint response (Su, 2006).

Nucleotide Excision Repair-NER

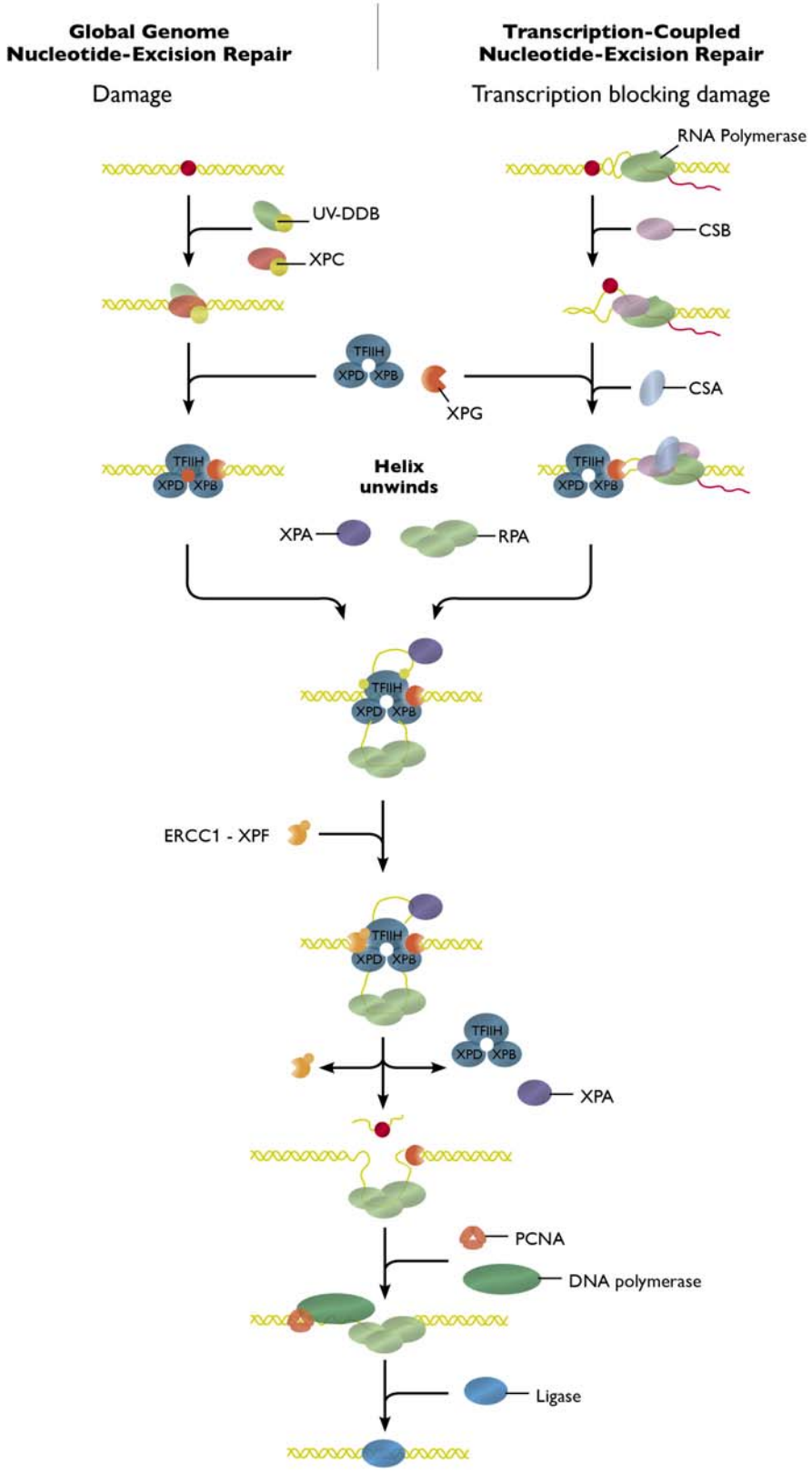
The nucleotide excision pathway removes a variety of helix distorting lesions. The NER pathway is generally divided into two subpathways, Global Genome NER (GG-NER) and Transcription-Coupled Repair (TCR). TCR is also called Transcription-Coupled NER (TC-NER). As the name implies, GG-NER functions throughout the genome, while TCR is active at transcribed regions. Apart from the DNA regions where these two pathways are active, there is a difference in the DNA damage recognition step. In GG-NER, the lesion is recognized by the XPC/hHR23B, and sometimes, UV-DDB complexes. The latter is needed for some lesions, such as cyclobutane pyrimidine dimers (CPDs) caused by UV irradiation (Tang et al., 2000). In TCR, the repair is triggered by damage-mediated blockage of an elongating RNAPII. Next, the two pathways converge; first, the DNA is unwound to form an open complex. This is performed by two helicases, XPD and XPB, which are subunits of the combined basal transcription factor and repair factor TFIIH. XPB and XPD have opposing polarities: XPB is active in the 3' to 5' direction and XPD in the 5' to 3' direction (van Brabant et al., 2000). Other factors involved are XPA, which is thought to bind to the damage, and RPA, which binds to single-stranded DNA. The binding of these factors to the damaged site recruits the endonucleases XPG and XPF

which perform the incisions necessary for the removal of the damaged site (Ma et al., 1995). The lesion is removed by single-stranded incision on both sides of the damage. A 24-32 nucleotide stretch of DNA is removed and the resulting gap is filled by DNA polymerase δ/ε followed by ligation of the nicks by DNA ligase I (Andressoo et al., 2005; Svejstrup, 2002).

Two factors are specific for transcription-coupled repair: CSA and CSB, which associate with the RNA polymerase when it is stalled by a lesion (van Gool et al., 1997). Cells deficient in the CSA and CSB proteins have an abolished ability to repair damages in transcribed DNA but are proficient in the repair of damages within the whole genome (Venema et al., 1990). For an overview of the GG-NER and TCR pathways, see fig 5.

Transcription-Coupled Repair in Mammalian Cells

DNA damage has the potential to block the elongating RNA polymerase during transcription. As described above, these damages are removed by a specialized repair pathway called transcription-coupled NER or transcription-coupled repair. In TCR, the stalled RNA polymerase is displaced from the damaged site so that repair can take place. It is well established that transcribed regions of the genome are repaired faster than non-transcribed regions (Bohr et al., 1985; Fousteri and Mullenders, 2008; Mellon et al., 1986). The reason for this is most likely that these regions contain genes that need to be undamaged to maintain the integrity and function of the cell, and thereby the entire organism. The faster repair rate is not solely due to the fact that transcribed regions of DNA are found in more open areas of chromatin, and is therefore more easily reached by repair proteins. This is demonstrated by the discovery that the transcribed strand of the DNA template is repaired more rapidly than the non-transcribed strand; in fact, the non-transcribed strand is repaired at rates similar to that of non-transcribed regions (Mellon, 2005; Svejstrup, 2002),. Evidence was put forward to support the notion that BER was also a part of TCR (Le Page et al., 2000). However, this was later retracted.


Global Genome
Nucleotide-Excision Repair
Damage
Transcription-Coupled
Nucleotide-Excision Repair
Transcription blocking damage
RNA Polymerase
UV-DDB
XPC
CSB
TFIIH
XPD
XPB
XPG
CSA
Helix
unwinds
XPA
RPA
ERCC1 - XPF
PCNA
DNA polymerase
Ligase

The Role of the CSB Protein in TCR

Evidence from experiments using cells deficient in CSA and CSB indicate that cells down-regulate their transcription levels when affected by DNA damage. Lower overall transcription levels have been shown in cells deficient in CSA and CSB (Balajee et al., 1997; Dianov et al., 1997). One reason for this might be that DNA damage leads to a hyperphosphorylation of RNAPII, which makes it unable to initiate transcription. Cells deficient in CSB remain hyperphosphorylated following DNA damage as opposed to wild type cells, which rapidly return to a state of non-hyperphosphorylated RNAPII (Rockx et al., 2000). Furthermore, cells deficient in CSA and CSB were unable to restart transcription following UV-irradiation, they were even unable to transcribe inserted undamaged plasmid DNA (Proietti-De-Santis et al., 2006). The CSB protein has also been found to stimulate transcription elongation by RNAPII (Selby and Sancar, 1997a) Another mechanism that might account for the diminished transcription levels after DNA damage has been demonstrated in *in vitro* experiments; the TATA box binding protein TBP was shown to bind directly to damaged DNA, thereby inhibiting transcription by making the damaged DNA inaccessible for the transcription machinery (Vichi et al., 1997). Furthermore, in yeast, NER and transcription was shown to compete for TFIIH, thus lowering the transcription level in the presence of DNA damage (You et al., 1998).

Fig 5. Overview of GG-NER and TCR. The lesion is recognised by XPC/hHR23B, alternatively UV-DDB in GG-NER, or CSB in TCR. The DNA is unwound by XPD and XPB helicases of TFIIH. XPA and RPA are also involved in damage binding. XPG and XPF are recruited and incise the DNA around the damage. The lesion is removed and the gap is filled and ligated. Adapted from (Hoeijmakers, 2009)

NER Deficiency Syndromes in Humans

The link between DNA repair and human health is made clear by several rare human disorders, for example xeroderma pigmentosum (XP), trichothiodystrohy (TTD) and Cockayne syndrome (CS). These three disorders all arise from mutations in the same gene; the gene encoding the helicase XPD (Lehmann, 2001). CS patients are deficient in the TCR specific proteins CSA or CSB (Dubaele and Egly, 2002). Since XPD is involved in both transcription and DNA repair in its capacity as a subunit of the transcription factor TFIIH, the symptoms seen in XP and TTD patients may stem from either impaired transcription, impaired DNA repair capacity, or from a combination of the two. Patients do have residual NER capacity (de Boer and Hoeijmakers, 2000), a fact that makes it unlikely that the NER defect alone is responsible for the symptoms seen in these patients.

The XPD gene encodes one of the ten subunits of the transcription factor TFIIH. The core of TFIIH consists of XPB, p62, p52, p44, p34 and TTDA. The core associates with the Cdk-activating kinase (CAK) complex via the XPD helicase (Oksenych and Coin). The XPD protein is a helicase that unwinds DNA in the 5' to 3' direction. On its own it has a quite low activity and requires the binding of the p44 part of TFIIH for activity (Coin et al., 1998). TFIIH has a dual function; it is required both for basal transcription initiation by RNA polymerases I and II and for NER (Zurita and Merino, 2003). The function of XPD in these two processes is similar: in transcription, it unwinds DNA at the promoter to allow RNA pol II to bind, and in NER it unwinds DNA at the damaged site allowing damage-specific endonucleases to cleave DNA at either side of the damage so that the damage can be removed (Frit et al., 1999).

The effect of XPD mutations is, however, quite different regarding DNA repair and transcription. It has been demonstrated in yeast that the helicase activity of RAD3, an XPD homologue, is essential for DNA repair but not for transcription (Guzder et al., 1994; Tirode et al., 1999). The RAD3 has also been found to bind preferentially to damaged over non-damaged DNA (Sung et al., 1994), indicating a role in damage

recognition. The main function of XPD in transcription seems to be to stabilise TFIIH. In this function it is rather tolerant to mutations and it has been shown that many mutations do not lead to decreased transcription levels (Winkler et al., 2000). However, in its role as a part of DNA repair there is a greater need for enzymatic activity. Thus, cells that have severe defects in NER often have quite normal levels of transcription (Lehmann, 2003). Mutations in the XPD gene give rise to three distinct phenotypes; xeroderma pigmentosum (XP), trichothiodystrophy (TTD) and XP with features of Cockayne syndrome (XP/CS).

XP was the first disorder identified that is caused by a defective DNA damage repair gene (Lehmann, 2003). It is a rare autosomal recessive disorder characterised by a hypersensitivity to ultraviolet radiation (UV). Patients suffering from XP are extremely susceptible to skin cancer, with the risk of contracting skin cancer approximately 2000 times greater than for the general population (Vermeulen et al., 1997). The increased risk of skin cancer is caused by a deficiency in NER (van Hoffen et al., 2003), which makes damage caused by UV-light difficult to repair. Most XP patients have pigmentation changes, such as freckling. More severely affected XP patients also exhibit neurological abnormalities due to neuronal death. XP patients can be divided into eight complementation groups, XP-A to XP-G and XP variant. XP patients defective in the XPD gene belong to the XP-D complementation group (Lehmann, 2001).

Another disorder characterised by sun sensitivity, but not an increased risk of contracting skin cancer, is Cockayne syndrome (CS). CS patients suffer from dwarfism, mental retardation, microcephaly, skeletal abnormalities and features of premature ageing (Nance and Berry, 1992). Unlike XP patients, where neurological abnormalities are caused by neuronal death, the abnormalities in CS patients are caused by demyelinisation. There are a few cases where patients have combined features of XP and CS (XP/CS). These patients belong to the XP-D complementation group (Bohr et al., 1998). Trichothiodystrophy (TTD) is characterised by brittle hair due to sulphur deficiency in the hair. TTD patients also exhibit mental retardation. Some TTD patients are sensitive to sunlight but unlike XP patients they do not have pigmentation changes. Even though the

clinical features of XP and TTD patients are quite different, most patients diagnosed as suffering from TTD have been shown to belong to the XP-D complementation group (Bergmann and Egly, 2001).

Transcription-Associated Recombination -TAR

Background

Transcription enhances recombination in all kinds of cellular organisms, from bacteria to man. The first evidence for this phenomenon was observed in the λ phage (Ikeda and Matsumoto, 1979), and later it was also observed in *E.coli* (Thomas and Rothstein, 1989a).

In eukaryotes, the first evidence for TAR was found via the discovery of the recombination hotspot *HOT1* in Saccharomyces cerevisiae. This DNA fragment was found to stimulate recombination also when inserted in other locations in the yeast genome than where it was originally found, and to stimulate both interchromosomal and intrachromosomal mitotic, but not meiotic, recombination (Keil and Roeder, 1984). Later, the Roeder group discovered that two DNA sequences were needed for the enhancement of recombination. One of the sequences contained the transcription initiation site for the 35S ribosomal RNA transcript (I element) and the other contained an enhancer of RNAPI transcription (E element). Stimulation of recombination was observed only when *HOT1* was oriented so that a transcript initiating in the I region would proceed towards the recombining regions. The orientation specificity was only of importance concerning the I element, the E element would function regardless of its orientation. They showed that transcription by RNAPI indeed stimulates genetic recombination by inserting a termination signal for RNAPI transcription between *HOT1* and the sequences following. The stimulation of recombination was then abolished, showing that transcription is responsible for the enhancement of recombination, and that transcription needs to proceed through at least one repeat to stimulate recombination (Voelkel-Meiman et al., 1987). Mutations in *HOT1* that decrease its activity also decrease transcription, indicating that the recombination-stimulating activity of *HOT1* likely is

dependent on its ability to promote transcription (Stewart and Roeder, 1989). This finding was further corroborated by Huang and Keil (Huang and Keil, 1995).

Later, evidence that RNA polymerase II dependent transcription stimulated recombination emerged. Thomas and Rothstein showed that transcription of the *GAL10* gene in S. cerevisiae led to increased frequencies of recombination leading to deletions, but not gene conversions (Thomas and Rothstein, 1989a). The *RAD52* gene was shown to be necessary for transcription enhanced recombination, and also, to a lesser extent, the *RAD1* gene (Thomas and Rothstein, 1989b). The involvement of the *RAD52* gene in TAR was confirmed by Nevo-Caspi et al. They showed that in S. cerevisiae, the recombination frequency between a family of repeated sequences, the Ty family, could be increased by increasing the transcription levels (Nevo-Caspi and Kupiec, 1994). This event was found to be dependent on *RAD52* and *RAD1* (Nevo-Caspi and Kupiec, 1996). Saxe et al also showed that high RNAPII transcription levels stimulated recombination in S.cerevisiae. A *lys2* recombination substrate regulated by an inducible *GAL1-10* promoter or a low-level *LYS2* promoter was used (Saxe et al., 2000). Recently, also RNA polymerase III transcription has been shown to enhance recombination levels. The recombination levels were shown to correlate with head-on collisions with the replication fork (de la Loza et al., 2009). The connection between replication and TAR will be discussed in more detail below.

TAR in mammalian cells

TAR in mammalian cells was first described by Nickoloff et al. A plasmid based system where transcription could be controlled over heteroallelic neomycin resistance genes was used. Plasmid pairs were transfected into Chinese hamster ovary cells and the recombination frequency was determined as the number of neomycin resistant colonies. They found that when transcription was active and DNA double strand breaks were introduced, the recombination levels were six-fold over non-induced levels. When no DNA DSBs were introduced, no increase in TAR levels compared to control could be detected (Nickoloff and Reynolds, 1990).

Later, the effect of transcription on intrachromosomal homologous recombination was studied. A recombination system consisting of two neomycin resistance genes were used, where one allele was silent and the other regulated by an inducible promoter. Alleles that were transcribed at a high level had a recombination frequency that was two- to seven-fold higher than alleles that had a lower transcription level. Both direct and inverted repeats were used. The result of direct repeat recombination was mainly gene conversions, whereas inverted repeats produced a variety of recombination products (Nickoloff, 1992). The recent discovery of the human RECQL5 provides another link between transcription and recombination (Svejstrup). The RECQL5 helicase interacts directly with RNAPII, and inhibits transcription *in vitro* (Aygun et al., 2008; Aygun and Svejstrup; Aygun et al., 2009). Mouse *recq5-/-* cells exhibit elevated recombination levels and chromosomal rearrangements, suggesting that RECQL5 has a role in preserving genomic stability (Hu et al., 2007). RECQL5 also appears to play a role in preventing stalled replication forks after replication stress (Hu et al., 2009) Taken together, the involvement of the RECQL5 helicase in transcription and recombination makes it an interesting candidate for involvement in TAR.

The Mechanisms of TAR

There are two major, non-mutually exclusive, theories that attempt to explain the phenomenon of TAR, for a schematic overview, see figure 6.

I. Transcription facilitates the formation of recombinogenic structures by making the DNA more susceptible to DNA damage

II. Transcription interferes with replication fork progression, thus promoting recombination. The impaired replication fork progression can be due to the formation of R-loops, or the collision between replication and transcription complexes.

In favour of the hypothesis that TAR is caused by DNA damage is the fact that chromatin structure is altered when a gene is transcribed. The open chromatin structure of the transcribed DNA strands may facilitate access to the DNA for DNA damaging agents. Transcription promotes the formation of negative supercoils. Excessive supercoiling has been shown to induce hyper-recombination in yeast mutants of topoisomerase I, a protein that nicks one strand of DNA to release torsion. Yeast strains with a null mutation in the gene encoding topoisomerase I was shown to have greatly elevated frequencies of mitotic recombination (Christman et al., 1988; Trigueros and Roca, 2002). It has been proposed that negative supercoiling accumulating behind the elongating RNAPII may induce TAR, as negative supercoiling facilitates strand separation and thus increases the accessibility for DNA damaging agents to DNA. The resulting DNA damages would then require recombination to be repaired (Nickoloff, 1992; Saxe et al., 2000). The susceptibility of transcribed DNA to DNA damage was demonstrated by Garcia-Rubio et al that recombination induced by DNA-damaging agents was greatly increased by transcription, further strengthening the hypothesis that the higher recombination frequencies observed during active transcription may be a bi-product of the greater accessibility of DNA-damaging agents to the DNA. They introduced different recombination constructs introduced in yeast. They then used 4-nitroquinoline-N-oxide (4-NQO) or methyl methanesulfonate (MMS) to induce DNA damage. Damage and transcription combined increased recombination levels up to 12.800-fold for 4-NQO and 130-fold for MMS over levels observed in the absence of transcription or DNA damage. DNA damage alone increased recombination levels 193-fold for 4-NQO and 4. 5-fold for MMS over spontaneous levels, while transcription alone increased the recombination frequency 2-3 fold over spontaneous levels (Garcia-Rubio et al., 2003). A finding that supports this theory is that the mutation frequency increases with transcription, in fact, the mutation frequency in a gene is directly proportional to the amount of transcription on it (Jinks-Robertson and Petes, 1986; Kim and Jinks-Robertson, 2009; Lippert et al., 2004). The increase in TAR frequency in combination with DNA damage is not corroborated with findings from a mammalian system, however. When transcription and UV-damage was combined, increased transcription levels lowered the UV-induced intrachromosomal

recombination levels in a recombination system, although UV and transcription separately induced recombination (Deng and Nickoloff, 1994).

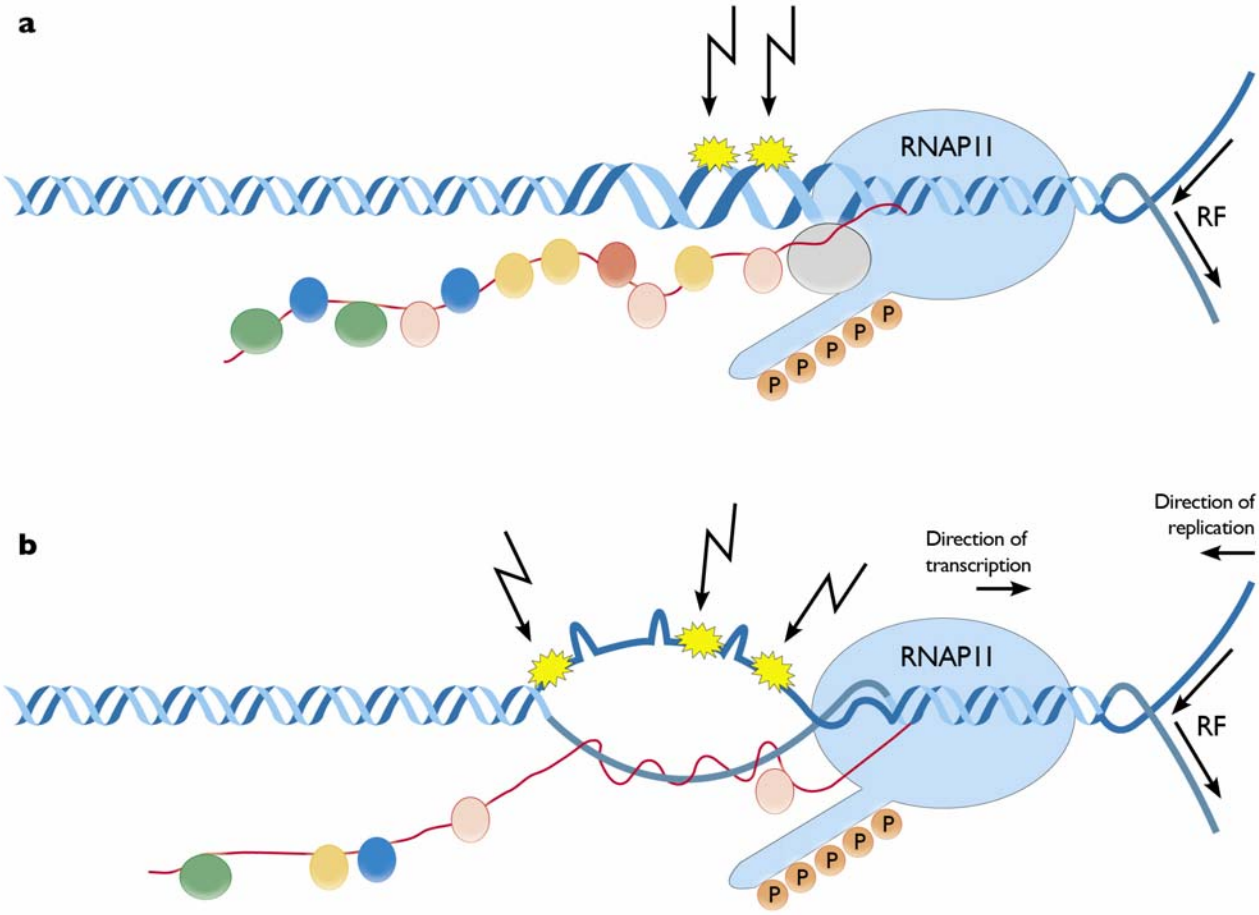


Fig 6. Possible inducers of TAR a) transcription elongation induces negative supercoiling behind the RNAPII, leading to accumulation of ssDNA which may be more easily damged, thus inducing TAR. b) DNA-RNA hybrids (R-loops) may form due to impaired mRNP biogenesis. The ssDNA region could be more susceptible to DNA damage, or form secondary structures that may impair RF progression. TAR is mainly observed in S-phase, indicating that TAR may be induced by collisions between replication and transcription complexes. Adapted from (Aguilera and Gomez-Gonzalez, 2008)

Taken together, these results provided evidence that high transcription levels are associated with genomic instability. Chavez et al proposed a model in which a stalled transcriptional elongation complex might induce breaks in the DNA, which would require recombination for its repair. Alternatively, stalled transcription elongation might cause a replication fork blockage. The blocked replication fork would then need to be bypassed by a recombination event (Chavez and Aguilera, 1997). A further link between transcription and DNA damage is that transcription might induce DSBs, which can serve as a substrate for recombination. Gonzales-Barrera et al compared DSB induced recombination and spontaneous recombination events in yeast under conditions of high and low transcription levels. They also examined whether different recombination products would arise when comparing recombination products from TAR or from DSBs. Yeast strains mutated in different proteins known to be involved in recombination were constructed, and it was shown that the effect of the respective mutations were the same regardless of whether the recombination products came from TAR or a DSB. As the recombination products from TAR and DSBs were the same, they hypothesised that transcription might induce the formation of DSBs (Gonzalez-Barrera et al., 2002a)**.**

In favour of the hypothesis that TAR is an effect of replication fork stalling is the fact that transcription and replication often occur simultaneously on the same DNA template. Collisions between the replication and transcription complexes are probably inevitable, as the two processes are active at the same time on the same template, and given that the replication fork moves faster than the elongating RNAP (Brewer, 1988). It is therefore likely that they at some point obstruct each other, leading to stalled replication forks, which may form a substrate for TAR. Replication fork progression is impaired by transcription in bacteria (French, 1992; Liu and Alberts, 1995) and yeast (Takeuchi et al., 2003). Deshpande and Newlon have reported that in tRNA genes polar replication pausing sites exist. These stall the replication forks only when they are in a head-on direction towards transcription. A mutant yeast strain, defective in initiation of transcription, did not stall replication forks, indicating a link between transcription and replication fork pauses (RFPs) (Deshpande and Newlon, 1996). In further support of this, it has been shown that highly transcribed genes act as RFP sites in *S. cerevisiae*

(Azvolinsky et al., 2009), highlighting the need to avoid collisions between transcription and replication complexes to preserve genomic stability. As impaired replication fork progression is known to induce HR, it is likely that TAR and replication fork stalling are connected. Evidence in support of the model that TAR is a consequence of a replication fork blockage caused by a collision between the transcription- and replication machineries was put forward by Prado et al. By using plasmid constructs they could show that RNA pol II transcription in the direction head-on to that of replication significantly increased recombination frequencies. In contrast, transcription co-directional to that of replication did not increase recombination frequencies. Head-on transcription and replication also impaired the progression of the replication fork, which may induce recombination. TAR was also shown to be associated with an RFP. Further, the helicase Rrm3 was shown to facilitate replication fork movement through the RFP (Prado and Aguilera, 2005). Rrm3 has previously been shown to facilitate replication fork progression at RFPs (Ivessa et al., 2000). The induction of recombination by collisions between the transcription and recombination machineries has also been demonstrated for RNA polI in *S.Cerevisiae* (Takeuchi et al., 2003). Findings from experiments in yeast indicate that nascent mRNA can impair transcription and induce TAR by the formation of DNA-RNA hybrids (so-called R-loops) behind the elongating RNAPII (Huertas and Aguilera, 2003). The role of R-loop formation on TAR will be discussed more thoroughly below.

The Role of the THO Complex in TAR

Insights about the possible formation of DNA-RNA hybrids can be gained from analyses of yeast THO mutants. The role of the THO complex in TAR will be discussed in more detail below.

In 1989 Aguilera and Klein isolated S.cerevisiae mutants exhibiting enhanced intrachromosomal recombination. One of these mutants, *hpr1*, showed almost only intrachromosomal crossovers resulting in deletions; the induction was 50-1000 fold over basal levels (Aguilera and Klein, 1990). The *hpr1* mutant was also shown to have a low

level of transcription, indicating that the hpr1 gene product has a function in this process (Fan and Klein, 1994; Zhu et al., 1995). The Hpr1 protein is part of the THO complex. This is a four-protein complex, non-essential for viability; apart from Hpr1, it consists of Tho2, Mft1 and Thp2. The THO complex together with the Thp1 protein connects the processes of transcription and recombination in yeast, as null mutations in either of the subunits of the THO complex confers a hyper-recombinant phenotype (Chavez and Aguilera, 1997; Chavez et al., 2000; Gallardo and Aguilera, 2001; Piruat and Aguilera, 1998). Chavez and Aguilera showed that *HPR1* is involved in transcription elongation. The defective transcription elongation in *hpr*Δ was shown to account for the high recombination levels observed. This showed a link between block of transcription elongation and TAR (Chavez and Aguilera, 1997). The THO complex has been show to associate with the messenger RNA export factors Yra1 and Sub2. This complex was designated TREX. TREX has been shown to be recruited to transcribed genes and travel along the gene together with RNA polymerase II (Strasser et al., 2002). Notably, mutants of *sub2* and *yra1*, as well as other mRNA export factors *mex67* and *mtr2*, show hyper-recombinant phenotypes and transcription impairment similar to those seen in THO mutants (Fan et al., 2001; Jimeno et al., 2002).

The hyper-recombination phenotype of *hpr*Δ cells has been shown to be linked to transcription elongation. Several direct repeat constructs were used to show that recombination events producing deletions were specific for constructs where transcription was initiated at a promoter that traverses particular regions of the DNA surrounding the repeats. Further, a hyper-recombining construct could be turned into a non-hyper-recombining construct by inserting a transcription terminator, showing that the transcription elongation is responsible for the elevated recombination frequencies observed (Prado et al., 1997). The Hpr1 protein was later shown to be required for transcription of either long or GC-rich sequences (Chavez et al., 2001). The requirement for the THO/TREX complex for transcription elongation and its involvement in mRNA metabolism was further demonstrated by Rondon et al (Rondon et al., 2003).

Interestingly, the hyper-recombination phenotype and transcription elongation defect in *hpr*Δ cells can be rescued by cleavage of the nascent mRNA, indicating a link between replication fork progression, transcription and mRNP biogenesis (Huertas and Aguilera, 2003). However, the requirement of the THO complex for transcription elongation was questioned by Jensen et al, based on the insensitivity of THO mutants to mycophenolic acid, a transcription elongation inhibitor (Jensen et al., 2004). The transcription impairment and hyper-recombination phenotypes observed in THO mutant strains might be interpreted as the formation of DNA-RNA hybrids during transcription and that the role of THO is to prevent this formation. When formed in the THO deficient cells, the DNA-RNA hybrids may act as a substrate for recombination. Evidence for impairment of transcription elongation by R-loops has also been shown *in vitro* (Tous and Aguilera, 2007). R-loop formation in THO mutants has also been confirmed by evidence that AID (a cytidine deaminase acting on ssDNA during somatic hypermutation and class-switch recombination in immunoglobulin genes) induces mutations and recombination in a transcription-dependent manner. AID appears to act on the ssDNA displaced at the R-loops (Gomez-Gonzalez and Aguilera, 2007). Other factors than R-loop formation appears to be involved in TAR, as demonstrated by the yeast THO point mutation *hpr1-101* does not show any hyper-recombinogenic phenotype or an increase in TAR. This THO mutant is impaired in transcription, however. R-loops did not appear to form in this mutant, indicating that the transcription defect in THO mutants are not solely due to R-loop formation. The impaired transcription in *hpr1-101* may be due to the accumulation of negative super coils which would increase the accessibility of the DNA strands to the action of AID, as AID induced hyper-recombination as well as hyper-mutation in these cells (Gomez-Gonzalez and Aguilera, 2009).

The recombination events observed in the *hpr*Δ cells were induced by transcription in the S-phase, but not G_2 phase of the cell cycle, providing evidence for the link between replication and TAR (Wellinger et al., 2006). Another link between replication and transcription elongation was put forward by Gomez-Gonzalez et al. They analyzed the interaction of THO mutants with genes involved in replication, S-phase checkpoints, DNA repair and chromatin remodeling. Interactions with S-phase checkpoint factors

were found, and, under replicative stress, THO mutants required functional S-phase checkpoints for survival. Double mutants of checkpoint factors and THO show increased genetic instability, as detected by RAD52 foci formation recombination frequency and gross chromosomal rearrangements (GCRs), indicating that recombinogenic DNA damage, sensed by S-phase checkpoints, is accumulated in THO null mutants (Gomez-Gonzalez et al., 2009). The THO complex has also been shown to be required for NER, particularly TCR, in yeast (Gaillard et al., 2007; Gonzalez-Barrera et al., 2002b), highlighting the interplay between TAR and DNA repair pathways

Present Investigation

The focus of this thesis has been to investigate the events that trigger TAR in mammalian cells, and to elucidate the proteins involved in this process. To this end, a recombination construct was stably integrated into Chinese hamster cells with well documented deficiencies in DNA repair proteins. The aims of the individual projects were

- *Paper I:* To develop a recombination construct and to establish a cell line stably expressing the recombination construct. This cell line was then utilised to investigate the basic mechanisms of TAR in mammals and to determine the prerequisites for its occurrence

- *Paper II*: Investigate the role of the RAD51 paralog XRCC2 and the HR protein BRCA2 on TAR and to determine the interplay between DSB induced recombination and TAR

- *Paper III*: Study the effect of deficiency in the XPD and CSB proteins on TAR in order to investigate the potential interplay between TAR and DNA repair pathways

- *Paper IV*: Investigate the connection between HR repair of UV damages and transcription.

Comments on Methodology

Cell Lines

V79

Wild type Chinese hamster lung fibroblasts (Ford and Yerganian, 1958).

Spd8- S8TofZM3, S8TofZM5 and S8TofZM24

Spd8 is derived from V79 Chinese hamster lung fibroblasts. Spd8 has a partial duplication of exon 7 in the *hprt* gene (Dare et al., 1996; Helleday et al., 1998). This duplication gives rise to a non-functional HGPRT protein; the function of the HGPRT protein can be regained by homologous recombination restoring the wild type *hprt* gene. The revertants can be selected for by addition of HAzT (hypoxanthine, L-azaserine and thymidine) to the growth medium (Helleday et al., 1998). The S8TofZM3, S8TofZM5 and S8TofZM24 cell lines were established from Spd8.

AA8TofZM7 and AA8TofZM16

The cell line used to establish these cell lines was AA8, a wild type Chinese hamster ovary cell line (Thompson et al., 1980)

UV5TofZM7 and UV5TofZM8

The UV5 cell line used to establish these cell lines is derived from AA8 and was isolated on the basis of its hypersensitivity to UV-irradiation (Thompson et al., 1980). The UV-sensitivity exhibited by this cell line is corrected by introduction of human XPD (Weber et al., 1988), a component of the transcription factor IIH (TFIIH) and an essential part of the NER pathway (Weber et al., 1990).

UV5TofZMX1 and UV5TofZMX4

The UV5TofZM7 cell line was stably transfected with an expression vector containing human XPD cDNA. Two clones showing restored NER activity and reversed UV-sensitivity was chosen.

V-C8TofZM24

Established from V-C8, a BRCA2 deficient cell line derived from V79 (Kraakman-van der Zwet et al., 2002). The BRCA2 deficiency renders this cell line deficient in homologous recombination.

irs1TofZM14 and irs1TofZM15

The original cell line is irs1, derived from V79. The irs1 cell line is deficient in XRCC2, a RAD51 paralog (Deans et al., 2000).

UV61TofZM2 and UV61TofZM24

These cell lines were established from UV61, derived from AA8 (Orren et al., 1996). This cell line is deficient in the CSB protein, a component of the transcription-coupled repair (TCR) pathway.

Irs1SF

This cell line is derived from AA8 and was isolated on the basis of their X-ray sensitivity (Fuller and Painter, 1988). They have later also been shown to be sensitive to a number of other DNA damaging agents, e.g. UV-irradiation and cross-linking agents (Caldecott and Jeggo, 1991). This cell line is mutated in the XRCC3 protein, a RAD51 family member and is impaired in HR (Tebbs et al., 1995).

CXR3

Irs1SF cells complemented with human XRCC3 (Tebbs et al., 1995).

For a summary of the cell lines used in this study, see Table 1.

Cell line	Genotype	Defect / modification	Origin	Reference
V79	Wild type	Wild type	lung	(Fuller and Painter, 1988)
Spd8	Wild type	*hprt* gene duplication	V79	(Dare et al., 1996)
S8TofZM3,5,24	Wild type	Spd8 carrying TARneo	Spd8	This study
AA8	Wild type	Wild type	ovary	(Thompson et al., 1980)
AA8TofZM7,16	Wild type	AA8 carrying TARneo	AA8	This study
UV5	XPD^{mut}	XPD mutation, deficient in NER	AA8	Thompson 1980
UV5TofZM7,8	XPD^{mut}	UV5 carrying TARneo	UV5	This study
UV5TofZMX1,4	XPD^{compl}	complemented with human XPD	UV5TofZM7	This study
V-C8	*BRCA2*$^{-}$	BRCA2^{-}, deficient HR	V79	(Kraakman-van der Zwet et al., 2002)
irs1	*XRCC2*$^{-}$	XRCC2^{-}, deficient in HR	V79	(Deans et al., 2000)
irs1TofZM14,15	*XRCC2*$^{-}$	XRCC2^{-}, deficient in HR, carrying TARneo	irs1	This study
Irs1SF	$XRCC3^{mut}$	$XRCC3^{mut}$ deficient in HR	ovary	(Fuller and Painter, 1988)
CXR3	$XRCC3^{compl}$	Irs1SF complemented with human XRCC3	AA8	(Tebbs et al., 1995)
UV61	CSB^{mut}	CSB^{mut} deficient in TCR	AA8	(Orren et al., 1996)
UV61TofZM2,24	CSB^{mut}	CSB^{mut} deficient in TCR, carrying TARneo	UV61	This study

Table 1. Summary of cell lines used in this study

The Recombination Construct

The recombination substrate consists of two non-functional neomycin resistance genes. The 3' neo copy contains an inserted cleavage site for the rare-cutting endonuclease *I-SceI*. This neo copy is controlled by a bi-directional tet-off promoter that also controls a luciferase gene, see fig 7.

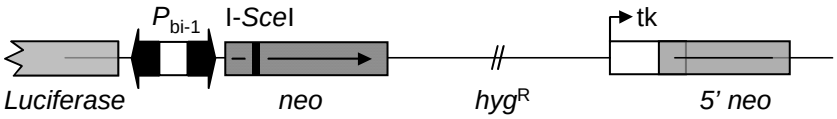


Fig 7. The TARneo recombination construct

This makes it possible to quantitatively measure the level of transcription. The insertion of this cleavage site functions as a stop codon and renders the gene non-functional. The 5' neo copy of the neo gene is truncated and thereby also non-functional. The recombination substrate also contains a hygromycin resistance gene in order to make it possible to select the cells that have integrated the substrate. The tet-off gene expression system is a system in which the gene expression of a certain gene of interest can be turned off by the addition of tetracycline or doxycycline, a tetracycline derivative, to the growth medium.

Spontaneous HR or inducing a DSB at the *I-SceI* site can yield either short tract gene conversion (STGC), long tract gene conversion (LTGC) or sister chromatid exchange (SCE). STGC yields a reversion of the upstream neo copy, controlled by the inducible promoter, to its functional form. LTGC will revert the downstream neo repeat, controlled by the tk promoter, to its functional form.

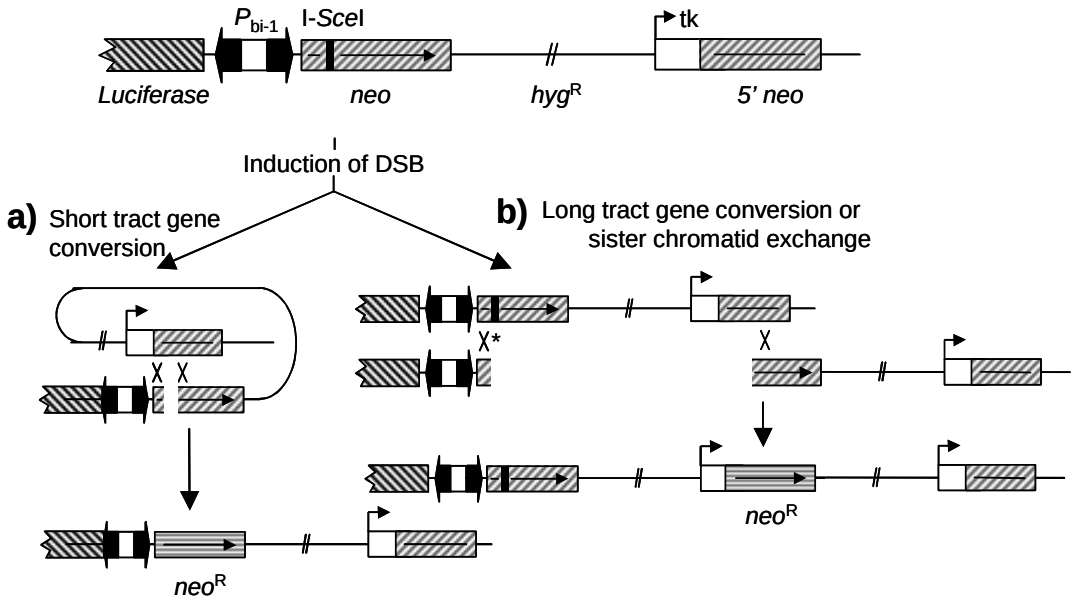


Fig 8. The Pbi-1 promoter is negatively controlled by the presence of the tetracycline derivative doxycycline (DOX). Transcription is turned off in the presence of doxycycline and turned on in its absence. Figure of the pathways for conversion of the inactive neomycin resistance genes to their active form. In a STGC event the upstream neo repeat is reverted to a functional form. In the second type of recombination event, the downstream neo repeat is reverted, resulting in either LTGC or SCE. The recombination products are selected for by addition of neomycin to the cell culture medium.

Adding doxycycline to the growth medium will produce only the products of LTGC, since the tet-off promoter is inactive.

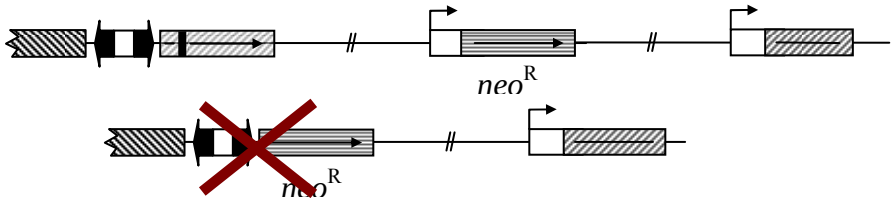


Fig 9. With dox during selection- the tet-off promoter is turned off and only products of LTGC and SCE are selected

In the absence of doxycycline in the medium, both promoters are active and products of STGC and LTGC will both be selected for. This makes it possible to identify the type of recombination event.

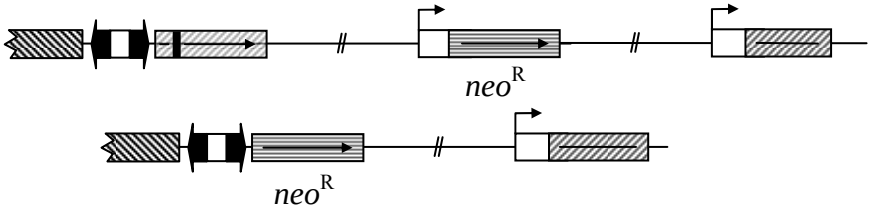


Fig 10. No dox during selection - products of STGC and LTGC/SCE are selected

The Tet-Off System:

In the Tet-Off system, the transcription of a gene is turned on when doxycycline, a tetracycline derivate, is absent from the growth medium. The system consists of a regulatory plasmid and a response plasmid, and the cells consequently have to be double stable, transfected with both of the plasmids for the system to work. The regulatory plasmid, pTet-Off zeo, encodes a trancsriptional activator which is functional in the absence of doxycycline. The plasmid also contains a zeocin resistance gene to make it possible to select successfully transfected cells. The response plasmid, pTre luc, expresses a luciferase gene. The luciferase gene is controlled by the tetracycline response element (TRE), and is expressed when the transcriptional activator expressed from the first plasmid binds to this site in the absence of doxycycline. The gene expression can be measured using a luciferase assay. This transfection is transient and this step is done in order to screen for clones with high inducibility and low background.The next stable transfection is done with the response plasmid pBI-L TARneo (the recombination substrate), which has a hygromycin resistance gene. This plasmid can express two genes controlled by the same TRE, in this case the neomycin resistance gene and luciferase gene. This makes it possible to select clones with high expression of the neomycin resistance gene based on luciferase activity. Clones resistant to hygromycin are selected and screened for luciferase inducibility (for info. On the Tet-Off system, see BD Tet-Off and BD Tet-On Gene Expression Systems User Manual)

Summary of Publications

Paper I: Transcription-Associated Recombination Is Dependent on Replication in Mammalian Cells

Transcription is known to enhance recombination levels in all cellular organisms so far studied. However, this phenomenon has been little studied in mammalian cells and the underlying factors and mechanisms influencing TAR are still largely unknown. To further investigate TAR in mammalian cells, a novel recombination construct was developed. The construct, TARneo, consists of two inactive repeats of the neomycin phosphotransferase gene. The transcription over the over the upstream neomycin resistance repeat can be tightly controlled by addition or removal of doxycycline to the growth medium. As the neomycin resistance repeats are inactive and a recombination event is required for neomycin resistance, the recombination frequency can be measured as the number of neomycin resistant clones in a TAR recombination assay. An efficient enhancement of homologous recombination frequency was observed in the absence of doxycycline, i.e. without transcription over the recombination construct. The increase in recombination frequency was not due to a general increase in recombination frequency, as homologous recombination at the *hprt* gene was not increased. Further, the effect of transcription on DSB-induced HR was examined by exploiting the I-SceI site in the TARneo construct. HR was increased by the induction of a DSB; however, transcription did not further increase HR frequencies. This implies that transcription has no further effect on HR frequencies once a HR event has been initiated. Using the TARneo construct, the recombination products arising from short-tract gene conversion (STGC) or long-tract gene conversion/sister-chromatid exchange (LTGC/SCE) can be scored. Examining the recombination products revealed that the outcome of TAR is a gene conversion event including both STGC and LTGC/SCE.

Inhibiting transcription elongation by 2, 3-dichlororibobenzimadazole (DRB) or actinomycin D had no significant effect on recombination levels, in contrast to evidence in yeast, where transcription elongation inhibition has been shown to be a potent inducer of TAR (Chavez and Aguilera, 1997; Prado et al., 1997). In yeast, TAR has been shown

to be S-phase and replication dependent (Prado and Aguilera, 2005; Wellinger et al., 2006). In concordance with this, arresting the S8TofZM3 cells in G_2/M phase by nocodazole treatment, thus hindering them from entering S-phase, abolished TAR. To test if this was a consequence of the collision of replication and transcription complexes, cells were treated with replication inhibitors. However, this did not have any effect on TAR frequencies, in contrast to what was expected. In yeast, TAR has been shown to be a result of head-on collisions between replication-and transcription complexes (Prado and Aguilera, 2005). To investigate whether inhibiting replication and transcription elongation simultaneously, thus lowering the number of collisions may decrease TAR frequencies, cells were treated with replication and transcription elongation inhibitors. Co-treatment did indeed decrease TAR frequencies, strengthening the findings from yeast that TAR may be dependent of replication-and transcription machinery collisions. ATM and ATR kinases have been shown to be activated by replication stress (Abraham, 2001). ATM and Chk1 has further been shown to be involved in signalling for HR at stalled replication forks (Bolderson et al., 2004; Sorensen et al., 2005). Also, DNA-PKcs and ATR has been shown to be required for response to replication stress (Yajima et al., 2006). To investigate the role of these kinases for TAR signalling, TAR frequencies were measured after inhibition of these. There was no effect on TAR frequencies after inhibition of either of these kinases, suggesting that TAR is independent of these signalling pathways, at least at a single locus.

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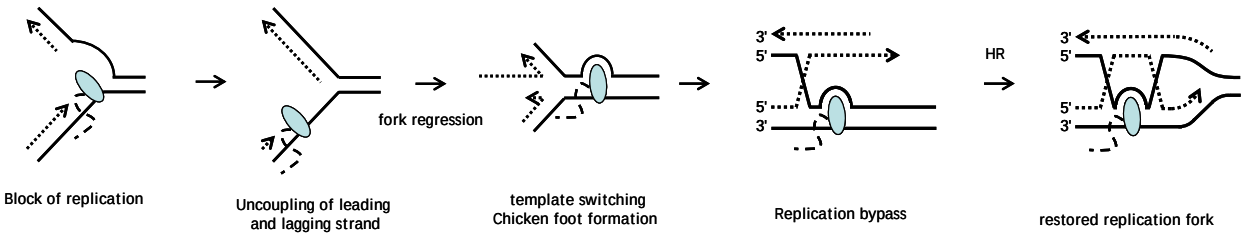
Paper II: Transcription-Associated Recombination is independent of XRCC2 and mechanistically separate from homology-directed DNA double-strand break repair

In this paper, we investigated the role of the DNA repair proteins BRCA2 and XRCC2, both required for homologous recombination, on TAR. BRCA2 and XRCC2 deficient hamster cell lines were stably transfected with the TARneo recombination construct. TAR assays were performed; we could show that BRCA2 is needed for TAR, showing that TAR utilised some HR proteins. The BRCA2 deficient cell line was in addition impaired in RAD51 foci formation following ionising radiation as well as thymidine treatment, showing that the BRCA2 protein is required for HR repair of both replication-associated lesions and two-ended DSBs. In contrast, XRCC2 was not needed for TAR, or for lesions occurring as a result of thymidine treatment, but was still required for repair of homology-directed DNA double strand breaks. To verify that XRCC2 had no effect on TAR, the XRCC2 deficient cell line was transfected with an expression vector containing wtXRCC2. The expression of XRCC2 protein was checked by Western blot, and TAR assays were performed. We found that expression of wtXRCC2 did not affect the TAR frequency. However, the defect in homology-directed DSB repair was reverted, showing that the expressed XRCC2 was indeed functional. We concluded that the TAR recombination pathway is mechanistically separate from the recombination repair pathway used for repair of two-ended DNA DSBs. Further, we presented a model in which the RNA polymerase is bypassed during replication.



Paper III: The XPD subunit of TFIIH is required for transcription-associated but not DNA double-strand break-induced recombination in mammalian cells

The *XPD* gene encodes one of the two helicase subunits of the transcription factor TFIIH. TFIIH has a dual function: it is required both for basal transcription by the RNA polymerases I and II (RNAPI and RNAPII), as well as for nucleotide excision repair (NER). The importance of a functional XPD protein is demonstrated by rare NER deficiency syndromes in humans; xeroderma pigmentosum (XP), trichothiodystrophy (TTD) and combined features of XP and Cockayne syndrome (XP/CS). These symptoms seen in patients suffering from these disorders are very divergent; despite this the disorders arise from mutations in the same gene; the *XPD* gene. We could show that the XPD subunit of TFIIH is required for TAR, but not for repair of two-ended DNA DSBs. We further found a reduced level of spontaneous HR in XPD defective cells, which could indicate that transcription is responsible for a portion of spontaneous HR events. Cell lines expressing XPD proteins with XP, TTD or XP/CS mutations were generated. The TAR deficiency was not rescued in these cells, indicating that the XPD protein needs to be fully functional for TAR to be induced. We also investigated the role of the CSB protein, essential for transcription-coupled repair, in TAR. We found that the CSB deficient cells were impaired in TAR, but not in recombination in general. However, we also found a transcription defect in these cells, and thus cannot determine whether the TAR defect is related to a downregulation of transcription or a genuine role in TAR.

Paper IV: Transcription-associated UV-induced DNA damage triggers futile homologous recombination repair in mammalian cells

In this paper, we investigated the connection between HR repair of UV damages and transcription. We found that repair of UV damages by HR requires transcription, but not via the transcription-coupled repair (TCR) pathway. Rather, transcription is required to form a factor promoting HR after UV damage. To investigate the effect of transcription elongation inhibition on HR, Spd8 cells carrying a substrate for HR in the *hprt* gene were treated with varying doses of 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB).

We observed a dose dependent increase in HR frequency, showing that a substrate for HR is formed when transcription elongation is inhibited, in line with results from *S.cerevisiae* (Chavez and Aguilera, 1997). Further, we investigated the effect on RAD51 foci formation of transcription elongation inhibition. We found that the number of RAD51 foci was increased after either 12 or 24 h treatments with DRB, further confirming that transcription elongation inhibition induces HR. Further, wild type AA8 cells and irs1SF cells, mutated in the HR protein XRCC3, were treated with varying dosed of DRB to investigate whether HR is triggered by a toxic lesion or if HR may be a result of increased accessibility for HR proteins to DNA as a consequence of DRB treatment. We found that the HR deficient irs1SF cells were markedly more sensitive to DRB treatment than wt cells, indicating that HR is triggered by a lesion formed by transcription elongation inhibition. Further, we found that complementing the irs1SF cell line with wt human XRCC3 reverted the sensitivity to DRB. The complemented cells were even more resistant to DRB than the AA8 cell line, perhaps as a consequence of overexpression of XRCC3. Further, we we treated wild type cells with increasing doses of UVC in presence or absence of the transcription inhibitor DRB. We found that DRB sensitised the cells to UV treatment, probably as a consequence of inhibited transcription-coupled repair. Next, we treated UV5cell line deficient in the XPD subunit of TFIIH, essential for NER, in the same manner. Surprisingly, UV5 cells maintained the increased sensitivity to DRB, as found in wild type cells, which is unexpected as UV5 cells are already defective in TCR. We then treated XRCC3 mutated and HR defective irs1SF cells with UVC in combination with DRB. We found that co-treating the irs1SF cells with DRB did not increase their sensitivity to UVC, indicating that the increased sensitivity to UVC by transcription inhibition observed in wild type and NER defective cells is mediated by HR. To investigate whether the increased sensitivity to UVC in combination with DRB is connected to replication, as HR is crucial for repair of replication associated lesions, cells were synchronised in the G1 phase of the cell cycle by serum starvation. They were then co-treated with DRB and UVC. The UV sensitivity caused by DRB treatment was decreased in all cell lines, showing that the sensitising effect of DRB is S-phase related. We went on to investigate the amount of γH2AX foci in cells co-treated with UVC and DRB in AA8 and irs1SF cells. We found that co-treatment

with UVC and DRB increases the amount of DNA damage compared to treatment with UVC alone, showing that DRB induces an increased amount of DNA damage. We further tested whether UVC induced RAD51 foci formation would be affected by DRB treatment. In agreement with the indication that DRB induces DNA damage repaired by HR, a synergistic increase in RAD51 foci formation was seen in AA8 cells after co-treatment with UVC and DRB. The HR deficient irs1SF cells were unable to form RAD51 foci as expected. Next, the HR frequency was measured by using the *hprt* recombination assay in Spd8 cells. Using UVC alone, a dose dependent increase was seen, as expected. Unexpectedly, co-treatment with UVC and DRB decreased recombination frequency. Further, we investigated whether transcription levels would influence UV-induced recombination by utilising cells stably transfected with the TARneo recombination construct. We found an additive effect on HR with increasing doses of UVC, indicating that UV induces the same number of recombination events regardless of transcription.

Future Perspectives

The establishment of a human cell line carrying our recombination substrate would be of great value for further investigations, as this would open up for the possibility of siRNA experiments. As there is no sequence for the hamster genome to date, this excludes the possibility of using this technique in hamster cells. Once a human TARneo cell line has been established, it would be interesting to further investigate the role of DNA repair proteins on TAR.

Establishment of a human TARneo cell line would also make it possible to explore the involvement of novel proteins in TAR, such as the newly discovered RECQL5 helicase (Aygun and Svejstrup). It has been shown in yeast that R-loops are formed in mutants of the THO complex. It would be interesting to further investigate whether the putative formation of R-loops has an effect on TAR in mammalian cells, and to investigate whether R-loops form under normal conditions in mammalian cells. This can be achieved by introducing a plasmid encoding an RNAse, which can cleave the RNA part of the potential RNA-DNA hybrid of the R-loop. If TAR is due to R-loop formation in

mammalian cells, this would reduce the TAR frequency. Further, it would be interesting to investigate the mechanisms of TAR in more detail. We have seen indications of the mechanisms, for instance that stalling replication forks with thymidine gives rise to the same response in cells as high levels of transcription, but as yet we have no biochemical evidence to support our models.

Concluding Remarks

This thesis has focused on the mechanisms and factors influencing transcription-associated recombination (TAR) in mammalian cells. TAR is a phenomenon found in all organisms so far studied. However, the rationale for the occurrence of TAR remains to be found. The proteins required for catalysing TAR in mammals are not known, and neither is the possible connection to DNA repair pathways identified. The primary aim has been to investigate which pathways that TAR employs, and to elucidate which proteins that are involved in catalysing TAR. To achieve this, a number of cell lines based on well-described Chinese hamster cell lines, deficient in proteins known to be required for various DNA repair pathways, were established. These novel cell lines were stably transfected with a recombination construct in which transcription could be regulated and recombination frequency determined as the number of clones resistant to selective media. Using this approach, the involvement of several proteins required for TAR in mammals were revealed. Further, the discovery that TAR employs a recombination pathway distinct from DSB induced recombination was made. In addition, a connection between replication fork stalling and TAR in mammals was established. The investigations into the mechanisms and rationale for TAR in mammals are still virgin territory, and a lot remains to be explored further.

Acknowledgements

Tack till

Min handledare Thomas Helleday för roliga projekt, aldrig sinande entusiasm och för att du givit mig förtroendet att jobba självständigt, jag har lärt mig mycket under den här tiden

Tobias Cassel för att du startade upp mig på projektet

Klaus för expertis inom såväl stenslipning som DNAreparation

Hela GMT, framförallt Toxkorridoren, för att ni är en så trevliga att jobba med

Helleday och Jensengrupperna, det har varit ett sant nöje att dela lab med er, jag har verkligen uppskattat den öppna och trevliga stämningen, alla f.d toxare, Jesper, Anna och Cissi icke att förglömma

Alla proffsiga postdocs i Helledaygruppen för att ni är så generösa med att dela med er av era kunskaper till oss doktorander

Esther för att du hjälpt mig så mycket inför disputationen och för korrläsning, du har varit oumbärlig

Sushigänget; Anette, Anne. Lina och Natalia för utflykter utanför korridoren

Petra och Ingegerd för att ni är så bra rumskompisar och att ni delar med er av era datorkunskaper

Mina föräldrar för stöd, uppmuntran och dagishämtningar

Marie-Louise för hjälp med festen och förståelse för min eremittillvaro senaste tiden

Och framför allt: mina älsklingar Peter och Elsie, det är en ynnest att få dela livet med er, ni är fantastiska ♥!

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