Abstract of Dissertation

Scott D. McCulloch

The Graduate School
University of Kentucky
April 2002
IDENTIFICATION AND CHARACTERIZATION OF MULTIPLE DNA LOOP REPAIR PATHWAYS IN HUMAN CELLS

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Graduate School at the University of Kentucky

By
Scott D. McCulloch
Lexington, Kentucky

Co-Directors: Dr. Guo-Min Li, Professor of Pathology and Laboratory Medicine and Dr. Stephen Zimmer, Professor of Microbiology and Immunology

Lexington, Kentucky
April 2002
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IDENTIFICATION AND CHARACTERIZATION OF MULTIPLE DNA LOOP REPAIR PATHWAYS IN HUMAN CELLS

The stability of DNA is a critical factor for several diseases, the most prevalent of which is cancer. Several neurodegenerative and accelerated aging diseases are also characterized by genomic instability. The number and complexity of DNA repair pathways that human cells possess underscores the importance of genomic stability. These pathways ensure that damaged DNA is repaired and that a cell’s complement of DNA remains stable upon cell division. How one particular type of DNA alteration, a DNA loop, is processed in human cells was the focus of this study. We have employed an in vitro system to study defined DNA loop substrates by human nuclear extracts. The influence of either a 5’ or 3’ nick, the range of loop sizes processed, and the role of DNA mismatch repair, DNA nucleotide excision repair, and the Werner Syndrome helicase proteins were variables tested.

The results indicate that DNA loops containing between 5 to 12 nucleotides are processed in a strand-specific manner when either a 5’ or 3’ nick is present, with repair being targeted solely to the nicked strand. This repair occurs by both mismatch repair dependent and independent pathways. The processing of DNA loops containing 30 nucleotides in length is directed either by a 5’ nick, or by the loop itself, but not by a 3’ nick. The nick independent pathway results solely in loop removal. The large loop pathway is independent of mismatch repair, nucleotide excision repair, and the WRN helicase/exonuclease protein. Both of the 5’ nick directed pathways occur by excision that initiates at the pre-existing nick and proceeds
towards the loop along the shortest path between the nick and loop. DNA resynthesis occurs using either DNA polymerase $\alpha$, $\delta$, or $\epsilon$ and also initiates at the pre-existing 5’ nick. The 3’ nick directed intermediate loop repair pathway proceeds in a similar fashion, likely after a nick is made 5’ to the loop region on the strand that contained the pre-existing nick. DNA synthesis inhibition has only a minor affect on the nick independent loop removal pathway as only a short tract of DNA surrounding the loop site is processed. In total, the results point to at least 3 novel pathways that process DNA loops that likely contribute to total genomic stability.

**Keywords:** DNA Loop Repair, Genomic Stability, Mismatch Repair, Carcinogenesis, Nucleotide Excision Repair
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Dedicated to my parents Leslie Ruth McCulloch and (Late) William Alan McCulloch, who taught me the importance of thinking for yourself, while always thinking about others.
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CHAPTER 1
Mechanisms Of DNA Repair In Human Cells

I. Introduction

The stability of the hereditary material in any organism is of critical importance. For multicellular organisms such as humans, every aspect of cellular function is ultimately dependent on the information carried by a cell’s total complement of DNA, or genome. Cells that divide by mitosis must ensure that daughter cells have a viable copy of the genome, or the RNA and proteins required for normal functioning may be made incorrectly or not at all. During production of gametes by meiosis the stability of the genome has enormous implications not only for the immediate offspring of an individual, but for the future of the species as well. For individuals, the rate of instability of DNA over a lifetime is a major factor in the probability of getting cancer (Loeb, 1991). Alterations to the DNA that cause activation of oncogenes and/or loss of tumor suppressor genes can allow unchecked proliferation of cells, a key event in carcinogenesis. Several hereditary cancer syndromes, as well as many sporadic cancers, are caused by loss or malfunction of pathways responsible for maintaining DNA integrity (Vogelstein and Kinzler, 1998). Several other diseases also have as a defining characteristic DNA instability (Mitas, 1997; Moses, 2001).

The number and complexity of pathways cells devote to ensuring genomic stability underscore its importance. Loss of any of these pathways can cause DNA instability in actively growing cells. Ironically, the loss of proteins that are involved in the metabolism of normal DNA can also cause instability. There are three excision repair pathways that can remove most types of altered DNA (Friedberg et al., 1995). Base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MR) each involve several proteins and multiple steps of processing. In addition to these pathways, there are several other mechanisms by which human cells process altered DNA, such as direct removal of damage, recombination, and bypass of DNA lesions by specialized DNA polymerases. There are also mechanisms that help to prevent DNA damage from occurring, including systems to decrease the oxidative burden of a cell and proteins that remove potentially mutagenic DNA precursors. Finally, multicellular organisms have the luxury of selective cell death to ensure the continued existence of the whole. Dedicated
II. Nucleotide Excision Repair

As one of the most versatile DNA excision repair pathways, NER is responsible for the removal of bulky, helix distorting lesions primarily caused by exogenous chemicals and other physical insults (Wood, 1997). It is the only known mechanism by which the cyclobutane pyrimidine (CPD) adducts caused by ultraviolet (UV) light are removed (Wood et al., 1988). Defects in this pathway can cause at least 2 different diseases, Xeroderma pigmentosum (XP) and Cockayne’s Syndrome (CS), and possibly a third, trichothiodystrophy (TTD). XP is characterized by a severe sensitivity to sunlight and an increased incidence of UV-induced skin cancers, and to a lesser degree by progressive deterioration of the skin, eyes and nervous system (Bootsma et al., 1998). Seven different complementation groups (XP-A through XP-G) have been identified using cells from patients with this disease and the genes responsible are named XPA through XPG (Table 1-1) (Bootsma et al., 1998; Friedberg et al., 1995). CS is characterized by sun sensitivity, neurological dysfunction and a host of other physical ailments (Bootsma et al., 1998). CS patients are deficient for only 1 of the 2 sub-pathways of NER (see below). Mutations in XPB, XPD, and XPG, as well as 3 other genes (CSA, CSB, XAB2) can cause CS. Specific mutations in both the XPB and XPD genes have been documented in patients with TTD, but there is currently some debate as to whether the disease is caused by a defect in NER. TTD patients exhibit sulfur-deficient brittle hair, brittle toe and fingernails, ichthyosis, and about half of all patients exhibit UV sensitivity (see Bootsma et al., 1998; Friedberg et al., 1995). TTD has been suggested to be a disease with subtle defects in both NER and transcription (de Boer et al., 1998; de Boer et al., 1999). The phenotype may be primarily caused by defects in transcription, since XPB and XPD are components of the general Transcription Factor II H (TFIIH) complex (see below), but a slight decrease in NER is also observed which could account for the UV sensitivity.

The ‘core’ NER system can be artificially divided into 4 categories: lesion recognition, DNA opening, DNA excision/damage removal, and DNA resynthesis. XPC, in combination with the human homolog of RAD23B (hHR23B), is the main lesion recognition factor, at least
Table 1-1. Factors required for nucleotide excision repair in human cells. Proteins required for the core NER reaction up to but not including DNA resynthesis and ligation.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>XPA</td>
<td>Lesion verification; Lesion recognition</td>
</tr>
<tr>
<td>XPC</td>
<td>Lesion recognition, GGR initiation (complexed with hHR23A)</td>
</tr>
<tr>
<td>HHR23A</td>
<td>Lesion recognition, GGR initiation (complexed with XPC)</td>
</tr>
<tr>
<td>DDB1$^\S$</td>
<td>Lesion recognition (forms complex with DDB2)</td>
</tr>
<tr>
<td>DDB2$^\S$</td>
<td>Lesion recognition (forms complex with DDB1)</td>
</tr>
<tr>
<td>TFIH$^*$</td>
<td>Lesion demarcation/bubble formation</td>
</tr>
<tr>
<td>XPB</td>
<td>3’ → 5’ helicase</td>
</tr>
<tr>
<td>XPD</td>
<td>5’ → 3’ helicase</td>
</tr>
<tr>
<td>RPA$^\‡$</td>
<td>Bubble stabilization; ssDNA protection; lesion verification?</td>
</tr>
<tr>
<td>XPF</td>
<td>5’ incision (forms dimer with ERCC1)</td>
</tr>
<tr>
<td>ERCC1</td>
<td>5’ incision (forms dimer with XPF)</td>
</tr>
<tr>
<td>XPG</td>
<td>3’ incision</td>
</tr>
<tr>
<td>CSA</td>
<td>Unknown; required for TCR</td>
</tr>
<tr>
<td>CSB</td>
<td>Unknown; required for TCR</td>
</tr>
<tr>
<td>XAB2</td>
<td>Unknown; required for TCR</td>
</tr>
</tbody>
</table>

$^\S$ DDB1 and DDB2 are mutated in some XP-E patients.
$^*$ TFIH core subunits p62, p44, p34, p52 and kinase subunits CDK7, CCNH, MNAT1 are also in this complex.
$^\‡$ The RPA protein has 3 subunits: p17, p24, and p70.

for UV photoproducts (Jones and Wood, 1993; Reardon et al., 1993; Sugasawa et al., 1998). Cells from the XP-E complementation group can be restored to normal by the addition of two related proteins, damaged DNA binding proteins 1 and 2 (DDB1 and DDB2) (Hwang et al., 1998; Treiber et al., 1992). The exact role of the XP-E complementing proteins in NER is not clear, although a recent publication demonstrated that they stimulate the removal of UV photoproducts from DNA by the rest of the NER system (Wakasugi et al., 2002). After a lesion has been recognized, the DNA duplex surrounding the lesions is partially unwound, creating a small ‘bubble’ structure. The next step ‘marks off’ the lesion site by creation of a larger bubble.
TFIIH, specifically the ATP hydrolysis dependent helicase activities of XPB (3'→5') and XPD (5'→3'), the human single stranded DNA binding protein replication protein A (RPA), and XPA are recruited to create and stabilize this expanded bubble region (Coverley et al., 1992; Schaeffer et al., 1994; Shivji et al., 1992; Volker et al., 2001; Wood, 1997). Next, XPG and the dimer of XPF and the excision repair cross complementing protein 1 (ERCC1) are recruited to the growing DNA/protein complex. Incisions are made at the 3' (XPG) and 5' (XPF/ERCC1) junctions of the bubble structure on the strand containing the damaged base (Matsunaga et al., 1995; O'Donovan et al., 1994). This creates a 24-32 nucleotide (nt) long fragment that contains the damaged DNA (Huang et al., 1992). Both of these activities are structure specific endonucleases that nick DNA at junctions of double stranded and single stranded DNA (Friedberg et al., 1995). The fragment containing the damage is released from the NER complex (exactly how is unknown) and then a PCNA dependent DNA polymerase (Pol) fills the gap (Shivji et al., 1992). It is presumed that either Pol ε or δ, with replication factor C (RFC), is responsible for this step, with DNA ligase I presumably sealing the nick, restoring the DNA to normal (Bootsma et al., 1998; Wood, 1997; Wood and Shivji, 1997).

There are 2 sub-pathways of NER: global genome repair (GGR) and transcription coupled repair (TCR). The XPC protein is the primary lesion recognition factor for GGR (Sugasawa et al., 1998), which is a slower process than TCR, but acts on the entire genome. TCR is the rapid and preferential removal of lesions from the transcribed strand of active genes (Mellon et al., 1986; Mellon et al., 1987). This mechanism is thought to ‘tag’ the active genes required for cell survival for preferential repair, and a stalled RNA polymerase is a possible damage sensor. The CSA, CSB and XAB2 proteins are involved in this process (Nakatsu et al., 2000; Troelstra et al., 1990; van Hoffen et al., 1993), as are at least 2 MR proteins, MutS homolog 2 (MSH2) and MutL homolog 1 (MLH1) (Mellon et al., 1996). The breast cancer susceptibility gene BRCA1 has also been implicated in TCR of oxidative damage (Gowen et al., 1998). XPA, which does have the ability to recognize damaged DNA (Robins et al., 1991), is required for both GGR and some TCR, and is now thought to be involved in ‘lesion verification’ and coordination of the NER events downstream of lesion recognition. As mentioned above, the role of XPE in either of these processes is unclear. How the multitude of proteins required for TCR interact with each other and how the process is coordinated with transcription is unclear.
With so many of the major DNA repair systems involved in some aspect of TCR, it is clear that the process is very critical to total genomic stability.

II. Base Excision Repair

The base excision repair pathway has a more defined set of DNA substrates. In general, lesions and mispairs acted on by BER are expected to arise under normal cellular conditions. Specifically, adducts caused by the normal decay of DNA bases and those created by reactive byproducts of cellular reactions. For example, deamination of cytosine (C) creates a uracil (U):guanine (G) basepair, deamination of 5-methyl C creates a thymine (T):G mispair, the 8-oxoguanine (8-oxoG) adduct is created when a hydroxyl radical (⋅OH) interacts with G, and lipid peroxidation of adenine (A) causes the 1-N6-ethenoadenine adduct (Friedberg et al., 1995). Interestingly, the BER pathway can process two different 8-oxoG lesions. It can remove the 8-oxoG from a C:8-oxoG basepair, and also remove the A from an A:8-oxoG mispair. The latter arises as a result of replication of 8-oxoG adducts, which can form a stable basepair with A (Michaels and Miller, 1992). Several other adduct specific BER enzymes exist in other organisms but have yet to be identified in human cells.

The specificity of BER comes from the initiator of the reaction, proteins known as DNA glycosylases. There are currently 8 identified glycosylases in human cells (Table 1-2) (Wood et al., 2001). Each one specifically recognizes certain lesions. Four of these proteins act on adducts caused by oxidative or alkylation damage to DNA: methyl purine DNA glycosylase (MPG), MutY Homolog 1 (MYH1), endonuclease III homolog 1 (hNTH1), and the human 8-oxoguanine DNA glycosylase 1 (hOGG1). The other four remove uracil from DNA: methyl CpG binding protein 4 (MBD4), single-strand selective monofunctional uracil DNA glycosylase (hSMUG1), thymine DNA glycosylase (TDG), and the uracil DNA glycosylase (UNG). Mispairs containing U can occur either by misincorporation during DNA replication or from the spontaneous breakdown of normal DNA (see Friedberg et al., 1995). The one similar feature of all DNA glycosylases is that they catalyze the breakage of the N-glycosylic bond between the base and the deoxyribose sugar backbone, resulting in the release of a free base (Friedberg et al., 1995).
Table 1-2. Factors required for base excision repair in human cells. Proteins required for the complete BER reaction in human cells.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosylases</td>
<td></td>
</tr>
<tr>
<td>MBD4</td>
<td>Removes T or U paired with G</td>
</tr>
<tr>
<td>MPG</td>
<td>Removes 3-methyl adenine and ethenoA adducts</td>
</tr>
<tr>
<td>MYH1</td>
<td>Removes A paired with 8-oxoG</td>
</tr>
<tr>
<td>hNTH1</td>
<td>Removes T and C glycols, dihydrouracil, and altered pyrimidines</td>
</tr>
<tr>
<td>hOGG1</td>
<td>Removes 8-oxoG paired with C</td>
</tr>
<tr>
<td>hSMUG1</td>
<td>Likely removes U caused by deamination of C</td>
</tr>
<tr>
<td>TDG</td>
<td>Removes ethenoC paired with G</td>
</tr>
<tr>
<td>UNG</td>
<td>Removes U paired A</td>
</tr>
<tr>
<td>General BER Factors</td>
<td></td>
</tr>
<tr>
<td>APE1/HAP1</td>
<td>Cleaves sugar-phosphate backbone 5’ to AP site</td>
</tr>
<tr>
<td>Pol β</td>
<td>Inserts correct base into AP site; removes dRP residue</td>
</tr>
<tr>
<td>DNA Ligase I</td>
<td>Seals 3’ nick after Pol β action</td>
</tr>
<tr>
<td>DNA Ligase III</td>
<td>Seals 3’ nick after Pol β action with binding partner XRCC1</td>
</tr>
<tr>
<td>XRCC1</td>
<td>Seals 3’ nick after Pol β action with binding partner DNA Ligase III</td>
</tr>
</tbody>
</table>

The result of the glycosylase reaction creates an apurinic/aprymidinic (AP) site in the DNA. AP sites do not carry base pairing information and are strong inhibitors of several important cellular pathways (Parikh et al., 1998). They occur frequently in mammalian cells due to spontaneous hydrolysis of the N-glycosyl bond (Lindahl, 1993). Consequently, the next enzyme in the pathway, AP endonuclease (APE1 or HAP1) is of critical importance to cells. APE1 recognizes AP sites and cleaves the sugar-phosphate backbone of the DNA 5’ to the AP site (Doetsch and Cunningham, 1990; Robson and Hickson, 1991; Robson et al., 1992). The resulting nicked DNA strand is a substrate for DNA polymerase β (Pol β), which inserts the correct base by addition to the 3’ hydroxyl group created by APE1 (Bennett et al., 1997). Pol β also removes the deoxyribosephosphate (dRP) sugar group located 5’ to the original AP site.
The final step in the pathway involves DNA ligase III and the x-ray repair cross-complementing protein 1 (XRCC1), which close the nick and restore the DNA to its normal state (Kubota et al., 1996; Marintchev et al., 1999). Alternately, there is a ‘long patch’ BER pathway that has also been described. Specifically, instead of insertion of a single base and cleavage of the dRP residue, Pol β or Pol δ insert several consecutive nucleotides (Dianov et al., 1999; Fortini et al., 1998). The flap endonuclease 1 (FEN1) protein then cleaves the displaced strand and DNA ligase 1 (LIG1) seals the nick (Klungland and Lindahl, 1997; Wu et al., 1996).

III. Mismatch Repair

The last of the triumvirate of excision repair pathways is mismatch repair. Its main function is repairing DNA that contains normal DNA constituents in non-standard conformations (Modrich and Lahue, 1996). The human MR reaction can act on all 8 possible base:base mismatches (A:A, C:C, G:G, T:T, A:C, A:G, C:T, and G:T) as well as short regions of unpaired nucleotides (Holmes et al., 1990; Parsons et al., 1993), where one strand of DNA contains extra bases relative to the complementary strand. These structures are called DNA loops. The primary role of MR is correcting the occasional errors generated during DNA replication (reviewed in Jiricny, 1998b; Modrich, 1997; Wood et al., 2001). This is evident by the microsatellite instability (MSI) seen in cells that lack MR activity. Microsatellites are DNA regions that contain short repetitive sequences repeated many times (Parsons et al., 1993). Cells that lack MR activity show a large variation in the number of repeat units present. For example, a population of normal cells may have 20 units of a GT dinucleotide repeat, whereas a population of cells that lack MR activity will be comprised of subpopulations that contain anywhere from 16-24 repeat units (the actual numbers are given for example purposes only). MSI is a hallmark of patients with hereditary non-polyposis colorectal cancer (HNPCC) (Li et al., 2001). This phenotype led to the discovery that HNPCC patients contained germline defects in one allele of one of many MR genes, but primarily in MLH1 and MSH2 (see Peltomaki and Vasen, 1997). HNPCC, also called Lynch Syndrome, is defined by the existence of 3 family members in successive generations with colorectal cancer (CRC), at least 1 of which who was diagnosed before the age of fifty (Lynch et al., 1993). By contrast, CRC in the general population is diagnosed at an average age of 70 (Kinzler and Vogelstein, 1996). HNPCC
patients are also more susceptible to cancers of the endometrium, ovary, brain, urinary tract, stomach, and most other organs of epithelial origin (Lynch et al., 1991; Watson and Lynch, 1993). Several other spontaneous cancers have also been found to have MSI$, suggesting loss of MR (Boland et al., 1998). The decreased replication fidelity caused by loss of MR likely allows the accumulation of mutations required for a cell to become cancerous.

MR in human cells involves at least 5 different proteins that are homologous to MR proteins found in bacteria, as well as several other protein involved in other pathways of DNA metabolism (Table 1-3). Recognition of mismatches and loops is performed by two different heterodimers of proteins which are homologous to the bacterial MutS protein. MSH2 is the common partner of both MSH6 and MSH3 (Acharya et al., 1996). The complex of MSH2/MSH6 is termed human MutS$\alpha$ (hMutS$\alpha$) and is responsible for recognition of all 8 base:base mismatches, as well as DNA loops (Drummond et al., 1995; Palombo et al., 1995). The complex of MSH2 and MSH3 is called hMutS$\beta$. hMutS$\beta$ binds primarily to DNA loops (Acharya et al., 1996; Palombo et al., 1996; Wilson et al., 1999), although a recent report suggests it can recognize at least one base:base mismatch (Berardini et al., 2000).

Table 1-3. Factors required for mismatch repair in human cells. Proteins identified as being involved in the MR reaction in human cells

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSH2</td>
<td>Mispair/loop binding as a dimer with either MSH3 or MSH6</td>
</tr>
<tr>
<td>MSH3</td>
<td>Loop binding as a dimer with MSH2 (MutS$\beta$)</td>
</tr>
<tr>
<td>MSH6</td>
<td>Mispair/loop binding as a dimer with MSH2 (MutS$\alpha$)</td>
</tr>
<tr>
<td>MLH1</td>
<td>Coordination of repair steps? Forms a dimer with either PMS2 or MLH3</td>
</tr>
<tr>
<td>MLH3</td>
<td>Coordination of repair steps? Forms a dimer with MLH1 involved in MutS$\beta$ mediated repair</td>
</tr>
<tr>
<td>PMS2</td>
<td>Coordination of repair steps? Forms a dimer with MLH1 (MutL$\alpha$)</td>
</tr>
<tr>
<td>PCNA</td>
<td>Increases specificity of MutS$\alpha$ and MutS$\beta$; Processivity factor of DNA Pol $\delta$</td>
</tr>
<tr>
<td>ExoI</td>
<td>Exact role currently unknown; The protein has 5' $\rightarrow$ 3' exonuclease activity</td>
</tr>
<tr>
<td>DNA Pol $\delta$</td>
<td>DNA resynthesis</td>
</tr>
<tr>
<td>RPA</td>
<td>Protection of single stranded DNA after excision step</td>
</tr>
</tbody>
</table>
There is an approximately 10:1 ratio of hMutSα:hMutSβ in normal cells (Genschel et al., 1998). Another dimeric complex, hMutLα, is comprised of MLH1 and the post-meiotic segregation 2 (PMS2) proteins (Li and Modrich, 1995). Despite its name, PMS2 is a homolog of bacterial MutL as well. hMutLα is required for both mismatch and loop repair, although its exact role is unclear. Other complexes of MutL related proteins also exist in the cell (Kondo et al., 2001). Their exact role, if any, in MR is not currently understood. There are suggestions that hMutLα plays a role as a ‘molecular matchmaker’ to coordinate the different steps of the MR reaction (Jiricny, 1998a). The binding of hMutSα and/or hMutSβ to substrate DNA requires ATP, and the complex then moves along the DNA (Modrich, 1997). Whether this movement is dependent on ATP hydrolysis or simple diffusion is unclear, with two hypotheses currently presented in the literature (Gradia et al., 1997; Blackwell et al., 1998; Fishel, 1998; Iaccarino et al., 1998; Gradia et al., 1999, Gradia et al., 2000). Regardless, ATP hydrolysis by both hMutSα and/or hMutSβ is required for a complete MR reaction.

At this step in MR in *E. coli*, a helicase is brought in at a nick, the DNA unwound and the displaced strand is degraded by 1 of 4 redundant exonucleases (Dao and Modrich, 1998; Yamaguchi et al., 1998; Burdett et al., 2001). Unfortunately, the human system is not as well characterized. Other proteins that have been demonstrated to be involved in eukaryotic MR include PCNA, RPA, Exo1, and DNA polymerase δ (Pol δ). PCNA is implicated not only in DNA resynthesis (as a processivity factor for Pol δ), but also at a step prior to excision (Gu et al., 1998; Umar et al., 1996). It has also been demonstrated to increase the specificity of substrate binding of both yeast and human MutSα and MutSβ (Clark et al., 2000; Flores-Rozas et al., 2000). Exo1 has been shown to bind to MSH2 in both human and yeast cells, and yeast strains deficient in Exo1 show a slight mutator phenotype (Schmutte et al., 1998; Tishkoff et al., 1998). The spectrum of mutations is similar to those found in MR mutant strains (Amin et al., 2001). Very recently, hExo1 was demonstrated to be required for MR *in vitro* (Genschel et al., 2002). RPA is suspected of protecting the single stranded DNA in between excision and resynthesis, although this has yet to be directly demonstrated in human cells (Lin et al., 1998; Umezu et al., 1998). It is, however, a required component of the *in vitro* MR reaction (Ramilo et al., 2002). DNA polymerase δ is known to contribute to DNA resynthesis *in vitro*, but involvement of DNA Polymerase α was not completely ruled out in this study (Longley et al., 1997).
One striking difference between the *E. coli* and human MR mechanism is the strand discrimination signal. Transient hemi-methylation of the N\(^6\) of adenine at d(GATC) sequences is used in *E. coli* to designate ‘parent’ and ‘daughter’ strand immediately following replication (Modrich, 1989). The MutH protein contains an endonuclease activity that nicks the unmethylated strand at this sequence and the nick serves as the entry point for either Helicase II (Dao and Modrich, 1998; Yamaguchi et al., 1998). This nick can be located either 5’ or 3’ to the mismatch site (Cooper et al., 1993). In human cells, a homolog of MutH has not been identified, and the methylation of other sequences does not affect repair *in vitro* (Drummond and Bellacosa, 2001). However, a pre-existing nick does serve to direct repair to the nicked strand, and this nick can be located either 5’ or 3’ to the nick and up to several hundred base pairs away (Fang and Modrich, 1993; Holmes et al., 1990). Substrates that do not contain a nick undergo much lower levels of processing, and there is no discrimination between which strand is repaired. One current idea regarding strand discrimination in eukaryotes is that MR is physically linked to the DNA replication machinery through PCNA. The end of the leading or lagging strands, as well as the nicks created by Okazaki fragments in the lagging strand would then serve as the strand discrimination signal. Direct support of this hypothesis is still lacking however.

**IV. Other mechanisms of DNA stability**

Although the three excision repair pathways can process most types of DNA alterations, there are other pathways involved in the stability of DNA. Double strand breaks (DSB), which can be caused by ionizing radiation, are processed by either homologous or non-homologous recombination (Lindahl and Wood, 1999). Each of these pathways involves multiple activities (see Wood et al., 2001). There is at least one mechanism of direct damage reversal. Methylation of the O\(^6\) position of guanine is a common reaction in cells, with both environmental contaminants and endogenous byproducts contributing reactive methyl groups (Sedgwick, 1997; Vaughan et al., 1993). Consequently, a dedicated protein is made to restore the O\(^6\)-methylguanine adduct to normal (Moore et al., 1994). Methyl guanine methyltransferase (MGMT) directly removes the methyl group from the DNA and transfers it to a cysteine on the protein. This is a ‘suicide reaction’ because the S-methylcysteine protein adduct is very stable, thereby inactivating each protein as it performs its evolved function. Recently, it has been realized that human cells possess no less than 15 DNA polymerases (Lindahl and Wood, 1999).
Many of the newly discovered enzymes have very low processivity, but are capable of inserting bases opposite DNA lesions that would halt polymerases $\alpha$, $\delta$, and $\varepsilon$. One of these, Pol $\eta$, has been identified as the cause of XP-variant cases (Masutani et al., 1999). These patients display XP-like symptoms, but have normal levels of NER. Pol $\eta$ has been shown to preferentially insert A when it encounters thymine-dimer lesions (i.e the correct base) (Washington et al., 2001). It is thought that the loss of this activity causes XP-like symptoms by channeling thymine dimer lesions from the error free Pol $\eta$ lesion bypass pathway to the error prone polymerase $\zeta$ pathway (Wood, 1999). Lastly, there is a poorly defined pathway for the repair of DNA interstrand crosslinks. This pathway involves some NER proteins (XPF/ERCC1), some MR proteins (MutS$\beta$), a polymerase, and proteins that when missing cause Fanconi’s Anemia (FA) (Li et al., 2000; Wang et al., 2001; Zhang et al., 2000; Wood et al., 2001). There are at least eight complementation groups in cells from patients FA (FA-A through FA-H), suggesting that this pathway is at least as complicated as NER (Buchwald, 1995; Joenje et al., 1997).

In addition to pathways that deal directly with damaged DNA, there are consequences of losing proteins that are thought to be involved in normal DNA processing. One example is the family of RecQ helicase homologs (Chakraverty and Hickson, 1999; Shen and Loeb, 2000). At least 3 separate diseases are caused by the loss of one of these proteins. Each disease exhibits signs of accelerated aging (Ellis et al., 1995; Yu et al., 1996; Kitao et al., 1999). Cells from patients with Bloom’s syndrome (BS), Werner syndrome (WS), and Rothmund-Thompson syndrome (RTS) all display genomic instability and sensitivity to DNA damaging agents. Of interest here is the Werner’s Syndrome (WS) protein WRN. This protein is a 3’ $\rightarrow$ 5’ DNA helicase that also contains a 3’ $\rightarrow$ 5’ exonuclease activity (Shen et al., 1998). The WRN protein can bind to bubble and loop structures of DNA (Machwe et al., 2002). It has also been shown to interact with DNA Pol $\delta$ during replication of complex DNA structures (Kamath-Loeb et al., 2000; Kamath-Loeb et al., 2001).

V. Research Objectives

The extent of DNA damage processing pathways and the number of diseases that are caused by defects in some aspect of DNA metabolism are evidence of the importance of understanding DNA stability. One type of structure that has not been extensively studied in regards to DNA repair are the DNA loops. These structures are of particular interest in human
cells because of the abundance of repeat sequences present in the human genome (Hamada et al., 1982; Tautz, 1989; Beckmann and Weber, 1992). Mistakes during replication and recombination of this type of DNA can lead to looped structures that have the potential to cause severe changes in DNA. The early studies of human MR demonstrated that small loops (1-4 nt) could be processed by MR, but subsequent work has demonstrated that loops larger than this are subject to MR independent mechanisms of removal (Umar et al., 1994; Genschel et al., 1998). That they are processed has been known for over a decade (Ayares et al., 1987; Weiss and Wilson, 1987), but which pathways are involved is not well understood. Recent work in yeast shows that repair of large loops (> 25 nt) occurs by pathways other than those described above (Corrette-Bennett et al., 1999; Corrette-Bennett et al., 2001). Whether the same holds true for human cells is the focus of the work presented here. We have used a well characterized *in vitro* system that utilizes a defined DNA substrate and human cell nuclear extracts to investigate the repair of DNA loops ranging in size from 5 to 30 nt. This information will fill a crucial gap in the understanding of total genomic stability.
CHAPTER 2
Materials And Methods

I. General Techniques
I.A. Preparation of solutions

All solutions and cell culture media were prepared using 18 MOhm, deionized distilled water (ddH₂O). Solutions were sterilized either by an autoclave for 15 min at 121° C and 15-18 pounds per square inch of pressure or by filtering through a 0.22 µm filter. Stock solutions of buffers were prepared to give the desired pH for the working concentration of buffers. Unless otherwise noted, reagent grade chemicals were purchased from Sigma (St. Louis).

I.B. Agarose electrophoresis

Unless otherwise indicated, agarose gel electrophoresis was performed using ultrapure agarose (Gibco-BRL, Rockville) and Tris/Acetate/EDTA (TAE) running buffer that contained 40 mM Tris·Acetate (pH 8.5) and 2 mM ethylenediamine tetraacetic acid (EDTA) using standard methods (see Ausubel et al., 1987). DNA samples were prepared for analysis by the addition of 0.2 volumes (vol) of 6X agarose gel loading buffer that contained 40% (w/v) sucrose, 0.05% (v/v) xylene cyanol, 0.05% (v/v) bromophenol blue, 20 mM EDTA, and 0.2% sodium dodecyl sulfate (SDS). Gels were stained for 15-20 min using a solution of 0.5 µg/ml ethidium bromide (EtBr) in ddH₂O and then rinsed for 15-20 min in ddH₂O. Gels were visualized on an ultraviolet (UV) transilluminator and images captured using a Kodak DC120 digital camera (Kodak, Rochester). Band intensities of gel images were analyzed using Kodak Image 2.0.2 software.

I.C. Dialysis

Dialysis of both protein and DNA solutions was performed using Spectra/Por dialysis membrane (Spectrum Laboratories, Inc.). The membrane molecular weight cutoff was 6,000 – 8,000 Dalton (Da). Membranes were equilibrated in the appropriate dialysis buffer for at least 5 min prior to use. Unless otherwise noted, all dialysis was performed at 4° C with constant stirring in at least a 100-fold excess of the designated buffer relative to the sample volume.
II. Cell Culture

II.A. Bacteriophage and prokaryotic cells

II.A.1. Bacteriophage characteristics

The f1MR series of phage use the F pili to gain entrance into *E. coli* cells (Kornberg and Baker, 1992). The phage reproduction cycle allows for both double stranded DNA (dsDNA) and single stranded DNA (ssDNA) copies of the phage genome to be produced. For the experiments performed, the ssDNA was designated as the Viral (V) strand and dsDNA was composed of V and Complementary (C) strands.

II.A.2. Growth and storage techniques

Bacterial growth and handling was performed using standard sterile technique (see Ausubel et al., 1987). All bacterial cultures were of the XL1-Blue strain of *Escherichia coli* (*E. coli*) (Stratagene, La Jolla). The liquid media used was 2X-TY, which contained 1.6% (w/v) tryptone, 1.0% (w/v) yeast extract and 0.5% (w/v) NaCl. The solid media used was Luria-Bertani (LB), which contained 1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 1 mM NaOH, and 1.5% (w/v) agar. Unless otherwise noted, liquid cultures were grown at 37°C and 250 revolutions per minute (RPM) in containers that had at least a 3 fold volume of air compared to the growth media volume. Solid cultures were grown for 16-24 hours in a 37°C incubator. When used, ampicillin (50 µg/ml) or tetracycline (12 µg/ml) was added to media immediately prior to use.

Stocks of bacterial strains were prepared by adding 1.0 ml of saturated culture to 0.5 ml of 7% (v/v) dimethylsulfoxide (DMSO). This solution was frozen by submersion in liquid nitrogen and stored at –80°C. Liquid stocks of bacteriophage were prepared from 50 ml of 2X-TY that was inoculated with 0.5 ml saturated XL1-Blue culture that had been infected with the phage of interest and the culture was grown for 7-8 hr. The culture was incubated for 10 min in an ice-water slurry and centrifuged for 10 min at 2000 g at 4°C. The cell pellet was discarded and the supernatant was re-centrifuged under the same conditions. The supernatant was transferred to a sterile 50 ml glass vial and 1 drop (22.9 cm Pasteur pipette) of glacial (37%) formaldehyde was added. The vial was stored at 4°C. The titer of the phage was determined as described (see Ausubel et al., 1987).
II.A.3. Preparation of competent cells

A 100 ml culture (2X-TY) of *E. coli* was grown at 37°C and 250 RPM and until the optical density at 595 nm (OD$_{595}$) reached 0.6. The cells were harvested by centrifugation for 5 min at 2000 g at 4°C. The supernatant was discarded and the cell pellet was suspended in 5 ml of 2X-TY (pH 6.1) that contained 10% (w/v) polyethylene glycol (PEG) 3350, 5% (v/v) DMSO, 10 mM MgCl$_2$ and 10 mM MgSO$_4$. Aliquots of 100-200 µl were frozen by submersion in liquid nitrogen and stored at -80°C.

II.A.4. Transformation of competent XL1-Blue cells

Frozen aliquots of competent cells were thawed on ice. DNA to be transformed (generally 50-200 ng) was added to 100 µl KCM solution that contained 100 mM KCl, 30 mM CaCl$_2$, and 50 mM MgCl$_2$ and placed on ice. Equal volumes of KCM/DNA solution and competent cells were mixed and incubated on ice for 10-20 min. The cells were incubated at room temperature (RT; 20-23°C) for 10 min and then 1 ml of SOC media that contained 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, and 20 mM glucose was added to the cells. The cells were incubated for 1 hr at 37°C. For transformations with pDG117IIA plasmid DNA, duplicate aliquots of 1, 10, 50, and 100 µl were added to SOC media to make 200 µl total volume and were then spread onto LB plates that contained ampicillin. The plates were then incubated overnight at 37°C. For transformations with phage DNA, duplicate aliquots of 1, 10, 50, or 100 µl were added to 3 ml of LB top agar that contained 1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, and 0.7% (w/v) agar. Top agar was melted in a microwave and cooled to 45-50°C prior to addition of the transformed cell solution. The top agar/cell mixture was poured onto LB plates, allowed to solidify by incubation at RT for 2 hr, and then incubated overnight at 37°C.

II.B. Mammalian cells

II.B.1. Growth and storage techniques

Standard eukaryotic cell culture techniques were used for all manipulations (Freshney, 1994). Cell culture media and reagents were purchased from Gibco-BRL (Rockville). HeLa cells were grown in Roswell Park Memorial Institute (RPMI) 1640 media with 5% fetal bovine
serum (FBS) (Hyclone, Logan) and 4 mM glutamine. HCT15, NALM6, and GM02345 cells were grown in RPMI 1640 with 10% FBS. HCT116 and HEC-1A cells were grown in McCoy’s 5A media with 10% FBS. WS780 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS and 2 mM glutamine. AG08802 cells were grown in DMEM with 10% FBS, 1X non-essential amino acids and 1X vitamins. All cultures contained 10 U/ml penicillin and 10 µg/ml streptomycin. All cells were grown at 37° C with a 95% air/5% CO₂ atmosphere.

Cells grown in suspension (HeLa, NALM6, GM02345) were split 1:4 by addition of fresh media after the cell density reached 1 x 10⁶/ml. Cells were initially grown in culture flasks with a surface area of 75 cm² (Sarstedt, Newton) and then transferred to flasks of 175 cm² as dictated by the volume of the culture. Culture volumes larger than 200 ml were grown in 1 and 6 L spinner flasks (Bellco Glass; Vineland). Frozen stocks of suspension cells were prepared from 10-20 ml of culture that had a density of approximately 1 x 10⁶ cells/ml. Cells were harvested by centrifugation for 5 min at 300 g at RT. The supernatant was aspirated and the cell pellet was suspended in growth media that contained 20% FBS and 10% DMSO so the final concentration of cells was between 5 x 10⁶ and 2 x 10⁷ cells/ml. One (1) ml aliquots were cooled from RT to -80° C at a rate of -1° C/min using a “Mr. Frosty” freezing container (Nalgene, Rochester) and then stored in liquid nitrogen.

Cells grown as a monolayer (HCT15, HCT116, AG08802, WS780) were initially grown in culture flasks with a surface area of 75 cm² (Sarstedt, Newton), then transferred to flasks of 175 cm², then transferred into roller bottles (850 cm² surface area) and rotated on a roller rack (Bellco Glass) at 0.25 RPM. At each stage, cells were grown to confluence, dislodged by trypsin digestion (see Ausubel et al., 1987), then split into culture flasks to give a total surface area of 3-5 times greater than when starting (i.e. 1 bottle was split into 3-5 bottles). Frozen stocks of monolayer cultures were prepared from 2 culture flasks (75 cm² surface area). Cells were dislodged using trypsin, suspended in 10-20 ml media and the cell density determined. Cells were harvested by centrifugation for 5 min at 300 g at RT. The supernatant was aspirated and the cell pellet was suspended in growth media that contained 20% FBS and 10% DMSO so the final concentration of cells was between 5 x 10⁶ and 2 x 10⁷ cells/ml. One (1) ml aliquots were cooled from RT to -80° C at a rate of -1° C/min using a “Mr. Frosty” freezing container (Nalgene) and then stored in liquid nitrogen.
III. Protein Techniques

III.A. Phage fd Gene II Protein

III.A.1. Overexpression and purification

Plasmid pDG117IIA, which allows overproduction of the phage fd gene II product (gpII) (Meyer and Geider, 1981) was a kind gift of Dr. Paul Modrich, Duke University. Selection for cells that contained pDG117IIA after transformation (section II.A.4) was performed using 50 µg/ml ampicillin in the culture media. The gpII purification protocol was modified from Greenstein and Horiuchi (Greenstein and Horiuchi, 1987). Unless otherwise noted, all steps were performed on ice or at 4°C using solutions, bottles, and centrifuge rotors that were pre-cooled to 4°C. One (1) L of 2X-TY was warmed to 37°C and was inoculated with 10 ml of exponentially growing XL1-Blue culture, then incubated at 37°C and 250 RPM until the OD$_{595}$ reached 0.3 (0.75 ml aliquots were measured every 15 min, starting at 60 min post-inoculation). Isopropyl-β-D-thiogalactoside (IPTG) was added until the final concentration was 2 mM and the cells were incubated for an additional 7 hr at 37°C and 250 RPM.

The culture was chilled using an ice-water slurry and the cells were harvested by centrifugation for 15 min at 5000 g. The cell pellet was suspended in 45 ml of buffer gp-A that contained 100 mM maleic acid·NH$_4$ (pH 6.8 at 23°C), 10% (v/v) glycerol, 1 mM EDTA, 5 mM β-mercaptoethanol (β-ME), 0.1% (v/v) phenylmethylsulfonylfluoride (PMSF), and 1 µg/ml leupeptin. PMSF was prepared as a saturated stock (~ 0.1 M) in isopropanol at RT. Leupeptin stock (10 mg/ml) was prepared in 0.1 M potassium phosphate (K$_2$P$_4$) (pH 7.4). The cell suspension was evenly divided into two aliquots and each was sonicated (Fisher Scientific Model 550 Sonic Dismembrator; Pittsburg) on ice as follows: five cycles of 30 s on/30 s off at 10% power; five cycles of 30 s on/30 s off at 15% power; five cycles of 30 s on/30 s off at 20% power. Cell lysis was monitored at each step by light microscopy. More than 90% of the cells were lysed by this treatment.

The cell lysate was centrifuged for 1 hr at 100,000 g using a fixed angle rotor (T-865; Sorvall, Newton). The supernatant was discarded and the pellet was suspended in 17 ml buffer gp-A using the narrow end of a 22.9 cm Pasteur pipette that had been sealed with a Bunsen burner. The solution was stirred on ice for 1 hr while 3 ml of 7 M guanidine hydrochloride (GuanHCl) was added dropwise. The solution was re-centrifuged for 1 hr at 100,000 g as described for the previous spin. The supernatant was discarded and the pellet suspended in 5 ml
buffer gp-A. While stirring at RT for 1 hr, solid Guan-HCl was slowly added until the final concentration was 7 M and the solution was centrifuged as described for the previous spin. The supernatant was retained and stored on ice. A gel filtration column packed with Sephacryl S400 beads (Pharmacia, Piscatawy) equilibrated at RT in buffer gp-B that contained 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM β-ME, and 7 M Guan-HCl. The column diameter was 1.5 cm and the resin bed height was 90 cm, which gave a resin bed volume (BV) of 159 ml. The bed volume is the total volume of the resin: the volume of solution contained within the resin beads and the volume of solution outside the beads. The void volume, defined as the volume of the solution outside the resin beads, was 82-84 ml as measured using Blue Dextran dye with a molecular weight (MW) of 2 x 10^6 Da (Sigma, St. Louis).

Two (2) ml of the supernatant stored on ice above was loaded onto the S400 column and was eluted at RT with a continuous addition of buffer gp-B at a flow rate of ~13 ml/hr. Fractions (3.5 ml each) were collected until 140 ml (40 fractions) of buffer passed through the column. The gpII protein was expected to elute near the void volume (Greenstein and Horiuchi, 1987). A 100 µl aliquot of each fraction was concentrated by adding 400 µl methanol, 100 µl chloroform and 300 µl water, vortexing the solution briefly, and centrifuging for 2 min at 19,600 g in a benchtop microfuge. The upper, aqueous phase was aspirated and 300 µl of methanol was added to the tube. The solution was then vortexed, centrifuged as in the previous step, and the supernatant aspirated. The pellet of precipitated protein was air dried and suspended in 15 µl SDS loading buffer that contained 62.5 mM Tris-HCl (pH 6.8), 2.0% (w/v) SDS, 10 % (v/v) glycerol, 42 mM dithiothreitol (DTT), and 0.01% (v/v) phenol red. The protein solution was then heated to 95°C for 5 min and separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using a 5% stacking gel and 12% separating gel (29:1 acrylamide:bis-acrylamide). Protein size markers were from New England Biolabs (Beverly). Gels were stained for 20 min in a solution that contained 50% (v/v) methanol, 10% acetic acid and 0.05% Coomassie Brilliant blue. Fractions collected from the S400 column that contained gpII were identified by the presence of a band of MW = 46,000 Da.

The fractions that contained the highest amount of gpII (as judged visually from the MW = 46 kDa band intensity) were individually dialyzed using 0.5 L buffer per fraction saved. The first dialysis step was using buffer gp-C that contained 25 mM imidazole-HCl (pH 6.8 at 23°C),
10% (v/v) glycerol, 400 mM KCl, 1 mM EDTA, 5 mM β-ME and 1 M Guan-HCl for 2 hr. Fractions were then dialyzed for a total of 25 hr in buffer gp-C that lacked Guan-HCl, with buffer changes at 5, 10, 15, and 20 hr. The dialysates were cleared of aggregated protein by centrifugation for 10 min at 12,000 g. Aliquots (250 µL) of each dialyzed fraction were frozen in liquid nitrogen and stored at -80° C. An aliquot (10 µl) of each dialyzed fraction was analyzed by SDS-PAGE. The purity was estimated to be 80-95% as judged visually using Coomassie blue stained gels. Protein concentrations were determined by the Bradford method (see Ausubel et al., 1987).

To increase the total yield of gpII isolated, after the first set of S400 fractions were collected, the column was washed by passage of 10 BV of buffer gp-B through it. Another 2 ml aliquot of the supernatant was then loaded onto the column and the steps described above repeated with a second set of S400 fractions.

III.A.2. Determination of gpII activity

The gpII activity from dialyzed fractions was determined using 2 µL of serial 1:10 dilutions (10⁰ through 10⁻³) of the dialyzed fractions in reactions that also contained 250 ng MR1 supercoiled dsDNA (Su et al., 1988), 20 mM Tris-HCl (pH 8.0), 5 mM CaCl₂, 5 mM DTT, and 80 mM KCl. Reactions were incubated for 30 min at 30° C. The DNA was separated through 1% agarose gels. One unit (U) of gpII activity was defined as the amount (in µl) of gpII fraction required to convert 250 ng of DNA from the supercoiled to the nicked form in 30 min at 30° C (Greenstein and Horiuchi, 1987). The supercoiled and nicked forms of the DNA correspond to the faster and slower migrating bands, respectively.

III.B. Nuclear Extract Preparation

Extracts of nuclear protein from mammalian cells were prepared as previously described (Holmes et al., 1990). All steps were performed on ice or at 4° C using ice-cold solutions and pre-cooled bottles and centrifuge rotors. All solutions contained 0.1% (v/v) PMSF and 1 µg/ml leupeptin. For cells grown in suspension, cultures of 5-6 L were grown to a density of 1.0 x 10⁶ cells/ml and then harvested. The cell suspension was chilled to ≤ 10° C with an ice-water slurry, then collected by centrifugation for 10 min at 3000 g. The supernatant was discarded and the
cell pellet suspended in buffer ne-A (20 ml per L of culture) that contained 20 mM Hepes-KOH (pH 7.5), 5 mM KCl, 0.5 mM MgCl₂, 0.5 mM DTT, and 0.2 M sucrose. When cells were grown as a monolayer, roller bottles (10–40 bottles/extract) were emptied of growth media and 20 ml buffer ne-A added per bottle. The cells were dislodged using a cell scraper (Bellco Glass, Vineland). The slurry of cells was transferred to a centrifuge bottle and the roller bottle rinsed with an additional 15 ml buffer ne-A. Whether from suspension or monolayer cultures, steps after the cells were suspended in buffer ne-A were the same.

Cells were then harvested by centrifugation for 5 min at 3300 g, the supernatant decanted, and the wet cell mass was measured. The cell pellet was then suspended in buffer ne-B (2.78 ml per gram of cell pellet) that contained 20 mM Hepes-KOH (pH 7.5), 5 mM KCl, 0.5 mM MgCl₂, and 0.5 mM DTT and then incubated on ice for 10 min. Cells were lysed by Dounce homogenization using a type B pestle (Bellco Glass, Vineland) (see Ausubel et al., 1987). Progress of cell lysis was monitored with a light microscope observing the decrease in intact cells and increase in free nuclei. The number of strokes required to lyse approximately 80% of cells (with ≤ 10% of nuclei lysed) was 3–15 and varied depending on cell line. Nuclei were recovered by centrifugation of cell lysates for 5 min at 2000 g. The nuclear pellet was suspended in buffer ne-C (1.39 ml per gram of starting cells) that contained 50 mM Hepes-KOH (pH 7.5), 10% (w/v) sucrose, and 0.5 mM DTT. The volume of the nuclei solution was measured and 0.031 vol of 5 M NaCl added while swirling gently. This solution was then rotated on a LabQuake shaker (Barnsted-Thermolyne, Dubuque) for 60 min and then centrifuged for 20 min at 14,500 g. The pellet was discarded and the volume of the supernatant was measured. Ammonium sulfate (0.42 g/ml) was slowly added (spanning ~20 min) while the solution was stirred on ice, taking care to prevent frothing of the solution. The solution was stirred for an additional 20 min and then centrifuged for 20 min at 15,800 g.

The supernatant was decanted and the protein pellet was slowly suspended in a small volume (~20–30 µl per gram of starting cells) of buffer ne-D that contained 25 mM Hepes-KOH (pH 7.6), 50 mM KCl, 0.1 mM EDTA, and 2 mM DTT. The protein slurry was dialyzed in buffer ne-D until the conductivity of the extract was equivalent to approximately 100 mM KCl. The conductivity of the protein solution was measured using a 1:400 dilution of extract in ddH₂O until a value of ~50 µS/cm was obtained. The dialyzed extract was cleared of precipitated protein by centrifugation for 10 min at 19,600 g in a microfuge at 4°C. Aliquots (30–40 µl) of
the supernatant were frozen in liquid nitrogen and stored at −80°C. Protein concentrations were measured by the Bradford method (see Ausubel et al., 1987) using duplicate reactions from two different dilutions.

IV. DNA Techniques

IV.A. Bacteriophage construction and DNA isolation

IV.A.1. Construction of bacteriophage

All DNA manipulations were performed using standard molecular techniques (see Ausubel et al., 1987). Unless otherwise indicated all enzymes were purchased from New England Biolabs (Rockville). The bacteriophage used were derivatives of the MR series described previously (Su et al., 1988). Phage MR1 and MR24 were kind gifts of Dr. Paul Modrich, Duke University. The oligonucleotides used to create phage MR24 derivatives that contained 5-12 extra nucleotides were synthesized by Gibco-BRL (Rockville) (Table 2-1). MR24 was previously created using the method described below and the oligonucleotides listed. Pairs of oligomers were mixed in equimolar amounts in 25 mM Tris–HCl (pH 8.0) and 100 mM NaCl, incubated for 5 min at 95°C, cooled at −0.5°C/min to 25°C, then placed on ice. To create each phage, MR1 (Su et al., 1988) dsDNA (100 ng) was digested with *Hin*DI and *Xba*I and the digestion products were separated on a 1% agarose gel. The large fragment (6,429 bp) was recovered and purified by gel extraction (Qiagen Inc., Valencia). Each annealed duplex was ligated to this fragment (separate reactions for each set of duplexes) using T4 DNA ligase as

<table>
<thead>
<tr>
<th>Phage Name</th>
<th>Insert Sequence (boldface indicates extra bases compared to MR24)</th>
<th>Restriction Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR24</td>
<td>3′-.....CGTCGGTGAGCTCACAAGACACACACGGATC C</td>
<td>XcmI</td>
</tr>
<tr>
<td></td>
<td>5′-AGCTGCAGCCAGCGGCTGTGTGGGC.... V</td>
<td></td>
</tr>
<tr>
<td>MR31 (+5)</td>
<td>3′-.....CGTCGGTGAGCTCAGCTGACACACACGGATC C</td>
<td>XhoI</td>
</tr>
<tr>
<td></td>
<td>5′-AGCTGCAGCCAGCTGAGCTGACACACACGGATC A</td>
<td></td>
</tr>
<tr>
<td>MR32 (+8)</td>
<td>3′-.....CGTCGGTGAGCTCACAAGACACACACGGATC C</td>
<td>XhoI</td>
</tr>
<tr>
<td></td>
<td>5′-AGCTGCAGCCAGCTGAGCTGACACACACGGATC A</td>
<td></td>
</tr>
<tr>
<td>MR53 (+10)</td>
<td>3′-.....CGTCGGTGAGCTCACAAGACACACACGGATC C</td>
<td>XhoI</td>
</tr>
<tr>
<td></td>
<td>5′-AGCTGCAGCCAGCTGAGCTGACACACACGGATC A</td>
<td></td>
</tr>
<tr>
<td>MR33 (+12)</td>
<td>3′-.....CGTCGGTGAGCTCACAAGACACACACGGATC C</td>
<td>XhoI</td>
</tr>
<tr>
<td></td>
<td>5′-AGCTGCAGCCAGCTGAGCTGACACACACGGATC A</td>
<td></td>
</tr>
</tbody>
</table>

Table 2-1. Sequence of the oligonucleotide inserts used to create phage MR1 derivatives.
recommended by the manufacturer (New England Biolabs). The resulting DNA was transformed into *E. coli* XL1-Blue competent cells and plated using top agar as described above (section II.A.4). Individual phage plaques (6-12 per ligation reaction) were used to inoculate 3 ml of 2X-TY, which was incubated overnight at 37° C and 250 RPM. Phage dsDNA was isolated from 1.5 ml of this culture using a miniprep spin column kit (Qiagen). Insertion of the annealed duplexes abolished the *Hin*DIII restriction sequence and added an *Xho*I restriction sequence to the DNA. Miniprep DNA (100-200 ng) was screened for oligonucleotide insertion by digestion with *Bsp*DI and *Xho*I. The digestion products were separated through a 1% agarose gel and samples that contained a doublet of DNA bands at 3.1 and 3.3 kilobasepairs (kbp) were kept (i.e. digested by both *Xho*I and *Bsp*DI).

Phage MR0 was created by digestion of MR1 dsDNA with *Nhe*I and *Xba*I followed by gel purification of the large fragment (6,423 bp) from a 1% agarose gel (Qiagen). The compatible cohesive ends of *Nhe*I and *Xba*I digested DNA were ligated together using T4 DNA ligase, transformed into *E. coli* XL1-Blue competent cells and plated using top agar as described above (section II.A.4). Twelve (12) phage plaques were used to inoculate 3 ml of 2X-TY, which was incubated overnight at 37° C and 250 RPM. Phage dsDNA was isolated from 1.5 ml of this culture using a miniprep spin column kit (Qiagen). The MR0 phage was predicted to have a 30 nucleotide deletion relative to MR24 that abolished both the *Nhe*I and *Xba*I sites. Two (2) sets of miniprep DNA (100 ng each) were digested with either *Bsp*DI and *Xba*I or *Bsp*DI and *Nhe*I. The digestion products were separated through a 1% agarose gel and samples that contained a single band at 6.4 kbp (i.e. digested only by *Bsp*DI) were retained. Plasmid DNA from at least 3 clones each of potential derivative phage was sequenced by the University of Kentucky Molecular Structure Analysis Facility for verification, and 1 clone for each correct phage (MR0, MR31, MR32, MR53, MR33) was randomly selected and a phage stock prepared (section II.A.2).

IV.A.2. Large scale isolation of phage double stranded DNA

Three (3) L of 2X-TY was pre-warmed to 37° C and inoculated with 30 ml of actively growing *E. coli* XL1-Blue cells that were screened for tetracycline (12 µg/ml) resistance. The culture was incubated at 37° C and 250 RPM until the OD₅₉₅ reached 0.3. The cell density at this OD was approximately 5 x 10⁸/ml, giving 1.5 x 10¹² total cells. A 10-fold excess of phage virions (1.5 x 10¹³) was added and the culture was incubated for an additional 8 hr at 37° C at
250 RPM. The culture was then chilled using an ice-water slurry for 20 min and centrifuged for 10 min at 4500 g. The supernatant was kept for later use (see section IV.A.3). The cell pellet was suspended in 60 ml of ice-cold buffer ds-A that contained 25 mM Tris⋅HCl (pH 8.0), 10 mM EDTA, 0.9% glucose (w/v) and 5 mg/ml lysozyme and was then vortexed for 1 min on medium. The cell suspension was incubated for at RT for 10 min and then on ice for 10 min. Freshly prepared buffer ds-B (120 ml) that contained 0.2 N NaOH and 1% SDS was added while stirring gently in a single direction with a rubber policeman attached to the end of 5 ml plastic pipette. The solution was then incubated on ice for 10 min. Buffer ds-C was prepared fresh by mixing together 60 ml of 5 M potassium acetate, 11.5 ml glacial (17.4 M) acetic acid and 28.5 ml ddH$_2$O. Buffer ds-C (90 ml) was added to the lysed cell mixture while stirring as described in the previous step, then the solution was incubated on ice for 10 min. This solution was centrifuged for 60 min at 16,000 g at 4°C.

The supernatant was decanted and filtered through 4 layers of cheesecloth, the volume measured, and 0.6 vol of isopropanol was added. The solution was mixed thoroughly by repeated inversion and then incubated for 30 min at RT. DNA was precipitated by centrifugation for 30 min at 16,000 g at 4°C with no brake after the spin was completed. The supernatant was discarded and the pellet was washed in 50-100 ml of ice-cold 70% ethanol, followed by centrifugation for 10 min as described for the previous spin. The supernatant was decanted and the pellet was allowed to air dry for 15-30 min. The DNA was suspended in 20 ml TE solution that contained 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, and the weight of the solution measured. CsCl (1.05 g/g solution) and 10 mg/ml EtBr (50 µl/g solution) was added and supercoiled DNA was isolated by CsCl/EtBr equilibrium centrifugation (see Ausubel et al., 1987). The centrifugation was for 18-24 hr at 45,000 RPM at 23°C using a near vertical rotor (NVT65; Beckman, Fullerton). All steps that involved EtBr were performed with the overhead lights turned off. The band of supercoiled dsDNA was generally located near the middle of the tube and was removed from the centrifuge tube using a syringe with an 18 gauge needle. EtBr was removed from the DNA solution removed from the centrifuge tube by the addition of 1.1 vol of H$_2$O-saturated n-butanol and gentle mixing by inversion. The water and n-butanol phases were separated by centrifugation for 2 min on setting 5 of a clinical centrifuge (International Equipment Company, Needham Heights) and the upper n-butanol phase was removed by aspiration. The extractions with n-butanol were repeated until no pink color was detected in the
upper butanol phase (4-6 times). The aqueous solution containing the supercoiled DNA was then dialyzed using TE (pH 8.0). The buffer was changed at least 3 times with a minimum of 6 hr in between changes. The concentration and purity of DNA was measured by ultraviolet absorption at 260 and 280 nm (see Ausubel et al., 1987).

IV.A.3. Large scale isolation of phage single stranded DNA

Phage particles were precipitated from the culture supernatant (see section IV.A.2.) by the addition of 36 g NaCl and 50 g PEG-8000 per L of supernatant. The solution was stirred at RT for 1 hr and then centrifuged for 30 min at 5500 g at 4°C. The supernatant was transferred to clean bottles and centrifuged for 15 min at 7000 g at 4°C. The pellets from both centrifugation steps were suspended in a combined total of 25 ml TE (pH 8.0), incubated for 1 hr at 37°C and 150 RPM, and centrifuged for 10 min at 14,500 g at 4°C. The pellet was discarded and the weight of the supernatant was measured. CsCl (0.4342 g/g solution) was added and the phage particles were concentrated by equilibrium centrifugation using the same conditions for dsDNA isolation (see section IV.A.2). The band of phage particles was removed using a syringe with an 18 gauge needle, then dialyzed in TE (pH 8.0). The buffer was changed at least 3 times with a minimum of 6 hr in between changes. DNA from the phage particles was isolated by extraction of the phage solution with heated (55°C) phenol (2 times), RT phenol (2 times), and ether (4-5 times). All phenol was buffered with TE pH 7.6-8.0. Centrifugation steps for all extractions were for 5 min at 12,000 g at 25°C. The ether phase was aspirated after the final extraction step and trace ether was evaporated by incubating the solution for 10-30 min at 37°C. The solution was dialyzed as described for dsDNA (see section IV.A.2). DNA concentration and purity was determined by ultraviolet absorption at 260 and 280 nm (see Ausubel et al., 1987).

IV.B. DNA loop substrates

IV.B.1. DNA loop substrate nomenclature

DNA loop substrates were named using the convention: (nick location)-(loop size)(loop strand). For example, 5’-12V designates a substrate with a 5’ nick that contained a 12 nucleotide (nt) loop on the V strand. For all substrates, the 5’ nick was located on the C strand and the 3’ nick was located on the V strand.
IV.B.2. DNA loop substrate construction and purification

Loop substrates were constructed and purified using a previously described method (Holmes et al., 1990). For 5-12 nt loop substrates, phage MR24 DNA and DNA from phage MR31 (+5), MR32 (+8), MR53 (+10), or MR33 (+12) was used. For 30 nt loop substrates, DNA from phage MR0 and MR24 was used (see Table 2-2). A flow diagram of the substrate preparation process can be seen in Figure 2-1. DsDNA (0.5 – 3 mg) was linearized with Sau96I (0.25U/µg DNA) using conditions recommended by the manufacturer (New England Biolabs, Beverly). The DNA was purified by phenol extraction and ethanol precipitation, and was then suspended in 1 ml TE (pH 7.6). A 5 fold excess (relative to dsDNA) of circular ssDNA was then added to the linear DNA. The combined DNA solution was brought to 50 mM Tris-HCl (pH 7.6), 10 mM NaCl, and 1 mM EDTA in a total volume of 2 ml by the addition of stock solutions of the chemicals. Depending on the amount and concentration of ssDNA required, reactions were scaled in 2 ml increments as needed. An aliquot that contained 100 ng of linear dsDNA was removed and stored on ice (‘pre-annealing’ sample).

Sixty (60) µl of fresh 10 N NaOH (for a 2 ml reaction) was added to the DNA solution and it was incubated at RT for 5 min. The solution was neutralized by adding 200 µl of 2.9 N acetic acid, and then 90 µl of 3 M KCl, and 248 µl 1 M K-Pi (pH 7.4 at 100 mM) was added (all values are for a 2 ml starting volume). The C strand of linear dsDNA was annealed to the

Table 2-2. List of phage DNA used to construct DNA loop substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>V Strand</th>
<th>C Strand</th>
<th>ssDNA</th>
<th>dsDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-5C</td>
<td>MR24</td>
<td>MR31</td>
<td>MR24</td>
<td>MR31</td>
</tr>
<tr>
<td>5'-5V</td>
<td>MR31</td>
<td>MR24</td>
<td>MR31</td>
<td>MR24</td>
</tr>
<tr>
<td>5'-8C, 3'-8C</td>
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<td>MR32</td>
<td>MR24</td>
<td>MR32</td>
</tr>
<tr>
<td>5'-8V, 3'-8V</td>
<td>MR32</td>
<td>MR24</td>
<td>MR32</td>
<td>MR24</td>
</tr>
<tr>
<td>5'-10C</td>
<td>MR24</td>
<td>MR53</td>
<td>MR24</td>
<td>MR53</td>
</tr>
<tr>
<td>5'-10V</td>
<td>MR53</td>
<td>MR24</td>
<td>MR53</td>
<td>MR24</td>
</tr>
<tr>
<td>5'-12C, 3'-12C</td>
<td>MR24</td>
<td>MR33</td>
<td>MR24</td>
<td>MR33</td>
</tr>
<tr>
<td>5'-12V, 3'-12V</td>
<td>MR33</td>
<td>MR24</td>
<td>MR33</td>
<td>MR24</td>
</tr>
<tr>
<td>5'-30C, 3'-30C</td>
<td>MR0</td>
<td>MR24</td>
<td>MR0</td>
<td>MR24</td>
</tr>
<tr>
<td>5'-30V, 3'-30V</td>
<td>MR24</td>
<td>MR0</td>
<td>MR24</td>
<td>MR0</td>
</tr>
</tbody>
</table>
Figure 2-1 – Flow diagram of substrate construction and purification.

A. Circular dsDNA (black) is linearized with *Sau*96I. B. Linearized dsDNA is then denatured and reannealed in the presence of excess circular ssDNA (gray) from derivative phage. The lollipop denotes extra bases in MR33. C. After the annealing step, 4 types of DNA are present: nicked circular heteroduplex, linear homoduplex, linear ssDNA and circular ssDNA. D. Hydroxyapatite is used to separate the majority ssDNA from dsDNA. E. *E. coli* ExoV digests linear DNA to short (1-5 nt) fragments which are separated from heteroduplex DNA by Sepharose S300 size exclusion chromatography. F. BND-cellulose resin is used to remove trace ssDNA, leaving the purified 5’ nicked substrate. G. Ligation in the presence of ethidium bromide and isolation of closed, supercoiled substrate, followed by introduction of a site specific nick by the fd phage gpII protein creates the 3’ nicked substrate, seen in H.
V strand of circular ssDNA by incubating the solution for 30 min at 65°C, gradual cooling in a large volume (1-2 L) water bath to 37°C that spanned 3-5 hr, and incubation for 30 min at 37°C. The solution was then stored on ice. An aliquot of 100 ng of dsDNA was removed (‘post-annealing’) and both pre- and post- annealing samples were separated through a 0.7% agarose gel. The solution at this point contained nicked circular heteroduplex DNA (the desired species), excess circular ssDNA, re-annealed linear homoduplex, and linear ssDNA of the displaced V strand (see Figure 2-1, step C). Linear dsDNA and ssDNA migrate at distinct positions in the gel, and both types of DNA migrate faster than nicked circular dsDNA. Therefore, the appearance of a third, slower migrating band in the ‘post-annealing’ sample indicated that heteroduplex substrate was produced.

Hydroxyapatite resin (Biorad, Hercules) (1-1.2 g/mg of total DNA) was equilibrated in 30 mM K-Pi (pH 6.9) by gentle swirling in 20-30 ml of buffer and then incubated for 10-15 min incubation at RT. The supernatant was decanted along with the ‘fine’ particles that did not settle. This process was repeated 3-4 times. A column with a diameter of 4 cm was poured and washed with 2 BV of 30 mM K-Pi (pH 6.9) at a flow rate of 1-1.3 BV/hr. The annealed substrate was diluted 1:1 with an equal volume of ddH2O and this solution was slowly (>60 min) loaded onto the column. The resin bed was then washed with 1 BV of 30 mM K-Pi (pH 6.9). The flow-through from the sample-loading and wash steps were kept as separate fractions. SsDNA was eluted from the column using 4-5 BV of 160 mM K-Pi (pH 6.9). Fractions of 7-10 ml were collected during this step. DsDNA was eluted using 420 mM K-Pi (pH 6.9). One (1) ml fractions were collected and the fractions that contained DNA were identified using an “EtBr spot test”: Three (3) µl of each fraction was mixed with 7 µl of EtBr (1 µg/ml) on plastic wrap and viewed on a UV transilluminator. Fractions that contained DNA had greatly increased fluorescence compared to fractions that contained only buffer. An aliquot (10 µl) of the fractions collected at each step (but only the 420 mM K-Pi elution fractions that contained DNA as identified by the EtBr spot test) were separated through a 0.7% agarose gel to visualize the separation of ssDNA from dsDNA. Fractions from the 420 mM K-Pi (pH 6.9) elution that contained the highest concentrations of dsDNA were combined and concentrated 3-4 fold by vacuum centrifugation and then dialyzed in TE (pH 7.6) at 4°C. The buffer was changed 3 times with at least 6 hr between changes. The DNA concentration of the dialysate was measured.
by UV absorbance at 260 nm and an aliquot of 200 ng DNA was removed and placed on ice ('pre-exo' sample).

Linear homoduplex dsDNA was removed from the substrate preparation using *E. coli* Exonuclease V (ExoV) (United States Biochemical, Cleveland). The dialysate from the hydroxyapatite column fractions was brought to 66.7 mM glycine (1 M stock pH = 9.4), 5 mM MgCl₂, 8.3 mM β-ME, and 0.5 mM adenosine triphosphate (ATP). *E. coli* ExoV was added to 0.2U/µg total DNA and the solution was incubated for 60 min at 37°C, and was then placed on ice. An aliquot of 200 ng DNA was removed ('post-exo') and both the 'pre-exo' and 'post-exo' samples were separated through a 1% agarose gel using Tris/Borate/EDTA (TBE) buffer that contained 89 mM Tris base, 89 mM boric acid and 2 mM EDTA. The faster migrating linear dsDNA band generally was undetectable after 60 min of incubation, but additional incubation of the reaction (in 30 min increments) at 37°C was sometimes required. Once the linear dsDNA was completely removed, the reaction solution was extracted once with phenol and the volume reduced to between 0.5 and 1.0 ml by vacuum centrifugation or *n*-butanol extraction.

Sephacryl S300 resin (Pharmacia) was used to separate the circular DNA substrate (6.4 kbp) from the ExoV digestion products (1-5 nt oligomers). The beads were equilibrated in buffer sub-S that contained 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 0.3 M NaCl. A long (45 cm), narrow diameter (1.2 cm) glass column was then poured. The resin bed was washed using 3 BV of buffer sub-S. The buffer reservoir above the top of the resin bed was allowed to fully enter the resin then a 200 µl aliquot of the concentrated ExoV digestion reaction was added to the top of the resin bed and allowed to enter the resin. This process was repeated until the entire volume of the concentrated ExoV digestion reaction had entered the resin bed and then 3 ml of buffer sub-S (in 200 µl aliquots) was added to the column using the same technique. A reservoir (1-2 ml) of buffer sub-S was added to the top of the resin bed and then a continuous flow of buffer sub-S into the column was used to elute the DNA from the column. The flow rate through the S300 column was 10-15 ml/hr. One (1) ml fractions were collected starting from the time of the DNA sample being loaded onto the column. Groups of 10 fractions were tested as they became available by the EtBr spot test described above. At least 15 fractions were collected after DNA was no longer detected by this method. These later fractions were tested for ExoV digestion products by absorbance at 260 nm. Values of 1.5 or greater from undiluted samples indicated the presence of the oligonucleotide DNA fragments produced by ExoV. A 10 µl aliquot of each
fraction that was positive for DNA by the EtBr spot test was separated through a 1% agarose gel to determine purity of the sample. If only a single band was evident in the gel (which corresponded to nicked circular dsDNA), the fractions were pooled, reduced in volume 8-10 fold by vacuum centrifugation and dialyzed in TE (pH 7.6) with 3 changes of buffer. The volume reduction and dialysis steps were repeated until the DNA concentration was approximately 0.1 mg/ml, as measured by UV absorbance at 260 nm.

If the fractions from the S300 column revealed 2 bands after agarose gel electrophoresis (which corresponded to nicked circular dsDNA and circular ssDNA), the fractions with the highest concentration of DNA were combined and 5 M NaCl was added to a final concentration of 1 M, and benzoylated napthoylated diethylaminoethyl cellulose (BND-cellulose) resin was used to remove ssDNA from the solution. The BND-cellulose resin was prepared for chromatography by mixing 15 g of powdered BND-cellulose resin with 200 ml of 20 % (v/v) ethanol (EtOH) and stirring for at RT for 2 hr. The resin was then filter washed as follows: (1) two cycles of 2 M NaCl in 20 % (v/v) EtOH (600 ml) followed by ddH2O (1000 ml), (2) two cycles of 1 M NaCl in 20 % (v/v) EtOH (600 ml) followed by ddH2O (1000 ml), (3) 0.5 M NaCl in 20 % (v/v) EtOH (500 ml), (4) ddH2O (2000 ml). The resin was then suspended in 100 ml of buffer sub-S. A column with 2-3 ml of the BND-cellulose resin slurry (in buffer sub-S) was poured using a 10 ml syringe. The column was washed with 5 BV of buffer sub-S that was allowed to drain from the column until the top of the resin bed was just covered with buffer. The solution of combined and concentrated S300 column fractions was added to the top of the resin bed and allowed to flow through the column. After the DNA sample had entered the resin bed, the column was developed with a buffer that contained TE (pH 7.6) and 1 M NaCl. One (1) fraction equal in volume to the combined S300 fraction volume was collected starting when the sample was added to the column. Fractions of 0.5 ml were then collected until 3-5 BV of buffer had passed through the column. A 10 µl aliquot of each fraction was separated through a 0.7 % agarose gel to identify the fractions that contained dsDNA and to verify ssDNA removal. Fractions that contained dsDNA were combined, reduced in volume 2-3 fold, and then dialyzed as described for S300 fractions that contained only 1 band. The S300 column purification (and the BND-cellulose column, if required) provided a 5’ nicked substrate that was free of both ssDNA (either circular or linear) and linear homoduplex dsDNA.
Substrates that contained a 3’ nick were constructed using 5’ nicked substrates as starting material. A reaction with at least 300 μg DNA, 20 mM Tris-HCl (pH 7.6), 0.5 mM EDTA, 100 μM nicotinamide adenine diphosphate (NAD⁺), 10 mM (NH₄)₂SO₄, 50 μg/ml BSA, 0.125 mM DTT, 5 mM MgCl₂, 29 nmoles of EtBr/100 μg DNA, and 27 U E. coli Ligase per 100 μg DNA was prepared. The precise EtBr concentration (in mM) was determined using Beers Law¹ from the absorbance at 480 nm of a 1:20 dilution of a 1 mg/ml solution. All steps that contained both DNA and EtBr were performed with the overhead lights turned off. The reaction was incubated for 2 hr at RT and then placed on ice while the extent of ligation was checked on a 0.7 % agarose gel. Ligated, supercoiled DNA migrated much further in the gel than the unligated form. The weight of the ligation reaction solution was measured and CsCl (1.05 g/g solution) and 2 mg/ml EtBr (50 μL/g solution) was added. The solution was subjected to equilibrium centrifugation as previously described (section IV.A.2) and the lower of the two bands was removed using a syringe and an 18 gauge needle. EtBr was removed from the recovered DNA solution by H₂O-saturated n-butanol extraction (see section IV.A.2.) and the DNA solution was then dialyzed in TE (pH 7.6) at 4°C for 24 hr with 3 changes of buffer. The concentration of supercoiled DNA was measured by UV absorption at 260 nm.

After dialysis, the supercoiled DNA solution was brought to the conditions described previously (section III.A.2) and digested with purified gpII (25 U/μg DNA) for 30 min at 30°C. The reaction was placed on ice and the conversion of the DNA from the supercoiled to the nicked form was monitored with a 0.7 % agarose gel. Conversion from the faster, ligated form to the slower, nicked form was desired at this step. After complete conversion to the nicked form, the gpII nicking reaction was terminated by the addition of stock solutions to obtain the final conditions of 75 μg/ml proteinase K (Sigma), 0.5 % SDS, and 20 mM EDTA. The reaction was incubated for 15 min at 37°C and the protein (gpII, proteinase K) was removed by 2 extractions with phenol and 2 extractions with diethyl ether. The weight of the ligation reaction solution was measured and CsCl (1.05 g/g solution) and 2 mg/ml EtBr (50 μL/g solution) was added. The solution was subjected to equilibrium centrifugation as previously described (section IV.A.2). The single band of nicked DNA was removed using a syringe with an 18 gauge needle.

¹ A = (ε)(c)(l) where A is absorbance, ε is the extinction coefficient, c is the concentration and l is the path length used to measure the absorbance. The ε₄₈₀ nm for EtBr is 5.6 mM⁻¹ cm⁻¹. The path length used was 1 cm.
EtBr was removed from the recovered DNA solution by H₂O-saturated n-butanol extraction (see section IV.A.2.) and the DNA solution was dialyzed in TE (pH 7.6) at 4°C for 24 hr with 3 changes of buffer. The DNA solution was dialyzed and concentrated as described previously for 5’ nicked substrates after S300/BND-cellulose columns until the DNA concentration was 0.1 mg/ml. This purification strategy (ligation, gpII digestion) provided a substrate that contained a 3’ nick.

V. Experimental Protocols
V.A. Loop repair assays

The conditions to detect *in vitro* DNA loop repair were modified from previous reports (Holmes et al., 1990; Parsons et al., 1993). Single reactions (1X) contained 20 mM Tris-HCl (pH 7.6), 50 µg/ml bovine serum albumin (BSA) (New England Biolabs), 1.5 mM ATP, 1 mM glutathione, 0.1 mM each of dATP, dCTP, dGTP, and dTTP (United States Biochemical), 5 mM MgCl₂, 110 mM KCl, 75 µg nuclear extract (section III.B.) and 100 ng (24 fmol) of substrate DNA (section IV.B.). The nuclear extract was added last to initiate the reaction. When it was used, the final concentration of Aphidicolin (Sigma) was 0.1 mM. Dideoxynucleotides (ddNTP) (Gibco-BRL) were added to 0.1 mM final concentration each for ddATP, ddCTP, ddGTP, and ddTTP, when used. Reaction volumes were typically 10-25 µl and were assembled on ice. Negative control reactions were prepared with extract and without substrate DNA, heated to 95°C for 5 min and spun briefly in a microfuge. The DNA substrate was then added and the reactions were incubated and processed identically to other repair reactions. After a 30 min incubation at 37°C, 2 reaction volumes of freshly-prepared protease digestion solution that contained 0.67% SDS, 25 mM EDTA, and 90 µg/ml proteinase K was added and the reactions were incubated an additional 15 min at 37-50°C. The substrate DNA was purified from the reaction by adding 1.1 vol TE buffered phenol (pH 7.6-8.0), vortexing for 30 s and spinning for 2 min at 19,600 g in a microfuge. The aqueous upper phase was then transferred to a clean tube. The lower phenol phase was back-extracted with 1 reaction volume of TE (pH 8.0), vortexed for 30 s and spun for 2 min at 19,600 g in a microfuge. The second aqueous phase was removed and added to the first. The combined aqueous phases were extracted with 2 volumes of diethyl ether, vortexed for 30 s and spun for 2 min at 19,6000 g in a microfuge. The upper ether phase was removed by aspiration and the ether extraction process was repeated on the lower aqueous phase.
Trace ether was then evaporated by incubation for 5-10 min at 37° C. The nucleic acids were precipitated by adding 0.1 volume of 3 M sodium acetate (pH 7.0) and 2.5 volume 100% ethanol, incubating for 20 min at -80° C and centrifuging for 10 min at 19,600 g at 4° C. The supernatant was decanted and ice-cold 70% EtOH (500 µl) was added to the pellet, the tube was vortexed briefly and was then centrifuged for 6 min at 19,600 g at 4° C. The supernatant was removed by aspiration, the DNA pellet dried by vacuum centrifugation and then suspended in 10 µL TE (pH 8.0). All repair reactions were performed at 2X volume and the DNA was divided evenly into 2 aliquots after isolation. At this point one of two different methods was performed to detect DNA repair.

V.A.1. Restriction digest sensitivity and agarose gel analysis

Repair of substrates that contained 5, 8, 10, or 12 nt loops (excluding 3’-8V and 3’-12V) was monitored by restriction enzyme sensitivity and agarose gel electrophoresis. One (1) aliquot of the purified substrate DNA was digested with 2U each of BspDI and XhoI while the other aliquot was digested with 2U each of BspDI and XcmI. All restriction digest reactions were performed in HXB buffer that contained 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM DTT, 10 µg/ml BSA, 12 mM MgCl₂ and 15 µg/ml RNaseA. The digestion reactions were incubated for 1 hour at 37° C and were then separated through a 1% agarose gel. A 13.5 cm long gel was typically run for 5-6 hr at 70 volts (V) or for 14-16 hr at 25-35 V. Sensitivity to digestion by either XhoI or XcmI ideally occurred only after processing of the substrate by proteins in the nuclear extract that use one strand of the DNA as template for DNA synthesis. Therefore, restriction digestion that produced bands that 3.1 and 3.3 kbp in size indicated repair (i.e. digested by both BspDI and the scoring enzyme), whereas a product 6.4 kbp in size indicated no repair, since only BspDI cut the DNA. Which enzyme (XcmI or XhoI) was able to cut indicated whether the nicked or continuous strand was processed (Table 2-3). The repair percentage was calculated as the intensity (see section I.B.) of repair bands (3.1 + 3.3 kbp) divided by the intensity of all DNA bands (6.4 + 3.3 + 3.1 kbp).

Repair directed to the nicked strand of 5’-30V and 3’-30C and to the continuous strand of 5’-30C and 3’-30V was monitored by digestion with 2U each of BspDI and XcmI as described above. Due to the sequence of the non-looped strand in these substrates, repair directed to the
Table 2-3. Restriction enzyme sensitivity for 5-12 nucleotide loop substrates.

<table>
<thead>
<tr>
<th>Substrate Class</th>
<th>Nicked strand processing</th>
<th>Continuous strand processing</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-C</td>
<td>XcmI</td>
<td>XhoI</td>
</tr>
<tr>
<td>5’-V</td>
<td>XhoI</td>
<td>XcmI</td>
</tr>
<tr>
<td>3’-C</td>
<td>XhoI</td>
<td>XcmI</td>
</tr>
<tr>
<td>3’-V</td>
<td>XcmI</td>
<td>XhoI</td>
</tr>
</tbody>
</table>

continuous strand of 5’-30V and 3’-30C, as well as repair directed to the nicked strand of 5’-30C and 3’-30V was unable to be determined using this assay.

All loop repair experiments were repeated at least 3 times and the results averaged. Statistical analysis was performed using StatView ver 4.5 (Abacus Concepts, Inc.).

V.A.2. Southern blot analysis of strand sizes

Due to extensive digestion (50-85%) by both XcmI and XhoI of 3’-8V and 3’-12V nt loop substrates in the absence of exposure to nuclear extract, a Southern blot assay was performed to visualize repair products. Due to the lack of restriction enzyme sequence on the non-looped strand of the 30 nt loop substrates, this same type of assay was also used to score repair to both strands for these substrates. For 8 and 12 nt loop substrates, both aliquots of purified substrate DNA were digested with 2U each of NheI and Sau96I. Thirty (30) nt loop substrates were digested with 2U each of SspI and BanII. Both sets of digestion reactions (NheI/Sau96I; BanII/SspI) were performed in buffer 2 of the manufacturer (New England Biolabs) supplemented with 10 µg/ml BSA and 15 µg/ml of RNaseA. The digestion reactions were incubated for 1 hr at 37°C and terminated by adding 0.5 vol of 3X formamide loading buffer that contained 80% deionized formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue. The samples were heated for 5 min at 95°C, incubated on ice for 5 min, and then separated by denaturing PAGE with gels that contained 7 M urea, 19:1 acrylamide:bis-acrylamide, and TBE buffer. Acrylamide gels (10% for 8 and 12 nt substrates; 5% for 30 nt substrates) were 0.75 mm thick, 16 cm long and run at 450 V until the fragments of interest (Table 2-4) were ~3-5 cm from the end of the gel. The region of the gel that was expected to contain the desired fragments of DNA was transferred onto a piece of filter paper,
and the DNA was transferred to nylon membrane by electrotransfer for 3-3.5 hr at 35 V at 4°C. The buffer used was 0.5X TBE that contained 44.5 mM Tris base, 44.5 mM boric acid and 1 mM EDTA. After the electrotransfer, the membrane was submerged briefly in 2X SSC buffer that contained 0.3 M NaCl and 0.03 M sodium citrate (pH 7.0), air dried, and the DNA was cross-linked to the membrane by a 5 min exposure to UV light (the membrane was placed 5-6 cm above the surface of a UV transilluminator with the DNA side facing down).

The membrane was pre-hybridized in buffer that contained 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 2% SDS, 1 mM EDTA, 0.5% polyvinylpyrrolidone, and 0.2% heparin for at least 3 hr at 42°C. Oligonucleotide probes (15-20 nt) were 5’ end-labeled with \( \gamma^{32P} \)-ATP (DuPont, Wilmington) using T4 polynucleotide kinase, as recommended by the manufacturer (United States Biochemical). For 8 and 12 nt substrates, probes V5744-5768 (5’-TTGATTAGGGTTGGTTCACGTAG) and C5765-5746 (5’-CGTGAACCATCACCCTAATC) were added to the hybridization solution to probe the C and V strands, respectively. For 30 nt loop substrates, probes V5216-5235 (5’-ATTGTTCTGGATATTACCAG) and C5259-5235 (5’-GAAGAACTCAAACTATCGGCCCTTG) were added to the hybridization solution to probe the C and V strands, respectively. The duplicate repair assay and digestion reactions were separated on separate gels and each was probed for either the C or V strand. All hybridization and wash steps were performed using a HB-1D hybridization oven (Techne, Princeton). Between 8 and 10 ng of labeled probe was incubated with the membrane for 12-16 hr at 42°C. The membrane was washed twice for 5 min each using 25 ml of 2X SSC that contained 0.1% SDS, then twice for 5 min each using 25 ml of 0.2X SSC with 0.1% SDS. All wash steps were at 25-30°C. The membranes were patted dry using paper towels, wrapped in plastic and exposed to film (Fujifilm Super RX). Autoradiographs were scanned into Kodak Image 2.0.2 software using a flatbed scanner (Umax Powerlook II, Fremont) and the band intensities measured. The expected size for C and V strands depended on which phage contributed each strand to the substrate being analyzed (Tables 2-2 and 2-4). Repair of substrates exposed to nuclear extract was defined as the conversion of one strand length into the complementary strand length. Repair to both strands was calculated as the intensity of the ‘converted’ band divided by the combined intensity of the ‘converted’ and ‘unconverted’ bands.

All loop repair experiments were repeated at least 3 times and the results averaged. Statistical analysis was performed using StatView ver 4.5 (Abacus Concepts, Inc.).
Table 2-4. Probes used for Southern blot analysis of complete repair reactions and the expected sizes of each strand.

<table>
<thead>
<tr>
<th>Phage</th>
<th>Probe Name</th>
<th>V Strand Size (nt)</th>
<th>C Strand Size (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR24</td>
<td>MR32</td>
<td>MR33</td>
<td></td>
</tr>
<tr>
<td>V5744-5768</td>
<td>148</td>
<td></td>
<td></td>
</tr>
<tr>
<td>156</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>160</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MR24</td>
<td>MR32</td>
<td>MR33</td>
<td></td>
</tr>
<tr>
<td>C5765-5746</td>
<td>149</td>
<td></td>
<td></td>
</tr>
<tr>
<td>157</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>161</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MR0</td>
<td>MR24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V5216-5235</td>
<td>444</td>
<td></td>
<td></td>
</tr>
<tr>
<td>474</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MR0</td>
<td>C5259-5235</td>
<td>448</td>
<td></td>
</tr>
<tr>
<td>478</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

V.B. Inhibition of mismatch repair (MR) during in vitro loop repair

Loop repair reactions by HeLa nuclear extracts under conditions of MR inhibition were performed similar to those described above (section V.A.). Paired reactions contained 250 ng (60 fmol) of DNA loop substrate DNA and 75 ng (18 fmol) of either phage fd homoduplex DNA or a hybrid DNA that consisted of one strand of phage fd DNA and one strand of phage M13 DNA, as previously described (Wu et al., 1999). The fd homoduplex and fd:M13 hybrid DNA was prepared as described for 5’ nicked substrates (section IV.B.2). The fd:M13 heteroduplex DNA was used to sequester mismatch binding complexes required for MR. The fd homoduplex DNA was used to control for non-specific inhibition of MR by the extra DNA present in these reactions (compared to reactions in section V.A.). Substrate and inhibitor DNA was isolated after exposure to nuclear extract (see section V.A.) and digested with 2U each of BseRI and either XcmI or XhoI (depending on the substrate), separated through a 1% agarose gel and the repair percentage measured as described above (section V.A.1). Loop repair that occurred when MR was inhibited was calculated as the percent repair observed in reactions that contained heteroduplex DNA divided by the percent repair in reactions that contained homoduplex DNA. The substrates tested using this method were: HeLa extract: 5’-G/T, 5’-2V, 3’-4C, 5’-5V, 5’-5C, 5’-8V, 3’-8C, 5’-12V, and 3’-12C; HCT15, HCT116, and HEK-1A extracts: 5’-8V, 3’-8C, 5’-12V, and 3’-12C. The 5’-G/T, 5’-2V, and 3’-4C substrates contain a G/T mismatch, a 2 nt and a
4 nt loop respectively and are the same as described previously (Su et al., 1988, Parsons, 1993 #177). They are otherwise identical to the substrates described above (see section IV.B.2) in terms of nick location and sequence.

V.C. Mapping of intermediates from DNA synthesis inhibited loop repair assays

Loop repair reaction intermediates were trapped during in vitro repair assays by inhibiting DNA synthesis. Repair reactions were performed as described above (see section V.A.) either in the absence of exogenous dNTP, in the presence of aphidicolin (0.1 mM), or in the presence of 0.1 mM each of ddATP, ddCTP, ddGTP, ddTTP in addition to the normal amount of dNTP’s (0.1 mM each). Substrate DNA was purified as described for normal repair assays (see section V.A.) and digested with 2U SspI and 15 µg/ml of RNaseA in SspI unique buffer as recommended by the manufacturer (New England Biolabs). The DNA was then separated through 6% denaturing PAGE and further processed for Southern blot analysis as described above (section V.A.2). The probes used for these experiments (V5216-5235, C5259-5235, V5744-5768, and C5765-5746) were the same as described above (section V.A.2).

The analysis of 8 and 12 nt loop substrate intermediates was also performed using purified DNA that was digested with 2U each of SspI and Sau96I in buffer 2 of the manufacturer (New England Biolabs) supplemented with 15 µg/ml of RNaseA. The DNA was separated through an 8% denaturing PAGE, and processed for Southern blot analysis as described above (section V.A.2). The probes used were V5744-5768, and C5765-5746.

Size standards were created using MR24 dsDNA. Forty (40) µg of DNA was digested with 20 U of SspI in buffer recommended by the manufacturer (New England Biolabs) for 2 hr at 37° C. The DNA was isolated by ethanol precipitation and resuspended in 100 µl ddH₂O and then divided into 4 equal aliquots. Three (3) of these aliquots were additionally digested with 20 U of either BanII, NheI, or XcmI in buffer recommended by the manufacturer (New England Biolabs) for 2 hr at 37° C. The DNA from these reactions was isolated by ethanol precipitation and resuspended in 25 µl ddH₂O. All 4 tubes of DNA were then pooled together (the 3 double digests and the SspI only digested sample). One (1) µl of this solution was used as a size marker in the experiments in this section. For experiments in which the purified DNA from loop repair reaction intermediates was digested with SspI and Sau96I prior to analysis, the marker DNA was
Table 2-5. DNA marker sizes for DNA synthesis inhibited intermediate analysis reactions.

<table>
<thead>
<tr>
<th>Probe Used</th>
<th>Marker size (nt) when digested with SspI and:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Ban</em>II <em>Xcm</em>I <em>Nhe</em>I</td>
</tr>
<tr>
<td>V5216-5235 (Probe 1)</td>
<td>757  477  428  405</td>
</tr>
<tr>
<td>C5259-5235 (Probe 2)</td>
<td>757  473  426  409</td>
</tr>
<tr>
<td>C5768-5744 (Probe 3)</td>
<td>757  280  329  352</td>
</tr>
</tbody>
</table>

Marked size (nt) when digested with *SspI* + *Sau*96I and:

<table>
<thead>
<tr>
<th>---</th>
<th><em>Ban</em>II</th>
<th><em>Xcm</em>I</th>
<th><em>Nhe</em>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5768-5744 (Probe 3)</td>
<td>555  77  127</td>
<td>149</td>
<td></td>
</tr>
</tbody>
</table>

Additionally digested with *Sau*96I immediately prior to use. The sizes of the marker bands depend on which probe was used (Table 2-5).

V.D. Gap formation assay

To test for 5'→3' excision that started from the nick of 5'-30C and 5'-30V substrates, repair reactions identical to those used for intermediate mapping experiments (section V.C.) were performed. The purified substrate DNA was digested with 2U each of *Ban*II and *Bse*RI and in buffer 4 of the manufacturer (New England Biolabs) supplemented with 15 μg/ml RNaseA and then the digestion products were separated through a 1% agarose gel. Excision that occurred from the nick towards the loop site was predicted to generate a single stranded region that would be resistant to digestion by *Ban*II, generating a linear 6.4 kbp molecule. Substrate DNA that did not undergo excision would be digested by both enzymes creating a doublet of bands at 2.8 and 3.6 kbp. The intensity of the 6.4 kbp band was measured as previously described (section I.B) and reported as a percentage of total DNA intensity (2.8 + 3.6 + 6.4 kbp bands).
CHAPTER 3
Bi-Directional Repair Of Intermediate Size DNA Loops In Human Cells

I. Introduction

Multiple pathways exist to restore damaged or otherwise altered DNA to its normal state, a fact that underscores the importance of genomic stability. Helix distorting, bulky base adducts are recognized and removed by the nucleotide excision repair (NER) system (Friedberg et al., 1995; Sancar, 1996). Less bulky base adducts are processed by base excision repair (BER), whereby the removal of adducts is initiated by different DNA glycosylases (Friedberg et al., 1995; Lindahl and Wood, 1999). Single base mispairs caused by misincorporation during replication and other aberrant DNA structures are corrected by the mismatch repair (MR) system (Harfe and Jinks-Robertson, 2000). Recently, it has been demonstrated that both of the MR DNA recognition complexes can recognize many of these adducts normally removed by NER and BER pathways (Duckett et al., 1996; Li et al., 1996; Mello et al., 1996; Mu et al., 1997; Ni et al., 1999; Wang et al., 1999; Wu et al., 1999; Yamada et al., 1997). However, it is not yet clear if MR is acting to remove these adducts from the DNA. Instead, it may be acting as a damage sensor (Li, 1999). In addition to these adducts and the 8 base:base mismatches that give the pathway its name, MR also recognizes short regions of unpaired DNA, or DNA loops (Fishel and Wilson, 1997; Jiricny, 1998; Kolodner and Marsischky, 1999; Modrich, 1997). However, it is not the only pathway that is capable of processing these types of structures (Ayares et al., 1987; Bishop et al., 1989; Bishop and Kolodner, 1986; Corrette-Bennett et al., 2001; Corrette-Bennett et al., 1999; Kirkpatrick and Petes, 1997; Littman et al., 1999; Umar et al., 1994; Weiss and Wilson, 1987; Weiss and Wilson, 1989).

It has been shown that MR is the predominant pathway for the correction of loops containing 1-4 nucleotides (nt) in both prokaryotic and eukaryotic cells (Drummond et al., 1995; Modrich and Lahue, 1996; Parsons et al., 1993). These loops can be produced during DNA replication of microsatellite repeats units when one or more of the repeat units slips out of the DNA polymerase active site, then reanneals out of register, causing ‘looping’ of one strand of DNA. If left unrepaired, these intermediates cause an increase or decrease in the number of repeat units on the newly synthesized strand, depending on whether the loop occurred in the daughter or parental strand, respectively (Friedberg et al., 1995; Sia et al., 1997). In fact, the
instability of small repeat units is a hallmark for cells that have lost the ability to perform MR. Genetic studies in *S. cerevisiae* indicate that MR affects the stability of repeat units up to 13 nt long, giving rise to a distinction between micro- (1-13 nt) and mini- (> 13 nt) satellites (Sia et al., 1997). The MR DNA binding complexes are MutSα, comprised of a heterodimer of MSH2 and MSH6, and MutSβ, a heterodimer of MSH2 and MSH3 (Acharya et al., 1996). Biochemical studies using complexes purified from both yeast and human cells have demonstrated that MutSα preferentially binds to base:base mismatches and smaller (1-4 nt) loops, while MutSβ preferentially binds larger loops (up to 24 nt), but not to base:base mismatches (Acharya et al., 1996; Marra et al., 1998; Marsischky and Kolodner, 1999; Palombo et al., 1996; Wilson et al., 1999). However, a recent report has shown that MutSβ can bind to a G/T mismatch in a gel shift assay, although subsequent competition experiments suggested the binding was no stronger than binding to homoduplex or methylated DNA (Berardini et al., 2000). The addition of purified hMutSα and hMutSβ to MR deficient extracts can increase the repair of loops up to 8 nt (Genschel et al., 1998). Hence, studies performed using yeast and human systems indicate that MR can recognize and process loops larger than 4 nt.

There is accumulating evidence that there is some redundancy in the repair of loops. An activity in *S. cerevisiae* has been identified that binds to DNA loops up to 9 nt, and is present in extracts from strains deficient in MSH2, a required MR gene (Miret et al., 1996). Strand specific repair of loops from 5-16 nt has been demonstrated in MR deficient human cells, although only 5’ nicked substrates were studied for loops larger than 5 nt (Genschel et al., 1998; Umar et al., 1994). MR independent correction of very large loops (> 25 nt) by yeast cells (Corrette-Bennett et al., 2001; Corrette-Bennett et al., 1999) and human cells (Littman et al., 1999) has also been reported. Another report described a pathway that corrected a 26 nt loop and required MutSβ, as well as the NER endonuclease complex Rad1/Rad10 (Kirkpatrick and Petes, 1997). It is not clear if the entire MR pathway or only MutSβ is required for this pathway. To study intermediate loop repair in human cells, we constructed DNA substrates containing loops of 5, 8, 10, or 12 nt and characterized their repair in MR+ and MR− cells extracts. For 8 and 12 nt loops, substrates differed in the location of the nick (5′ vs. 3′) and location of the loop (nicked vs. continuous strand). We also analyzed reaction intermediates to investigate the molecular mechanism of the MR independent loop repair pathway.
II. Results

II.A. Bi-directional, nick directed repair of loops up to 12 nucleotides by MR+ and MR- cell extracts

To examine the repair of different sized loops and the involvement of MR in their processing, we created derivatives of the f1 series of phage commonly used for in vitro studies of MR (Su et al., 1988; Fang and Modrich, 1993; Parsons et al., 1993). Using DNA isolated from these phage, we constructed substrates with 5, 8, 10, or 12 nt loops. Paired substrates containing a loop in either the C or V strand and a 5’ nick (in the C strand) were created for all four loop sizes (Figure 3-1A). Paired substrates with the loop in either strand with a 3’ nick (in the V strand) were also created for loops of 8 and 12 nt. Both nicks are located approximately the same distance from the loop site (130 and 186 bp for 5’ and 3’ nicks, respectively). The loop sequences (Figure 3-1B) were not expected to form secondary structure, a variable that can affect repair (Bill et al., 2001; Corrette-Bennett et al., 2001; Moore et al., 1999). This set of substrates allowed us to test for bi-directional nick directed repair that can involve either loop addition or loop removal by monitoring the processing of both strands.

Most of the data was obtained from a restriction digest sensitivity based assay that has been well characterized in MR studies (Figure 3-2A) (Drummond et al., 1995; Fang and Modrich, 1993; Su et al., 1988). Each strand of the DNA substrate contains the recognition sequence for a different endonuclease at the site of the loop. Ideally, the substrate is resistant to cutting by both enzymes, and only after repair of one strand using the complementary strand as template, is sensitivity to one of the enzymes restored. In two of our substrates (3’-8V and 3’-12V), extensive digestion (50-85%) of the substrate by both XhoI and XcmI was observed after exposure to heat inactivated extract, making determination of repair by the assay described above impossible. To visualize the processing of these two substrates, we used a Southern blot assay to detect the size of the strands in Sau96I and NheI digested samples. For example, when the nicked, V strand of 3’-12V is probed, reactions containing heat-inactivated extract show only one band, corresponding to the 161 nt fragment created by the digestion (Figure 3-2B). However, in reactions that contained viable extract, the appearance of a shorter band is evident. The size of this band is consistent with loop removal. No loop addition to the continuous, C strand is evident, indicating that no processing occurred. Controls using 5’-8C and 3’-8C
Figure 3-1. DNA loop mispair substrate conformations and sequences. A. Schematic diagrams of the four possible substrate configurations. Location of unpaired DNA is indicated by a lollipop. A single strand nick in the Complementary (C) strand is located 130 bp, 5’ to the loop site. The nick in the Viral (V) strand is 186 bp, 3’ to the loop site. Substrates were constructed as described in Chapter 2. All loop sequences contain the XhoI recognition sequence and non-looped strands contain the XcmI recognition sequence. Substrates are named by the convention: Nick position-(loop size)(loop strand), i.e. 5’-8C has a 5’ nick and an 8 nt loop in the C strand. B. Sequence of relevant regions of MR24 and derivative phage used in this study. Boldface letters indicate unpaired loop sequences.
Figure 3-2. Examples of \textit{in vitro} loop repair assay results. A. Agarose gel showing nick directed repair of the 5’-10C substrate. In this example, the loop is removed from the nicked strand conferring \textit{XcmI} sensitivity after the continuous, non-looped strand is used as template for DNA resynthesis. B. Example of results from the Southern blot analysis used to score repair of some substrates. The schematic drawing depicts the region of the substrate probed. Gray bars indicate location of probe binding site. Nick directed repair is manifested as conversion of the longer V strand into the C strand length (see Table 2-4).
demonstrated that repair values given by the two different assays were similar (data not shown). Interestingly, a slightly larger band also appears in reactions that contained MR deficient extracts. Because DNA resynthesis immediately surrounding the loop region in 3’-12V requires addition of four consecutive TG repeats (see Figure 3-1B, C strand of MR24 for template sequence), we suspect that this band is the result of a polymerase slippage event that goes unrepaired in MR deficient extracts, resulting in a 2 bp addition.

We examined and combined results for repair targeted to the nicked strand for paired substrates of each nick position and loop size (Figure 3-3). For example, the category labeled 5’-5 is the average obtained combining the results from both 5’-5C and 5’-5V substrates. The most obvious result is that bi-directional, nick directed repair of loops up to 12 nt occurs independent of MR status. Nuclear extract from cells with mutations in the MR genes MSH6 (HCT15), MLH1 (HCT116), or PMS2/MSH6 (HEC-1A) all have repair activity on both 5’ and 3’ nicked substrates with loops up to 12 nt. We have also tested a subset of these substrates for repair by a cell line we have recently identified as missing MSH2, with similar results (data not shown). Importantly, repair of the continuous strand was not detected by either assay used. This indicates that the nick is the main signal for determining which strand is incorrect. With a few exceptions, no differences were seen with paired substrates of the same nick position and loop size. Hence, loop addition or loop removal had little if any effect on the absolute level of repair. This trend held true for MR+ and MR− extracts. For example, using HeLa cell extracts repair values of 19.3% and 21.8% were obtained for 5’-8C and 5’-8V, respectively. In HCT15 extracts, the values for these two substrates were 8.3% and 9.9%, respectively. The only difference in these two substrates is the presence of the loop in either the nicked or continuous strand, respectively. Even when paired substrates were repaired differently, there was still an overwhelming bias to repair of the nicked strand. For example, repair to the nicked strand by HCT116 extracts with the 5’-12C and 5’-12V substrates was 8.0% and 18.3%, respectively. However, both HeLa and HEC-1A extracts repaired both substrates to a similar extent, indicating the difference was not intrinsic to the substrate preparations.

These results confirm and extend previous studies (Ayares et al., 1987; Bishop and Kolodner, 1986; Genschel et al., 1998; Kirkpatrick and Petes, 1997; Umar et al., 1994). It has been previously demonstrated that cells lacking a functional MR system can process loops of 5, 8
Figure 3-3. Strand specific, nick directed loop repair in MR+ and MR- cell extracts. Repair assays were performed using 75 µg nuclear extract and 100 ng substrate DNA. The average repair percentage for both V and C strand loops for each nick/size combination is shown (i.e. group 5'-5 combines results for both 5'-5C and 5'-5V substrates). Each substrate was tested at least 3 times with each extract. Differences in the results when using separate preparations of extract were within 10%. Error bars represent standard error of the mean (SEM). Values for MR deficient cell lines with mutations in MSH6 (HCT15), MLH1 (HCT116) or PMS2/MSH6 (HEC-1A) were individually compared to MR proficient HeLa cells by the Students t test using StatView. Calculated p values are given in the data table. Star (*) indicates difference at a significance level of α = 0.05.
and 16 nt with a 5’ nick (Umar et al., 1994), but this is the first description that a 3’ nick can also
direct repair, independent of MR status for loops larger than 5 nt. Since HeLa cell extracts
presumably have both MR dependent and independent loop repair pathways, we compared the
repair values for each MR− cell extract individually against MR+ HeLa cell extracts using the
Students $t$ Test ($\alpha = 0.05$) to ascertain whether MR was processing the substrates tested. The
results of this analysis indicate that while the lower limit of MR-independent loop repair may be
5 nt, MR deficiency severely attenuates the repair of loops of this size. All three MR− extracts
exhibit reduced repair of 5 nt loops compared to MR+ HeLa extracts ($p$ values from the Students
$t$ test are given below graph in Figure 3-3). Even the HCT15 extract, which retains the ability to
process small DNA loops due to the presence of hMutSβ, is only slightly better at processing
these substrates than the HCT116 and HEC-1A extracts. The difference between HCT15 and
HeLa is due to hMutSα mediated loop repair. This supports the notion that hMutSα is the
primary DNA recognition complex in MR, since it is present in 10-fold excess relative to
hMutSβ (Genschel et al., 1998). Interestingly, both HCT116 and HEC-1A extracts repaired only
one of the two 5 nt loop substrates (5’-5C). This phenomenon has been described elsewhere
(Umar et al., 1994), and possibly indicates that sequence context can affect substrate selection by
these pathways.

The repair of loops of 8-10 nt clearly has both MR dependent and independent
components. For loops with 8 or 10 nt and a either a 3’ or 5’ nick, the ‘complete’ loss of MR
(base:base repair and loop repair loss) in both HCT116 and HEC-1A extracts causes a drop in
repair relative to HeLa. However, ‘partial’ MR loss (base:base repair loss only) in the MSH6
deficient HCT15 extract reduces repair of only 5’ nicked substrates of these sizes. Repair of the
3’ nicked, 8 nt loop substrates is statistically indistinguishable from that of the HeLa extract.
This may suggest that hMutSα is not involved in the processing of 3’ nicked substrates
containing intermediate sized loops. While the repair observed by the HCT15 extract could be
mediated by hMutSβ, the low amounts of repair of the 5 nt substrates argues that it is the MR
independent pathway instead. None of the MR− extracts had reduced repair of 3’ nicked, 12 nt
loop substrates, and only the $MSH6/PMS2$ double mutant extract (HEC-1A) was less active on 5’
nicked, 12 nt loop substrates compared to HeLa. These results indicate that there may be some
involvement of MR in the correction of 12 nt loop substrates, but under the conditions used, its
contribution is small.
II.B. The human mismatch repair pathway is capable of processing 12 nucleotide loops

The cell lines used in this study are not isogenic, hence it is possible that the higher levels of repair we observed using HeLa extracts were the result of higher levels of the MR independent pathway in HeLa cells, and not because MR acts on these substrates. Data suggesting that MR is involved in the repair of DNA loops larger than 5 nt comes from in vitro binding studies of purified hMutSα and hMutSβ (Acharya et al., 1996; Drummond et al., 1997; Marsischky and Kolodner, 1999; Wilson et al., 1999). These reports indicate that either or both of these complexes can recognize loops of up to 24 nt. To more directly test if MR is processing 5-12 nt loops, we used a hybrid DNA molecule consisting of one strand of fd phage DNA and another of the related M13 phage DNA to act as an inhibitor of MR. The DNA from these two phage share ~97% identity and the hybrid molecule contains ~180 mismatches of all possible combinations (van Wezenbeek et al., 1980; Worth et al., 1994). We have previously shown that this molecule can specifically inhibit MR, presumably by sequestering hMutSα, in our in vitro repair assay (Wu et al., 1999).

We studied the effects of adding either fd homoduplex or fd:M13 heteroduplex DNA on loop repair in HeLa extracts (Figure 3-4). The conditions used for this experiment (60 fmol substrate, 18 fmol of fd:M13 heteroduplex DNA) introduce ~50 fold more mismatches compared to loops on the target substrate. This amount of fd homoduplex DNA does not alter the amount of repair (data not shown). Repair of a G/T mismatch is completely blocked by addition of fd:M13 heteroduplex DNA, as expected. The 2 and 4 nt loop substrates used were identical to the 5-12 nt substrates except for loop size (Parsons et al., 1993). The loop sequences were GT or CACA for 5'-2V and 3'-4C, respectively. The repair of loops of 2-8 nt is significantly reduced, but not completely abolished under the same conditions. This indicates that MR is indeed contributing to the total repair of these substrates. Even though the absolute level of repair of all loops is similar in MR depleted HeLa extracts, there is evidence that this is not due to a single pathway that repairs loops of 2-12 nt when MR is absent. First, MR deficient HCT116 cell extracts have been shown previously to be incapable of repairing 2 and 4 nt loops (Parsons et al., 1993). Another potential explanation is that the residual repair is hMutSβ mediated MR. The hybrid DNA molecule has at most 2 small (1-2 nt) regions of unpaired DNA and may not fully sequester hMutSβ. The residual repair of both 2 and 4 nt loops is likely mediated by hMutSβ.
Figure 3-4. Analysis of the MR dependent and independent contributions to loop repair. A. HeLa nuclear extracts were proficient (black; fd DNA) or depleted (gray; fd:M13 DNA) for MR activity when the loop repair activity was measured using the restriction digest sensitivity assay. Comparisons between proficient and depleted extracts were by the Students t Test. Star (*) indicates a significant difference at $\alpha = 0.05$. B. Graphical representation of the contributions of the hMutS$\alpha$ dependent and independent loop repair pathways (data plotted is from Table 3-1).
However, the residual repair of 5-12 nt loops cannot be attributed solely to hMutS\(\beta\). Adding fd:M13 heteroduplex DNA to HCT15 extracts, which contains hMutS\(\beta\), does not affect the level of repair (Table 3-1). In fact, the addition of fd:M13 heteroduplex DNA to loop repair reactions had no effect on any of the MR extracts (data not shown). We think it is merely a coincidence that hMutS\(\beta\) mediated repair of 2 and 4 nt loops gave the same absolute value as hMutS\(\alpha\) independent 5-12 nt loop repair.

Since a majority of MR reactions are likely mediated by hMutS\(\alpha\), we can make some estimations on the contributions of the MR dependent and independent components of loop repair (Figure 3-4B). Due to the relative levels of hMutS\(\alpha\) and hMutS\(\beta\) in normal cells (Genschel et al., 1998), “hMutS\(\alpha\) independent” loop repair is likely to be a good estimate of “MR independent” loop repair. The repair of 5 nt loops drops by \(~\)50%, of 8 nt loops by \(~\)40%, and of 12 nt loops by 20-25% in MR depleted HeLa extracts. These last values are particularly interesting in light of the results presented earlier. They indicate that MR can in fact process loops up to 12 nt, but may not be the primary pathway involved. In other words, the contribution of MR to loop repair of 8-12 nt may be small, but the pathway is capable of processing them.

II.C. Analysis of the mismatch repair independent loop repair mechanism

We were interested in understanding the mechanism of the MR independent loop repair pathway identified in our study. To study loop repair reaction intermediates, we used conditions

<table>
<thead>
<tr>
<th>Substrate</th>
<th>HeLa (%)</th>
<th>HeLa (%)</th>
<th>(p) value</th>
<th>HCT15 (%)</th>
<th>HCT15 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ fd</td>
<td>+ fd:M13</td>
<td>(fd vs fd:M13)</td>
<td>+ fd</td>
<td>+ fd:M13</td>
</tr>
<tr>
<td>5'-G/T</td>
<td>52.23</td>
<td>0.00</td>
<td>(&lt;\ 0.001)</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
<tr>
<td>5'-2V</td>
<td>41.18</td>
<td>8.18</td>
<td>(&lt;\ 0.001)</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
<tr>
<td>3'-4C</td>
<td>40.17</td>
<td>9.08</td>
<td>(&lt;\ 0.001)</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
<tr>
<td>5'-5C</td>
<td>20.34</td>
<td>10.15</td>
<td>0.0477</td>
<td>4.57</td>
<td>4.43</td>
</tr>
<tr>
<td>5'-8V</td>
<td>17.43</td>
<td>10.17</td>
<td>0.0068</td>
<td>9.88</td>
<td>10.40</td>
</tr>
<tr>
<td>3'-8C</td>
<td>20.99</td>
<td>12.79</td>
<td>(&lt;\ 0.001)</td>
<td>19.50</td>
<td>19.23</td>
</tr>
<tr>
<td>5'-12V</td>
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<td>11.34</td>
<td>0.2363</td>
<td>14.80</td>
<td>14.36</td>
</tr>
<tr>
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<td>10.10</td>
<td>7.42</td>
<td>0.0337</td>
<td>12.07</td>
<td>11.71</td>
</tr>
</tbody>
</table>
of limited DNA synthesis on substrates in both MR+ and MR− extracts. By omitting dNTP’s from the reaction buffer, only a limited amount of DNA synthesis can occur using the small pool of endogenous DNA precursors present in the nuclear extract. Once this pool is depleted, the events prior to DNA resynthesis are ‘trapped’ and the strand lengths of reaction intermediates can be visualized by Southern blot (Fang and Modrich, 1993; Littman et al., 1999). We examined 4 different substrates using this method: 5’-8C, 3’-8C, 5’-12V, 3’-12V (Figure 3-5). The probes used bind to sites ~420 nt removed from the loop site, and 558 and 610 nt from the nick, for 5’ and 3’ substrates, respectively (Figure 3-5A). When DNA synthesis was prevented, fragments of DNA with endpoints near the vicinity of the loop site are evident (Figure 3-5B and C). This indicates that either excision from the nick back to the loop, or incision around the loop has occurred. Importantly, these bands occur solely on the nicked strand. Only the full length (757 nt) band was evident when the continuous strand was probed (data not shown). Also, no excision occurred along the long path from the nick to the loop was evident (data not shown). These results confirm that the nick directs repair to the nicked strand and also indicate that the region processed is confined to the short path between the nick and the loop.

Interestingly, we observed differences between the different substrates tested. Processing of 5’-8C and 5’-12V occurs over a more extensive tract of the DNA, with additional bands present well past the loop region, whereas processing of the 3’ nicked substrates is more confined to the area immediately surrounding the loop site (compare Figure 3-5, panels B and C). Also, DNA fragments that correspond in size to regions between the nick and loop are more evident with the 5’ nicked substrates. This suggests that excision starts from the nick and proceeds towards the loop in these substrates. These bands are absent when using the 3’ nicked substrates. Most interesting to us, however, is the abundance and location of the intermediate bands of 3’ nicked substrates processed by HCT116 extracts (Figure 3-5C, lanes F and H). The size of these bands corresponds to positions on either side of the loop. This occurs regardless of whether the loop is on the nicked or continuous strand. The decreased abundance of these bands in HeLa cells is possibly due to MR activities that excise DNA back from the nick to sites 90-170 nt past the mispair (Fang and Modrich, 1993). From these experiments, it is unclear if the bands observed are the result of excision back from the nick that terminates at the loop site, or if they are the products of incisions made close to the loop region.
Figure 3-5. Southern blot analysis of loop repair reaction intermediates.

A. Diagrammatic representation of the regions visualized in Panels B & C. Gray boxes indicate probe binding sites. Stars (*) indicates the location of the loop. Probe 1 is V5216-5235 and Probe 2 is V5259-5235 (see Chapter 2). Arrows indicate the location of single strand nicks in the starting substrates. B., C. Loop repair reactions were performed in the absence of exogenous dNTP’s to prevent DNA resynthesis. Purified DNA was digested with SspI and separated by 6% denaturing PAGE, followed by indirect end-labeling by Southern blot analysis using $^{32}$P end-labeled oligomers.
To test this idea, we also used a probe that binds in between the nick and the loop site to analyze the processing of 3’ nicked substrates (Figure 3-6). One factor that needs to be addressed is the DNA ligase activity in the extracts. Whether the nick is sealed by the extract influences both the extent of repair and the outcome of the present experiments. Very high ligase activity is inhibitory to repair, since the strand discrimination signal is sealed before repair can occur. For these experiments, if the nick is sealed the fragments of interest will be 144 nt longer than fragments from unligated substrates. We were unable to detect fragments that corresponded to unligated, processed substrates (around 186 nt) (data not shown). We did, however, detect DNA fragments that corresponded to ligated substrates that had endpoints very close to the loop site (around 330 nt) (Figure 3-6A, lanes B-D). The location of the probe binding site rules out exonucleolytic degradation of the DNA between the nick and the loop, supporting the idea that the bands are made by endonucleolytic activity. However, the intensity of the bands was decreased relative to the prior set of experiments. This suggests that at least a subset of the reactions do in fact undergo degradation of the DNA between the nick and loop. The reactions that contain HeLa extract can serve as internal controls for this idea, since MR is known to degrade back from the nick, thereby removing the DNA sequence that the probe binds to. The reactions containing HeLa extract do indeed have a lower abundance of these bands compared to HCT116 extracts, especially for the 3’ nicked substrates.

To further characterize the incisions made around the loop site, we processed the purified DNA in a way so that we could selectively analyze DNA that was not degraded between the nick and the loop. The recognition sequence for Sau96I is located between the pre-existing 3’ nick and the loop site, and since the restriction enzyme is not expected to digest ssDNA, substrate molecules that contained dsDNA between the nick and loop will be selectively analyzed. Much higher resolution was achieved in this experiment because the DNA fragments of interest are ~110-140 nt, compared to ~330 nt in Panel B. As would be expected from incisions made around the loop site, fragments with endpoints flanking the loop site are again evident in both the 3’-8C and 3’-12V substrates (Figure 3-6C). Size analysis of these bands map them to the sites immediately flanking the loop site (Figure 3-7). Most importantly, the bands occur only on the nicked strand, regardless of which strand the loop is on. As predicted, reactions that contained HeLa extract show reduced intensity of the bands compared to the HCT116 extract. Presumably,
Figure 3-6. Southern blot analysis testing for excision from a pre-existing nick.
A. Diagram of the regions analyzed in these experiments.  B. Loop repair intermediates produced by DNA resynthesis inhibition were probed by Southern blot analysis. The same membrane described in Figure 3-5C was stripped and re-probed with Probe 3 (C5765-5746) (see Chapter 2). Highlighted bands (arrows) were estimated to surround the loop site (~330 nt) C. Purified DNA generated by DNA synthesis inhibition during loop repair assays was digested with SspI and Sau96I to select for molecules that contain dsDNA in between the nick and the loop. DNA was separated by 8% denaturing PAGE and analyzed by Southern blot. The size of the labeled bands was estimated using Kodak 1D 2.0.2 software.
A. The autoradiograph shown in Figure 3-6C was scanned into Kodak 1D Image 2.0.2. The sizes (in bases) of the numbered bands were calculated using the marker DNA lane as a standard and map to the positions designated.

B. Repair assays (as described in Figure 3-2) were performed on the 3'-8C substrate using the conditions listed underneath the gel. The repair was visualized using the restriction enzyme sensitivity assay.

Figure 3-7. Mapping of excision intermediates of 3’ nicked substrates.

A. The autoradiograph shown in Figure 3-6C was scanned into Kodak 1D Image 2.0.2. The sizes (in bases) of the numbered bands were calculated using the marker DNA lane as a standard and map to the positions designated. B. Repair assays (as described in Figure 3-2) were performed on the 3’-8C substrate using the conditions listed underneath the gel. The repair was visualized using the restriction enzyme sensitivity assay.
the fully active MR system in HeLa cells is the cause of this. These results can be interpreted in
2 ways: 1) a key step in 3’ nicked, intermediate loop repair involves incision of the DNA near
the loop site, on the nicked strand, 2) 3’ nick directed repair occurs by a completely different
mechanism that creates a DNA gap that flanks the loop site. Evidence for the latter
interpretation comes from the fact that aphidicolin, an inhibitor of the processive DNA
polymerases α, δ, and ε does not affect the repair of the 3’-8C substrate (Figure 3-7B). Also, no
reaction intermediates were observed in HCT116 extracts when using aphidicolin to inhibit DNA
synthesis (data not shown). This suggests that at least a subset of 3’ nick directed repair events
occur by a unique mechanism, potentially involving DNA Pol β and a very short tract of DNA
resynthesis.

III. Discussion

The results presented here extend the range of nick directed bi-directional DNA loop
repair to at least 12 nucleotides. This repair has both MR dependent and MR independent
components. Focusing first on the MR dependent contribution, this work supports the idea that
stability of repeats up to 12 nt is MR dependent in human cells, similar to S. cerevisiae (Sia et
al., 1997). Using human cell extracts and purified proteins, Genschel et al. (Genschel et al.,
1998) demonstrated that hMutSα and hMutSβ were capable of supporting repair of loops up to 8
nt, but only 5’ nicked substrates were tested. Our data shows that MR can also act on 3’ nicked
substrates and extends to loops of up to 10 nt. In addition, while under some of the conditions
used here MR may not significantly contribute to repair of 12 nt loops, it is capable of processing
loops of this size. What factors determine repair in vivo is unclear. It should also be noted that
our own interpretation of the data of Genschel et al. is that repair of 12 base loops was increased
by the addition of hMutSα and hMutSβ.

Regarding individual protein contributions to MR dependent loop repair, experiments
using extracts from HCT15 cells suggest that hMutSα is less important for some types of loop
repair than hMutSβ. No statistically significant differences (α = 0.05) between HCT15 and
HeLa extracts were seen for 3’ nicked, 8 nt loops, whereas a difference was seen for the similar
5’ nicked substrates. Confounding this issue, however, are the results from the double mutant
HEC-1A which is deficient in both MSH6 and PMS2 (Risinger et al., 1998). These extracts
were the only ones to have a reduced ability to repair 5’ nicked, 12 nt loops. Given that both
HCT15 and HCT116 extracts are competent for repair of these substrates, the reduction in repair hints that there may be more complex interactions between the multiple hMutS and hMutL complexes than previously thought. Removal of either hMutSα or hMutLα does not affect repair, but loss of both apparently does. A recent report suggests that loss of both hMutLα and hMutSα has effects different than loss of hMutLα alone, similar to what we see here (Baranovskaya et al., 2001). Another possibility is that PMS2 is involved in a MR independent pathway specific for 5’ nicked loops.

One interesting point is the similarity in repair levels by MR+ and MR- extracts for the 3’ nicked, 12 nt loop substrates (Figure 3-3). This suggests that MR plays no role in the repair of these substrates. However, MR depleted HeLa cell extracts still show a drop in repair of ~25% for the 3’-12C substrate, similar to 5’-12V (Table 3-1). Unfortunately, these experiments use an agarose gel to score repair, so we were unable to verify that both 3’ nicked, 12 nt substrates showed decreased repair in the absence of MR. A possible explanation comes from the design of the experiment. In order to broaden the intensity range of the repair bands in these experiments, 2.5 times more substrate DNA was used with the same amount of extract (0.32 versus 0.8 fmol DNA/µg extract). It may be that while the MR system is able to process these substrates, the MR independent pathway is more active on them when DNA is limiting. When the amount of DNA is increased and the MR independent pathway becomes overwhelmed, MR processing of them becomes evident. While these loops may not be primarily repaired by MR, if needed, it can process them. It will be interesting to learn how these two loop repair pathways interact, especially in regards to whether the loop processing of MR is active during post-replicative MR, during recombination as a genetic sensor, or both (Evans and Alani, 2000).

Regarding the MR independent pathway, although this is not the first report of MR independent loop repair of substrates of this size, it is the first report of 3’ nick directed repair independent of MR (Genschel et al., 1998; Umar et al., 1994). The pathway appears to be somewhat active on loops of 5 nt, and its contribution to repair of 8 nt loops and larger increases with loop size. If this pathway is the same as that identified by Umar et al (Umar et al., 1994), the upper limit can be extended to at least 16 nucleotides. Since the fd:M13 hybrid contains at most 2 IDM of 1 or 2 nt (Worth et al., 1994), it can be argued that it will not sequester hMutSβ from the extracts and therefore is not a good inhibitor of ‘loop MR’. The 2 and 4 nt loops were only repaired at 20% of normal levels when hMutSα was sequestered, so a majority of the MR
dependent loop repair appears to be mediated by hMutSα. While there is no doubt that hMutSβ mediated repair is occurring in the presence of fd:M13 DNA, this hybrid DNA does not decrease repair of 8 or 12 nt loops by HCT15 nuclear extracts which also contain hMutSβ. So while we cannot rule out that a small amount of hMutSβ mediated MR is occurring in the HCT15 extracts, the fact that no reduction in repair occurs suggests that the MR independent pathway is responsible for a majority of the repair seen. In other words, it is at least as active as hMutSβ mediated MR or other hMutSβ-dependent loop repair reactions (Kearney et al., 2001; Kirkpatrick and Petes, 1997; Saparbaev et al., 1996; Sugawara et al., 1997).

The results from the analysis of reaction intermediates indicate that the MR independent pathway occurs by a slightly different mechanism than MR. They also suggest that processing of 5’ and 3’ nicked substrates may be different, possibly even by different pathways. Most importantly, the nicked strand is the target of repair events, with no processing of the continuous strand detected, irrespective of where the loop is located. For both 5’ and 3’ nicked substrates, the steps leading up to DNA re-synthesis produce DNA fragments with ends that are very close to the loop site. The span of DNA processed is defined on one end by the pre-existing nick, and on the other end by the loop site. When viewed along the short tract of DNA between the nick and the loop, the processing occurs just past the loop site, by no more than 20 nt. The results suggest that 5’ nicked substrates undergo 5’→3’ excision from the nick back towards the loop. One possibility is that the nick serves as the entry point for the helicase, similar to bacterial MR, and terminates once the loop binding activity marks the loop site. For 5’ substrates, the nick could also serve as the entry point for the DNA polymerase, with the end point of synthesis being near the loop site. This mechanism is similar to that proposed for MR, except for the specific proteins involved and the predominant excision stop site. Unfortunately, a parallel model for 3’ substrates cannot be invoked since the 3’ nick cannot support DNA re-synthesis along the short tract of DNA processed. Accordingly, excision originating at the nick does not appear to occur in 3’ substrates.

One possible explanation of the results is that excision still occurs 5’→ 3’, but that it starts near the loop and proceeds towards to nick. In this model, the loop would be recognized, the pre-existing nick identified to determine the incorrect strand, but then a nick is created near the loop site. Importantly, the reaction is still strand specific and nick directed. This is a critical point because whether the loop is removed or retained is dependent on the orientation of the nick.
and loop, and not on the loop itself. The property of using the nick as a strand discrimination signal but not excision point has also been described for the \textit{X. laevis} MR reaction (Varlet et al., 1996). The incision would have to be made 5’ to the loop site on the nicked strand (Figure 7, positions 1 and 4) to ensure the looped region was processed. Excision could then proceed 5’→3’ towards to the loop, with DNA re-synthesis initiating at the 5’ end created by the incision reaction and continuing towards the pre-existing nick. The DNA fragments that map to within the loop or 3’ to it (Figure 7, bands 2, 3, 5 and 6) could be the result of natural excision pause sites, or the products of a limited re-synthesis using dNTP’s endogenous to the extract preparation. The low abundance of bands observed Figure 3-6B might then be the result of a subset of reactions that were aborted after the incision step, but prior to excision that removed the entire tract of DNA between the nick and loop. Alternately, the 3’ nick directed pathway (or a subset of the reactions) could occur by an entirely different mechanism. This possibility is explored in detail in Chapter 5.

Clearly, the primitive models described require much more work to be validated or refuted. One point of interest is the apparent differences in MR involvement in 3’ nicked, 12 nt substrates. More limiting DNA conditions suggest MR is not acting, yet differences in reaction mechanism are evident, and consistent with MR involvement. While the apparent excision from the nick to the loop (and subsequent loss of probe binding sequence, Figure 3-6B) is not conclusive for MR, calling upon the classical MR mechanism provides an explanation for the results seen. Another possibility is interference of the two pathways, which could easily cause aberrant results that are difficult to interpret. Understanding how MR and this second pathway partition DNA loops between them will be critical to understanding the mechanism. Whether the MR system initially misses some loops during replication that this second pathway then repairs, or if MR independent correction is involved in entirely different processes (i.e. recombination) are questions that need to be answered. Although a picture of intermediate size loop repair is starting to emerge, a major task is to reconcile this body of evidence with the reports of large loop repair. While it is clear at least one pathway independent of MR is working for loops up to 16 nt, several reports exists of pathways for larger loop repair (> 25 nt) that show some different characteristics from the ones described here (Bishop and Kolodner, 1986; Clikeman et al., 2001; Corrette-Bennett et al., 2001; Corrette-Bennett et al., 1999; Kirkpatrick and Petes, 1997; Taghian et al., 1998; Umar et al., 1994; Weiss and Wilson, 1987; Weiss and Wilson, 1989).
identification and characterization of these pathways in human cells is the topic of the next chapter.
CHAPTER 4
Multiple Pathways Of Large Loop Repair In Human Cells

I. Introduction

The multitude of proteins and pathways devoted to DNA repair underscores the importance of genomic stability (Friedberg et al., 1995; Jiricny, 1998a; Jiricny, 1998b; Kolodner and Alani, 1994; Kolodner and Marsischky, 1999; Lindahl and Wood, 1999; Wilson and Kunkel, 2000). Exogenous chemical and physical insults can cause several types of damaged DNA. Assaults from reactive by-products of normal cellular metabolism can also modify DNA in deleterious ways. In addition to this, DNA metabolism can introduce structures that bear little resemblance to normal DNA. One such set of structures are regions of unpaired nucleotides (nt) on one strand of the DNA, or DNA loops. These structures can range in size from a single base to several thousand nucleotides. Smaller loops (< 20 nt) are generally formed during replication of repeat sequences (Henderson and Petes, 1992; Strand et al., 1993) and larger loops can arise during recombination events between divergent sequences (reviewed in (Petes et al., 1991). As described in the previous chapter, the repair of smaller loops (1-13 nt) occurs by both mismatch repair (MR) and another MR independent pathway. In this chapter we will focus on the repair of a larger, 30 nt loop.

The first description of large loop repair in eukaryotic cells came over a decade ago (Ayares et al., 1987; Weiss and Wilson, 1987). These reports used COS-1 monkey kidney cells that were transformed with plasmid DNA containing loops. The results indicated that unpaired loops of 12-283 nt were efficiently processed in mammalian cells. One of the reports (Weiss and Wilson, 1987) indicated that repair was biased 2:1 in favor of loop loss. A later report (Weiss and Wilson, 1989) from the same group indicated that a nick located up to 125 bp away from the loop only moderately influenced the outcome of repair. The strongest repair occurred when the nick and loop were on the same strand. It was not until much later that large loop repair in prokaryotes was identified (Fang et al., 1997). The results indicated that E. coli cells do have the ability to repair loops as large as 22 nt and that it was only partially dependent on MR function. However, the process was relatively inefficient when compared to MR correction of 1-nt loops. The nature of these experiments did not allow for any speculation on possible mechanisms or proteins that might be involved in the repair described. The recognition of DNA instability as an
early phenotype in carcinogenesis (Loeb, 2001) has led to a resurgence in research regarding the repair of these potentially mutagenic structures. Several recent reports in human and yeast cells have identified pathways for the processing of DNA loops ranging from 16 to over 5,600 nt (Bill et al., 1998; Bill et al., 2001; Corrette-Bennett et al., 2001; Corrette-Bennett et al., 1999; Kearney et al., 2001; Kirkpatrick and Petes, 1997; Littman et al., 1999). These pathways are separate from the one described in Chapter 3 and in other reports (Genschel et al., 1998; Umar et al., 1994).

In yeast cells, at least three separate repair activities have been described in vivo. One involves the MR proteins MutS homolog 2 (yMSH2) and post meiotic segregation 1 (yPMS1, the homolog of hPMS2) and is active on very large loops (2000+ nt) during double strand break (DSB) induced mitotic recombination (Clikeman et al., 2001). This possibly reflects the role of MR in recombination as a ‘genetic sensor’ (Evans and Alani, 2000). A separate activity involving the nucleotide excision repair (NER) protein RAD1 (the homolog of human XPF) and MSH2 that acts on a loop of 26 nt loop during meiotic recombination has also been described (Kirkpatrick and Petes, 1997). A third pathway also uses the RAD1 protein, but in combination with the MR protein MSH3 and possibly is active on rare DNA polymerase slippage errors of large size (90-100 nt) (Harfe and Jinks-Robertson, 1999; Harfe et al., 2000). There are also reports implicating MutSβ (a complex of MSH2 and MSH3) and the RAD1/RAD10 complex in both mitotic and meiotic recombination, although repair of loops was not specifically investigated (Saparbaev et al., 1996; Sugawara et al., 1997). Whether the complete MR and NER pathways are acting in any of the reactions is unclear. Also, an in vitro assay system for repair of large loops has recently been described (Corrette-Bennett et al., 1999). The results from in vitro experiments indicate that a 5’ nick directed pathway and a ‘nick-independent’ pathway that preferentially removes loops are active and that both pathways are at least partially independent of the MSH2, MSH3, RAD1, and RAD10 protein products. Which of the three in vivo identified pathways these correspond to, if any, is unclear.

There is also evidence of multiple pathways of large loop repair in human cells. Results from loops of 27-993 nt repaired in vitro indicated that only a 5’ nick located 114 bp from the loop could target repair to a specific strand (Littman et al., 1999). This repair was independent of MSH2, MSH6, MLH1, PMS2, and XPF in human cells, and independent of XPF(ERCC4) and ERCC1 in hamster cells (homologs of S. cerevisiae RAD1 and RAD10, respectively). Although
the repair described in that report is not likely to involve meiotic recombination, several other examples have implicated the recombination machinery in large loop repair in mammalian cells (Bill et al., 2001; Bollag et al., 1992; Taghian et al., 1998). Interestingly, Bill et al. (Bill et al., 2001) reported that palindromic loop sequences cause a shift in the repair bias from loop retention to loop loss as the loop size increases. This is contrary to other reports that loops with secondary structure are refractory to efficient repair (Bollag et al., 1992; Corrette-Bennett et al., 2001; Moore et al., 1999; Nag and Petes, 1991; Nag and Petes, 1993). The precise pathways that process very large loops (2000+ nt) in human cells are currently unknown. In this study, we used nuclear extracts prepared from cells that were proficient or deficient in either MR or NER to investigate the repair of 30 nt loops in a defined substrate. First, we were interested in determining if human cells have a pathway similar to the yeast ‘nick independent’ loop removal pathway. An activity that preferentially removes DNA loops seems to be a perfect candidate for the 2:1 bias towards loops loss originally reported in monkey cells (Weiss and Wilson, 1987). Second, we sought to understand the molecular mechanism of any repair pathways we were able to identify.

II. Results

II.A. Nick dependent and independent large loop repair pathways exist in human cells

In order to study the repair of a large DNA loop by human nuclear extracts, we created a set of substrates containing either a 5’ or 3’ nick with a 30 nt loop in either the complementary (C) or viral (V) strand. This encompasses all four possible combinations of nick position and loop location (Figure 4-1A). The substrate nomenclature used first identifies the nick orientation (as viewed along the shortest path between the nick and loop site), then the loop size, followed by the looped strand. For example, 5’-30V referred to a substrate that contained a 5’ nick and a 30 nt loop in the V strand. The distance between the nick and the loop is 115 and 175 bp for 5’ and 3’ nicks, respectively. For these substrates, the 5’ and 3’ nicks are located in the C and V strands, respectively. Therefore, the 5’-30C and 3’-30V substrates have the loop and nick in the same strand, while in 5’-30V and 3’-30C they are on opposite strands. The sequence of the loop is random, except for a run of 4 dinucleotide repeats, and was not expected to form any kind of secondary structure (Figure 4-1B).
Figure 4-1. Diagrammatic representation of the four 30 nucleotide loop substrates used. A. Phage MR24 dsDNA was digested with \textit{NheI} and \textit{XbaI}, gel purified, and religated (the recognition sequences of \textit{NheI} and \textit{XbaI} have compatible ends). The resulting DNA contains a 30 base deletion relative to MR24. Substrates containing the loop in either the C or V strand and containing either a 5’ (C strand) or 3’ (V strand) nick were prepared as described in Chapter 2. The distance from the nick to the loop site is 115 and 175 bp for the 5’ and 3’ nicks, respectively. Substrates are named as described in Chapter 2. B. Sequence of phage MR24 in the vicinity of the loop site. Loops (sequence in boldface) are generated when MR0 and MR24 DNA is paired. The sequence of the loop will depend on whether it is contained in the C or V strand. All loop sequences contain the \textit{XcmI} recognition sequence (underlined).
Initially, we tested for nick directed repair of the 5'-30V and 3'-30C substrates using the well described restriction enzyme based assay (Lu et al., 1983). As a negative control, HeLa nuclear extracts were heated to 95°C for 5 min prior to substrate addition. This treatment demonstrates that substrate molecules that do not undergo repair are resistant to digestion by \textit{XcmI}, since only the 6.4 kbp band is present in these lanes (Figure 4-2A, lane 2). After exposure to nuclear extracts from either MR proficient deficient cells, the recovered DNA becomes sensitive to \textit{XcmI}, indicating that the nicked, C strand was repaired using the V strand as template, thereby restoring a homoduplex \textit{XcmI} sequence to the substrate (Figure 4-2A, lanes 1, 3-6). This allows both enzymes to cut and creates a doublet of bands at 3.1 and 3.3 kbp. HeLa cells are proficient for MR, while NALM6, HCT15, HCT116, and HEC-1A cells are missing the MR proteins MSH2, MSH6, MLH1, or PMS2/MSH6, respectively, rendering them deficient for MR. The results from this type of assay indicated that human cells were capable of nick directed repair of the 5'-30V substrate, but not the 3'-30C substrate (data not shown). Unfortunately, because the non-looped strand contains no restriction site in this region, only repair causing loop addition (i.e. when the nick and loop were on opposite strands) could be scored by this assay. To study the other two 30 nt loop substrates (5'-30C and 3’-30V) and also to study processing of both strands of each substrate, we developed a Southern blot assay to visualize repair.

To follow the fate of each strand of the substrate, we took advantage of the 30 nt difference in size between looped and non-looped strands. A DNA fragment encompassing the loop region was separated by denaturing PAGE, transferred to a nylon membrane and sequentially probed for either the C or V strand. The size of each strand corresponded to whether the strand was looped or non-looped (see diagrams below gel for reference). For example, the C strands of 5'-30V and 5'-30C were 444 and 474 nt long, respectively, because in 5'-30V the C strand is non-looped, while in 5'-30C it contains the loop. The situation is reversed when the V strand is probed (478 and 448 bases for 5'-30V and 5'-30C, respectively). The V strand is 4 nt longer due to the 4 base overhang created by \textit{BanII}, but the difference between looped and non-looped strands is still 30 nt. The V strand probe (Probe 2) displays some non-sequence specific binding indicated by the minor band evident in heat-inactivated samples of all substrates. This same band is seen when the substrate is analyzed directly (no exposure to any
Figure 4-2. Visualization of loop repair reactions by human cell extracts. A. Loop repair reactions were performed using 75 µg nuclear extract and 24 fmol of 5'-30V substrate. Purified DNA was digested with XcmI and BspDI and the products separated through a 1% agarose gel and visualized using Ethidium bromide. B. Processing of both strands was performed using a Southern blot. Purified substrate DNA was digested with BanII and SspI, separated through a 5% denaturing acrylamide gel (7M urea, 1X TBE) and then transferred to a nylon membrane. The membrane was hybridized with 32P end-labeled oligonucleotide probes (gray boxes). Probe 1 is V5216-5235 and Probe 2 is C5259-5235 (see Chapter 2). Probing the V strand gave fragments 4 bases longer than the C strand due to the asymmetry of the BanII cut.
As expected, substrates exposed to heat-inactivated HeLa extract show only one major band (Figure 4-2B, lanes 1, 4, and 7, Probes 1 and 2). Repair directed by a 5’ nick is evident in substrates exposed to active nuclear extracts by the appearance of a second band evident when probing the nicked C strand (Figure 4-2B, Lanes 2, 3, 5, and 6, Probe 1). The size of the second band is consistent with loop addition (5’-30V) or loop removal (5’-30C). This confirms the results of Panel A, indicating that a 5’ nick can direct repair to the nicked strand regardless of whether the loop is in the nicked or continuous strand. Also consistent with a lack of 3’ nick directed repair, probing the nicked V strand of 3’-30C does not show a second band in either HeLa or MSH2 deficient NALM6 extracts (Figure 4-2B, lanes 8 and 9, Probe 2). These results are consistent with another report (Littman et al., 1999).

Interestingly, we also detected a second loop repair activity that is independent of a nick, but dependent on the presence of a loop. Probing the continuous V strand of 5’ nicked substrates shows that the looped V strand is converted to the shorter, non-looped strand, but that loop addition to the non-looped strand does not occur (Figure 4-2B, lanes 2, 3, 5, and 6, Probe 2). The results are slightly obscured by the background V strand probe binding, but correction for this (by subtraction of the heat-inactivated extract band intensity) gives values close to zero for 5’-30C but much greater than zero for 5’-30V. The only difference between these 2 substrates is the location of the loop in the nicked or continuous strand, respectively. Interestingly, we also detected a similar result in the 3’-30C substrate (Figure 4-2B, lanes 8 and 9, probe 1). In this substrate it is the C strand that is both continuous and looped. Loop removal (conversion from 474 to 444 nt) is evident. These results suggest that a nick independent, loop removal pathway similar to that described in yeast cells (Corrette-Bennett et al., 2001; Corrette-Bennett et al., 1999) is present in human cells as well.

Using the Southern blot based assay, we determined the repair values for each strand for all four substrates using HeLa nuclear extracts (Figure 4-3). Both 5’ nicked substrates show repair of the nicked strand at levels similar to that found for smaller loops (see Chapter 3), indicating that a 5’ nick is a signal for strand discrimination for these substrates. The repair values obtained for 5’-30V were similar in both assays. The results of 3’ nicked substrates are not as straightforward. Neither agarose nor Southern blot assays show 3’ nick directed repair for the 3’-30C substrate. Using the Southern blot assay, we do detect what appears to be 3’ nick
Figure 4-3. Repair values of 5’ and 3’ nicked substrates by HeLa nuclear extracts. Autoradiographs of repair assays (Figure 4-2, panel B) were quantitated after scanning using Kodak Image 1D version 2.0.2. Repair was calculated as the intensity of the ‘repaired’ band divided by the intensity of both bands. For example, ‘nick strand’ repair of 5’-30V was calculated as the intensity of the 474 base band divided by the intensity of both the 444 and 474 base bands (C strand probe). Values are the mean for at least 3 experiments for each substrate. Error bars show standard error of the mean. All values were corrected for background probe binding seen in substrate only controls.
directed repair of the 3′-30V substrate. However, in this substrate, the 3′ nicked V strand also contains the loop, meaning that nick directed repair and nick independent loop removal would have the same outcome. As noted above, the 3′-30C substrate does not undergo loop addition to the 3′ nicked strand, although nick independent loop removal from the continuous strand does occur. The level of nick independent loop removal in both 5′-30V and 3′-30C is similar to the level of nicked strand repair in 3′-30V, making it possible that what appears to be 3′ nick directed repair is actually nick independent loop removal. The 3′-30V substrate also undergoes a lower, but significant level of loop addition to the continuous strand. While it is not uncommon for substrates to undergo untargeted ‘background’ reactions in this type of assay, the level is higher than in all other substrates. The method of making 3′ nicked substrates uses the corresponding 5′ nicked molecules as starting material, so contamination of the 3′-30V substrate preparation with 5′-30V could account for the loop addition seen. Analysis of the 3′-30V repair products using the agarose based assay indicated that the resulting molecules are sensitive to XcmI, and during the course of other experiments, a small amount (≤ 5%) of 5′ nicked molecules were detected in the 3′-30V preparation but not the 3′-30C preparation (data not shown).

II.B. Large loop repair activity in MR, NER, and WRN deficient nuclear extracts

We assayed several cell lines deficient in various DNA repair proteins for repair activity of both the nick dependent and nick independent pathways using the 5′-30V substrate. This allows for detection of both pathways in a single reaction. Repair activities were compared to HeLa levels which is WT for both MR (Fang and Modrich, 1993) and NER (Wood et al., 1988), as well as WRN activity (Marciniak et al., 1998). Importantly, all cell lines tested showed some degree of activity for both pathways (Table 4-1). For the nick directed pathway, cell lines with mutations in the MR genes \textit{MLH1} (HCT116), \textit{MSH6} (HCT15) or \textit{MSH2} (NALM6), and also a cell line (WS780) mutated in WRN, the Werner Syndrome (WS) helicase/exonuclease, had levels of repair comparable to those of HeLa cells. The only MR mutant to have a different level of nick directed repair compared to HeLa cells (\(p = 0.031\)) was HEC-1A, which contains mutations in both \textit{MSH6} and \textit{PMS2}. Cells with mutations in the NER genes \textit{XPA} (GM02345) or \textit{XPG} (AG08802) show a similar drop in nick directed repair as HEC-1A, although neither value was
Table 4-1. Repair levels of nick dependent and independent large loop pathways in MR, NER, and WRN helicase deficient cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Relevant Mutation</th>
<th>5'-30V Repair Level (% ± sem)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nick Dependent</td>
</tr>
<tr>
<td>HeLa</td>
<td>None</td>
<td>22.37 ± 2.24</td>
</tr>
<tr>
<td>NALM-6</td>
<td>MSH2</td>
<td>23.19 ± 4.86</td>
</tr>
<tr>
<td>HCT15</td>
<td>MSH6</td>
<td>18.01 ± 2.28</td>
</tr>
<tr>
<td>HCT116</td>
<td>MLH1</td>
<td>19.84 ± 2.14</td>
</tr>
<tr>
<td>HEC-1A</td>
<td>PMS2; MSH6</td>
<td>11.87 ± 3.25</td>
</tr>
<tr>
<td>GM02345</td>
<td>XPA</td>
<td>13.08 ± 0.52</td>
</tr>
<tr>
<td>AG08802</td>
<td>XPG</td>
<td>13.60 ± 1.02</td>
</tr>
<tr>
<td>WS780</td>
<td>WRN</td>
<td>18.47 ± 1.67</td>
</tr>
</tbody>
</table>

Nuclear extracts from cell lines deficient in the indicated protein(s) were tested for repair activity using the 5'-30V substrate. Nick dependent repair is defined as repair to the C strand causing loop addition. Nick independent repair is defined as loop removal from the continuous V strand. Average and standard error of the mean (sem) are given for at least three independent reactions. Separate preparations of HeLa, NALM6, HCT116 and Hec-1A extracts were prepared with less than 10% variation in the results between preparations. HeLa cells are proficient for both MR and NER. NALM6, HCT15, HCT116 and HEC-1A are all deficient for MR using an *in vitro* assay. GM02345 and AG08802 contains mutations in the NER proteins XPA or XPG but show low levels of MR *in vitro*. WS780 contains a partially active form of the WRN helicase (the exonuclease activity is intact) and is proficient for MR using an *in vitro* assay.
deemed significant at $\alpha = 0.05$ ($p = 0.065$ and 0.081 for GM02345 and AG08802 extracts, respectively). All extracts displayed a similar activity level for the nick independent pathway. The 3’-30C and 5’-30C substrates were also assayed for repair by these cell lines with results similar to that of HeLa (data not shown). These results indicate that both repair pathways are largely independent of the MR and NER pathways, and also the WRN protein.

II.C. The 5’ nick directed pathway is sensitive to DNA synthesis inhibition

To study the mechanism of the 5’ nick dependent reaction, we mapped reaction intermediates when DNA resynthesis was inhibited by treatment of the extracts with aphidicolin, ddNTP’s, or in the absence of dNTP’s. Using the Southern blot assay, we found that all three conditions prevented repair, suggesting that extensive DNA synthesis is required to complete the reaction (Figure 4-A, compare lanes 2 with lanes 3-5 and lanes 2’ with lanes 3’-5’). As expected, the inhibition was not specific to loop addition or removal, indicating that it is the 5’ nick that is directing repair to a specific strand. Aphidicolin specifically prevents the more processive DNA polymerases $\alpha$, $\delta$, and $\varepsilon$ (DePamphilis and Wassarman, 1980) and addition of all four chain terminating ddNTP’s (ddATP, ddCTP, ddGTP, ddTTP) in equimolar concentrations compared to dNTP’s is expected to inhibit all DNA synthesis, as does a lack of dNTP’s. In order to visualize the intermediates formed by DNA synthesis inhibition, Southern blot hybridization was performed as previously described (Corrette-Bennett et al., 1999; Littman et al., 1999) Chapter 3). Under conditions of limited DNA synthesis, prominent DNA fragments with endpoints that correspond very close to the loop site are evident in both 5’-30V (Figure 4B, lanes 2-4) and 5’-30C (Figure 4C, lanes 2-4). This is more apparent when either no dNTP are present or ddNTP have been added. The reactions containing aphidicolin show less prominent, although similar sized bands. For both substrates, there are also less prominent bands that map to regions in between the nick and the loop. It is interesting that in reactions containing 5’-30C, a slightly different pattern of intermediate tracts is evident. This is possibly due to interference of the nick independent loop removal pathway, which would presumably be active on this substrate as well as the 5’ nick directed pathway. We also note that in both substrates, even under complete reaction conditions (Figure 4B and C, lane 1), a significant fraction (~10-20%) of the substrate remains unligated, consistent with previous observations (Littman et al., 1999) Chapter 3).
Figure 4-4. Analysis of the 5’ nick directed large loop repair mechanism. Repair assays were performed using conditions that limited DNA synthesis: removal of dNTP, addition of aphidicolin, or addition of ddNTP. Reactions were performed at 2X volume and divided evenly after substrate DNA isolation. A. Isolated DNA was digested with SspI and BanII and the extent of 5’ nick directed repair was analyzed as described in Figure 4-2. B and C. Isolated DNA was digested with SspI and separated through a 6% denaturing acrylamide gel. The DNA was transferred to a nylon membrane, followed by hybridized with 32P-end labeled oligonucleotide (V5216-5235, see Chapter 2). Schematic diagrams of the SspI fragment of interest (vertical lines) are shown next to each gel. Gray boxes indicate the binding site of V5216-5235. The gray line represents the unprobed strand. The marker is the same for Panels B and C.
If the intermediate bands observed (Figure 4-4B and 4-4C) were the result of incisions near the loop site on the nicked strand, a probe that binds between the nick and loop would cause bands of either ~120 or ~320 nt to be evident, depending on if the substrate were unligated and ligated, respectively. However, the only bands evident were 557 or 757 nt, presumably from unligated and ligated substrate molecules that were not processed, respectively (data not shown). These results suggested that the region of DNA between the nick and loop on the C strand was missing on substrates that had undergone incomplete repair reactions. To test this directly, we digested the recovered DNA with BseRI and BanII. The BanII site is located in between the nick and loop, 56 bp from the nick. Under conditions of limited DNA synthesis, 5’→3’ excision from the pre-existing nick back to the loop site will create a region of single stranded DNA, preventing BanII from cutting and creating a 6.45 kbp linear fragment (because of the BseRI cut). If both enzymes are able to digest the DNA, fragments of 3.68 and 2.77 kbp will be evident (Figure 4-5A). A control set of reactions using a G/T mismatch demonstrated that an increased prominence of the 6.45 kbp band was observed regardless of how DNA synthesis was inhibited (Figure 4-5B). This result indicates, as expected based on previous studies (reviewed in Modrich, 1997), that inhibition of DNA synthesis caused a ssDNA gap to be formed which is refractory to digestion by BanII.

Experiments using both the 5’-30C and 5’-30V substrates give somewhat surprising results. When DNA synthesis was inhibited because of a lack of dNTP’s, excision back from the nick was observed (Figure 4-5C, lanes 3 and 8), similar to the situation when using a G/T mismatch. However, reactions containing aphidicolin were indistinguishable from complete repair reactions (Figure 4-5C, compare lanes 2 with 5, 7 with 10). This observation suggests two possibilities: 1) the repair reaction was inhibited prior to excision; 2) the repair reaction did not require an aphidicolin sensitive DNA polymerase (i.e. the reaction went to completion). The latter possibility was eliminated because aphidicolin inhibited repair (Figure 4-4A, lanes 4 and 4’). Also, if an aphidicolin sensitive DNA polymerase was not involved in repair, no intermediate bands would be evident (Figure 4-4B lane 3 and Figure 4-4C, lane 3). These findings strongly suggest that one of the aphidicolin sensitive DNA polymerases (Pol α, δ, or ε) is involved in the excision step of nick directed loop repair. Interestingly, the addition of
Figure 4-5. Gap formation assay using 5’ nicked substrates. A. Diagrams of predicted DNA molecule for either incision or excision based processing of substrates. B. DNA from repair reactions containing HeLa cell extracts, a 5’ nicked G/T mispair, and the conditions listed was digested with BseRI and BanII and separated through a 1% agarose gel. Bands of 2.77 and 3.68 kbp indicate digestion by both enzymes, while a band at 6.45 kbp indicates that only 1 enzyme was able to digest the DNA. Controls reactions (not shown) demonstrated that BseRI cut to completion under all conditions used. C. The same analysis was performed on both 5’ nicked, 30 nt loop substrates (upper gel).
ddNTP’s allows more excision than aphidicolin treatment, but less than removal of dNTP’s (compare lanes 3 and 8 with lanes 4 and 9 in Figure 4-5C). This hints that base analogs that block DNA polymerase activity also inhibit the excision initiation reaction.

II.D. Nick independent loop removal is weakly affected by DNA synthesis inhibition

Taking advantage of the V strand loop in both the 5’-30V and 3’-30V substrates as substrates for the nick independent pathway, we performed similar analyses to those just described for the nick dependent reaction. While there is a drop in loop removal for both 5’-30V and 3’-30V substrates, repair is not completely inhibited by conditions of limited DNA synthesis. After subtraction of the non-specific ‘repair’ bands evident in the substrate only lanes (Figure 4-6A, lanes 1 and 6), the largest decrease in repair (~50% of normal levels) was observed with aphidicolin treatment (Figure 4-6A, lanes 4 and 9). Interestingly, a lack of dNTP’s in the reaction had very little effect on repair (compare lanes 2 and 7 with lanes 3 and 8 in Figure 4-6A). Parallel experiments using the 3’-30C substrate and probing for the C strand show similar results (data not shown). These results suggest that extensive DNA synthesis is not required for the nick independent repair reaction.

To understand the mechanism involved in nick independent loop removal, reactions performed under conditions of limited DNA synthesis were analyzed for reaction intermediates, as described for the nick directed pathway above. The results from these experiments indicate that a very small region of DNA centered around the loop is processed (Figure 4-6B and C). Reactions containing 5’-30V that have DNA synthesis inhibited (Figure 4-6B, lanes 2-4) show two bands (indicated by *) that correspond to regions flanking the loop site. These bands are not present under complete reaction conditions (Figure 4-6B, lane 1). The bands are most apparent in reactions lacking dNTP’s. It should be noted that a prolonged exposure to film (compared to the results presented in Figure 4-4) was required to see these bands. This indicates that nick independent loop removal is less sensitive to limited DNA synthesis conditions, in agreement with the repair results (Figure 4-6A). Similar results were evident in the 3’-30V substrate in the absence of dNTP’s (Figure 4-6C, lane 2). Similar to the 5’ nicked substrates, the nick in 3’-30V is incompletely ligated even under complete reaction conditions. Surprisingly, minor bands that map to regions in between the loop and nick (indicated by a vertical black bar) are present in
Figure 4-6. Analysis of the nick independent large loop repair mechanism. Repair assays were performed using conditions that limited DNA synthesis (see Figure 4-4). Reactions were performed at 2X volume and divided evenly after substrate DNA isolation. A. Isolated DNA was digested with SspI and BanII and the extent of loop removal was analyzed as described in Figure 4-2 by probing the V strand with probe C5259-5235 (see Chapter 2). B and C. Isolated DNA was digested with SspI, separated through a 6% denaturing acrylamide gel, transferred to a nylon membrane, and then hybridized with ³²P-end labeled oligonucleotide (C5259-5235, see Chapter 2). Schematic diagrams of the SspI fragment of interest (vertical lines) are shown next to each gel. Gray boxes indicate the binding site of C5259-5235. The gray line represents the unprobed strand.
3’-30V when aphidicolin or ddNTP’s are present in the reaction. This suggests that some type of processing of 3’ nicked substrate occurred that involved processive DNA synthesis and may indicate that processing of 3’ nicked substrates occurs in ways that we cannot detect using the methods described here.

III. Discussion

The results presented here describe at least two distinct pathways of large loop processing, one of which has previously been characterized only in yeast cells (Corrette-Bennett et al., 2001; Corrette-Bennett et al., 1999). Our results agree with those of Littman et al. (Littman et al., 1999) in that a 5’ nick is a strong strand discrimination signal for unpaired loops of 27 nt and larger. Whether the loop is removed or added is determined by the relative orientation of nick and loop. Also similar to that report, we fail to detect any influence of a 3’ nick in one of our substrates (3’-30C). However, we also detected a significant amount of repair targeted to the continuous strand in two of our substrates (5’-30V and 3’-30C), as well as what appeared to be 3’ nick directed repair in one substrate (3’-30V). In both cases where processing of the continuous strand was detected, the outcome was removal of the unpaired loop. Loop addition to a continuous, non-looped strand was not observed. For the 3’-30V substrate, the predicted outcome of nick directed repair is the same as that of nick independent loop removal. Given the lack of loop addition directed by a 3’ nick in the 3’-30C substrate, we think the loop removal activity seen in 3’-30V is not directed by the nick, but rather by the loop itself, similar to the processing of the V and C strands in 5’-30V and 3’-30C, respectively. Although we also observed loop addition to the continuous strand in 3’-30V, a low level (< 5%) of residual 5’ nick on the C strand was detected that could be the cause. Regardless, loop removal, directed either by a 3’ nick or by the loop itself was still the predominant reaction observed in this substrate.

Recently, several reports have described processing of very large loops (2,000-5,600 nt) in yeast cells (Clikeman et al., 2001; Kearney et al., 2001). Also in yeast systems, the MR protein MSH2 has been shown to interact with RAD1 and process a 26 nt loop (Kirkpatrick and Petes, 1997). Additionally, purified RAD1/RAD10 was able to increase (but not to wild type levels) in vitro repair of a 27 nt loop (Corrette-Bennett et al., 1999). Our results suggest that the repair described here is separate from these reports. First, the nuclear extracts used come from
immortalized cell lines, and would not expected to express meiosis specific repair and/or recombination pathways. Second, the *MSH2* mutant showed no difference in either repair pathway compared to wild type levels. Although we were unable to obtain a nuclear extract of sufficient quality to test the involvement of the XPF gene product (the homolog of yeast RAD1), another report has indicated that XPF is not required for 5’ nick directed large loop repair (Littman et al., 1999). Unfortunately, no mention of nick independent loop removal was made in this report. The fact that XPA or XPG deficient nuclear extracts are proficient for nick independent repair argue against the core NER being involved in this pathway, although we cannot rule out that XPF is involved separate from NER. Several recent reports suggest XPF can process DNA conformations other than dsDNA:ssDNA junctions (Kuraoka et al., 2000; Wang et al., 2001; Zhang et al., 2000).

One observation that warrants explanation is the reduced levels of nick directed repair activity in the *PMS2/MSH6* double mutant, as well as the *XPA* and *XPG* mutants. While this could reflect a role of these proteins in one of multiple pathways, we do not feel this is the case. Both of the NER deficient cell extracts had very low *in vitro* activity on a G/T mismatch (data not shown), while the WRN mutant line had very high MR activity. The mechanism we have proposed for the 5’ nick directed reaction is similar in many ways to the MR reaction, so the decrease in repair activity may be due to downstream events (DNA resynthesis, ligation) common to both pathways. This could be due to alterations in the cell lines or simply reflect lower quality nuclear extracts. Supporting the former hypothesis, we have also tested an XPG deficient fibroblast cell line for repair and obtained negative results for MR and both large loop pathways (data not shown). Clearly, however, the lymphoblast derived XPG deficient line (Table 4-1) is not defective for large loop repair. This indicates that the cell type and potentially the method of transformation can have an effect on the repair characteristics. A similar phenomenon has been described whereby transformation with SV40 causes a decrease in the amount level of NER activity in human fibroblasts (Bowman et al., 2000). Therefore, we cannot say for certain whether XPF is involved in the nick independent loop removal pathway, and it is possible that the decrease in 5’ nick directed repair observed in XPA, XPG, or MSH6/PMS2 deficient extracts is not due to involvement of these proteins in that pathway.

The analysis of reaction intermediates of the nick directed pathway allows for some speculation on the early steps of the pathway. Conditions of limited DNA synthesis cause a
DNA fragment with an endpoint very close to the loop site to accumulate. Similar intermediates from both yeast and human cells extracts have been reported previously (Corrette-Bennett et al., 1999; Littman et al., 1999), and they are also evident in 5’ nicked substrates with loops of 8 and 12 nt (Figure 3-5B). This fragment is only evident when the probe is located 3’ to the loop and nick, but not when the probe binding site is located in between the nick and loop. This observation is consistent with the idea of excision that initiates at the pre-existing nick and proceeds towards the loop site. This is similar to the MR mechanism (Modrich, 1997). Unlike MR, though, our results hint that excision initiation is dependent on an active DNA polymerase. In the MR reaction, the excision step is independent of DNA resynthesis, as none of the 3 treatments used to limit DNA synthesis had an affect on gap formation between the nick and loop. A single stranded region that is refractory to BanII digestion is created under all conditions (Figure 4-5B, lanes 2-4). This is in agreement with the E. coli MR mechanism where Helicase II acts to displace the nicked DNA strand, with excision then occurring on the resulting ssDNA (Dao and Modrich, 1998; Grilley et al., 1993). However, during 5’ nick dependent large loop repair, aphidicolin causes a complete loss of nick dependent repair (Figure 4-4A) as well as preventing any excision (Figure 4-5C, lanes 5 and 10). A lack of dNTP or presence of the chain terminating ddNTP are also inhibitory to repair, but excision still occurs under these conditions (Figure 4-5C, lanes 3, 4, 7 and 8). This suggests that a DNA polymerase competent for synthesis is required for the initiation of the excision step, but extensive synthesis is not required.

One model that incorporates this idea (discussed in detail in Chapter 5) could also explain the polarity of the nick dependent repair that we observed. If an aphidicolin sensitive polymerase (α, δ, or ε) were ‘linked’ to an exonuclease, excision from the nick followed immediately by DNA resynthesis would only work if the nick were 5’ to the loop (i.e. DNA resynthesis from the nick towards the loop must be 5’→3’). A nick 3’ to the loop would require excision/resynthesis along the long path between the nick and loop, which in these substrates would be ~6.3 kbp. Since the excision activity is 5’→3’, it cannot be from any known DNA polymerase (Kornberg and Baker, 1992). This model assumes that actual DNA resynthesis is not required for excision, since excision occurs in the absence of dNTP’s. It may be that the small pool of endogenous dNTP’s are enough to allow initiation of excision, but not complete resynthesis. It could also be that the inhibitors (aphidicolin and ddNTP) bind in the polymerase active site and alter the conformation enough to prevent nuclease interaction and/or initiation.
This would explain the slight drop in excision seen when ddNTP’s are present. Although Littman et al. (Littman et al., 1999) reported that 0.5 mM ddTTP was not inhibitory to 5’ nick directed repair, we used 0.1 mM of all four ddNTP. Such a high concentration is likely to inhibit all DNA polymerase activity, which could account for the slight drop in excision that we observed.

The nick independent loop removal pathway operates by a much different mechanism. The most obvious difference is that repair is reduced by a maximum of ~44% when DNA resynthesis is inhibited. This inhibition is greatest when aphidicolin is added, suggesting that polymerase α, δ, or ε may be involved in the resynthesis step of this pathway. Analysis of reaction intermediates under limited DNA resynthesis conditions suggests that a very small patch is involved in this repair. The appearance of bands flanking the loop site (Figure 4-6B and 4-6C) could possibly indicate that incisions on either side of the loop are made. Whether both are made in each reaction or if the placement (5’ or 3’) is arbitrary cannot be ascertained by these experiments. Interestingly, even though nick independent repair activity was only ~50% that of nick dependent repair, the intermediates seen in Figure 6 required a prolonged exposure compared to those of nick directed repair. In some experiments they were barely visible, even when repair levels were normal. One model to explain this would be that an XPG-like or XPF/ERCC1-like activity creates a strand break on one side of the loop in between the first unpaired base and the last paired base. This would create a flap DNA structure that could be processed by either Flap Endonuclease 1 (FEN1) or a similar activity with a preference for 3’ DNA ends. If excision removes only the looped sequence, no DNA synthesis would be required for repair. Even if a couple of the flanking homoduplex bases were removed because of local helix distortion, the repair patch could still be less than five bases. This could account for the residual repair seen even when dNTP are left out of the reaction buffer.

Another possibility is that a NER-like mechanism occurs, where the loop region is identified, and loop flanking incisions are made, followed by removal of a loop size oligonucleotide fragment. We were unable to detect any DNA fragments 15-50 nt long by Southern blot (data not shown). While we cannot rule out degradation of the fragment after excision or possibly covalent attachment to a polypeptide, similar experiments in NER studies have detected such a fragment (Matsunaga et al., 1995). A recent study in yeast showed that mutation of the yeast homolog of FEN1 (RAD27 or RTH1) did not affect total repair after
transfection of looped substrates into cells. Whether this is because of no involvement by this protein or whether other proteins compensate cannot be determined from these studies. Much more work is needed to fully elucidate the proteins involved and the mechanism of these pathways.
CHAPTER 5
Summary And Speculations

I. Summary of findings

The results presented here describe several different mechanisms for the repair of DNA loops in human cells. Pathways that process loops from 5 to 30 nucleotides (nt) were identified using an in vitro reaction containing extracts of nuclear proteins and a defined DNA substrate that contained a site-specific single strand break. From the overall results, we can artificially divide the repair observed into two classes based on loop size. The repair of loops from 5 to 12 nt was strand specific and utilized either a 5’ or 3’ nick. This repair occurred by both mismatch repair (MR) dependent and independent pathways. We now refer to the MR independent component simply as intermediate loop repair (ILR), even though MR can process these same sized loops. Specifically, we now know that MR is capable of processing loops of up to 12 nt. However, its contribution may be small. The contribution to total repair of ILR increases as loop size increases, being the dominant (> 75%) pathway for 12 nt loop repair. Interestingly, the results suggested that ILR is additionally divided into 2 separate pathways that process either 5’ or 3’ nicked substrates. The analysis of 5’ nicked ILR reaction intermediates demonstrated that the region of the DNA processed was confined to the short path between the nick and the loop site. The majority of this processing occurred by excision that originated at the nick and terminated within 20 base pairs (bp) past the loop site. The mechanism of 3’ nick directed ILR was less clear, with one of several hypotheses (discussed below) consistent with the data.

Large loop repair (LLR) was found to process loops of 30 nt, with only a 5’ nick as a strand discrimination signal (5’-LLR). This repair required extensive DNA synthesis. No evidence for comparable 3’ nick directed repair was found. Additionally, 30 nt loops were processed in a nick independent manner that preferentially removed the loop (ni-LLR). Both types of LLR activity were present in nuclear extracts from cells deficient in the MR proteins MSH2, MSH6, MLH1 or PMS2, the nucleotide excision repair (NER) proteins XPA or XPG, or the helicase/exonuclease WRN. Similar to the ILR reactions, the region of DNA processed in 5’ nick directed LLR was confined to the area between the pre-existing nick and the loop site. For these substrates, more direct evidence that the nick served as the entry point for a DNA exonuclease was obtained. Interestingly, the excision reaction was severely reduced in the
presence of a DNA synthesis inhibitor, suggesting the two processes are somehow linked. The level of the nick independent loop removal pathway was only partially reduced under conditions of limited DNA synthesis. The mechanism of the nick independent loop removal reaction is less clear, but possibly involves only a short tract of DNA immediately surrounding the loop as well as DNA Polymerase β (Pol β). In total, the results presented here describe at least 3 pathways for the processing of DNA loops (MR, ILR, and LLR). However, ILR may in fact be comprised of 2 separate pathways (5’-ILR and 3’-ILR), and LLR is almost certainly 2 separate pathways (5’-LLR and ni-LLR). Making matters even more complicated, the 5’ ILR and 5’LLR may represent a single pathway of loop repair!!! Regardless of the actual number, the results indicate that DNA loops up to 30 nt can be efficiently processed in human cells. The implications of the multiple pathways, putative models for reaction mechanisms, and speculations on future studies and discoveries constitute the remainder of this chapter.

II. 5’ nick directed loop repair

During the course of this work, the repair characteristics of intermediate and large loops were investigated separately. However, once both of the individual projects were completed, similarities between the 5’ nicked directed pathways prompted us to consider if maybe a single pathway was active on both types of loops. The results from 8 and 12 nt loops first indicated that separate pathways were active. Although both a 5’ or 3’ nick was able to direct repair specifically to the nicked strand, the intermediates formed by limited DNA synthesis were very different (Figure 3-4). The results from 5’ nicked substrates of 8 and 12 nt suggested excision initiated at the pre-existing nick and proceeded back towards the loop site. Similarly, 5’ nicked 30 nt loops were also processed in a manner consistent with this idea. A key point in attempting to explain the reaction mechanism is whether the 5’ and 3’ nick directed ILR reactions occur by the same or separate pathways. Assuming for the moment they are separate, a model that explains both intermediate and large loop 5’ nick directed repair can be proposed (Figure 5-1, right side). In this model, a protein or protein complex initiates the reaction by recognition of the loop. The next step involves location of the nick, which could occur by DNA bending or protein translocation along the DNA. Current debate about the mechanism of MR may prove instructive at this stage (Blackwell et al., 1998; Fishel, 1998; Fishel, 1999). After both the loop
Figure 51. Favored model for 5' nicked directed loop repair. Results from the mapping of loop repair reaction intermediates caused by DNA synthesis inhibition indicate that the processing end points are very close to the loop site. For 5' nicked substrates (right side), it is likely that excision initiates at the nick and proceeds back towards the loop. Substrates with a 3' nick (left side) would require a nick to be created 5' to the loop site on the nicked strand to allow both excision and DNA synthesis to occur 5'→3'. Single stranded DNA is likely protected by RPA, while DNA resynthesis and ligation will presumably require one of the PCNA dependent polymerases (α, δ, or ε). Loop recognition: gray cylinder; endonuclease: black lightning bolt; exonuclease: black pacman; DNA polymerase: gray arrowhead.
and nick have been identified, an exonuclease with 5′→3′ specificity could then be loaded onto the DNA at the nick, as this model is dependent on both DNA excision and DNA resynthesis originating at the pre-existing 5′ nick.

For large loop substrates at least, excision initiation requires an active polymerase complex. Adding aphidicolin and to a lesser extent ddNTP’s to the reaction not only prevented repair, but also inhibited the excision reaction to a degree. Due to these results, it is possible that either a preformed excision/re-synthesis complex exists in the cell, or that some level of control of the exonuclease occurs by the DNA polymerase. In principle, the reaction as proposed so far is similar to MR. The loop recognition protein(s) is analogous to hMutSα/hMutSβ. One difference, however, is the excision stop point. In human cells, the excision reaction can continue up to 70-190 bp past the mismatch (reviewed in Modrich, 1997). For 5′-ILR and 5′-LLR, however, the predominant band apparent upon DNA synthesis inhibition occurs very close to the loop site (Figure 3-5B; Figure 4-4B and 4-4C). In all these reactions, there is some variability in the stop point, but a single major band is present. The span of DNA degraded is defined on one end by the pre-existing 5′ nick and on the other by a site within 20 bp 3′ to the loop site. One possibility is that the loop recognition protein serves as the ‘stop sign’ for excision and/or DNA synthesis. Recent structural studies of the bacterial MutS protein suggest that it may not leave the mismatched complex during the reaction (Obmolova et al., 2000). Therefore, it is not inconceivable that a protein with similar properties is active in this pathway.

While the studies performed here did not explore the final steps of the reaction, analysis of the other major excision repair pathways allows us to speculate on several other proteins that may be involved. Replication protein A (RPA), the human single stranded binding protein, is likely used to protect the single stranded DNA in between excision and DNA re-synthesis. The extent of RPA involvement will be dependent on how closely excision and re-synthesis are linked. The size of the repair tract (> 100 nt) and the sensitivity of the reaction to aphidicolin predicts that DNA polymerases α, δ, or ε are involved in DNA re-synthesis, along with the processivity factor Proliferating Cell Nuclear Antigen (PCNA). The final ligation step is likely dependent on either DNA Ligase I or III. Clearly, much more work is needed to verify or refute this model. One point of interest will be the nick recognition activity. Whether it is also the loop binding protein or another is a key point. It is possible that after loop recognition, the same activity moves along the DNA and locates the 5′ nick, at which point the exonuclease is
recruited and the downstream events are started. Another interesting point of study will be the 5’ strand discrimination signal. The polarity of DNA polymerization makes the model described here the easiest explanation of the results, but where the 5’ nick comes from in vivo and the maximum distance is can be form the loop is of critical importance. Similar studies in yeast suggest that the upper limit is less than 800 bp (Corrette-Bennett et al., 1999).

III. 3’ nick directed intermediate loop repair

As noted above, a major question to be answered is whether 3’ nicked substrates containing intermediate size loops are processed in a similar fashion as the corresponding 5’ nicked substrates. It is not difficult to imagine a nearly identical reaction as the one described above. In this scenario, DNA excision would have to occur 3’→5’, and DNA resynthesis could not originate at the nick, but rather would have to start at the endpoint of the excision reaction (i.e. near the loop site). We found no evidence that excision from the pre-existing 3’ nick towards the loop was occurring, so we do not favor this model. Another potential model that is similar to 5’ nick directed repair is that an extra incision is made in the substrate, 5’ to the loop site (Figure 5-1, left side). If this were to occur, then the 5’→3’ excision and DNA resynthesis steps could conceivably be mediated by the same proteins as for 5’ nick directed repair, as the steps are nearly identical. The biggest drawback to this model is that DNA resynthesis would not be required to extend all the way to the pre-existing 3’ nick. One possible explanation is that a nick is made 5’ to the loop site, but then only a limited amount of excision and resynthesis occurs (Figure 5-2A). The discrete bands evident (Figures 3-5C and 3-6C) in the vicinity of the loop site could be from reactions that were aborted after the initial incision (Figure 3-7A, bands 1 and 4), and during/after the excision step (Figure 3-7A, bands 2, 3, 5, and 6). The results using aphidicolin, an inhibitor of DNA polymerases α, δ, and ε, support the idea that the repair resynthesis tract is of limited size. Additionally, excision that proceeds to the pre-existing 3’ nick is not supported, since the probe binding site is located between the nick and loop.

Another possibility is that dual incisions are made that flank the loop site, but always on the pre-existing nicked strand (Figure 5-2B and 5-2C). A major drawback of this model is the fact the when the loop is located in the continuous strand, a fragment of otherwise normal DNA must be removed (Figure 5-2C). An important consideration of all these models is the cross
Figure 5-2. Potential pathways for ILR involving nick directed incisions. A. A single incision made on the nicked strand on the 5’ side of the loop would allow 5’→3’ excision and short patch DNA resynthesis. B. A dual incision model when the nick and loop are on the same strand predicts the non-standard looped DNA is removed. C. Dual incisions on the nicked strand when the loop and nick are on opposite strands removes an otherwise normal fragment of DNA. D. A potential physical means to link the loop recognition, nick recognition, and incision steps for these models. The endonuclease activitie(s) could reside in either the loop or nick recognition proteins. Endonuclease: black lightning bolt; loop recognition: gray cylinder; nick recognition: lined rectangle. Gray triangle; DNA polymerase.
talk required by the loop and nick recognition proteins. The strand specific nature of the reaction precludes the idea of a loop recognition protein acting alone at the loop site. The distantly located pre-existing nick must be located and used to ‘tag’ which strand is to be repaired. The loop recognition protein cannot simply act based on the loop sequence. Models to explain how this can occur can be envisioned (Figure 5-2D), although no direct evidence has yet been obtained. One possible mechanism for this would be that one protein recognizes the loop, and another recognizes the nick. Either of these activities, or still another protein could then incise the DNA on the nicked strand, possibly after DNA bending to bring the two activities together. As mentioned, a drawback of this model is that the bands observed by Southern blot would have to correspond to substrates that were individually incised either 5’ or 3’ to the loop, but not both. Using Figure 3-6C as an example, bands 1 and 4 correspond to a nick 5’ to the loop. However, if dual incisions are made, then only the bands located 3’ to the loop should be evident, since the probe is also located 3’ to the loop. Conversely, in Figure 3-5C, the larger of the bands around 420 nt corresponds to a fragment with an end 3’ of the loop, even though the probe 5’ to the loop. If dual nicks are made, we are faced with having to explain why half of the reactions are aborted after only one of the incisions is made, while the other half continues on to make both incisions.

Variations of a ‘single incision’ model were also considered (Figure 5-3). Unfortunately, different mechanisms are required for whether the loop sequence is removed or retained. Repair causing loop removal (i.e. the nick and loop on the same strand) could occur by creating a nick 5’ to the loop (Figure 5-3A), which would immediately create a DNA flap-like structure that could be a substrate for the Flap Endonuclease 1 (FEN1) protein. Either the incision or excision activities could remove the loop sequence. Instability of repeat sequences (i.e. sequences likely to form looped structures) does occur when RAD27, the yeast homolog of FEN1, is mutated in S. cerevisiae (Tishkoff et al., 1997). A nick 3’ to the loop could conceivably be processed by an unidentified analogous activity with a preference for 3’ DNA ends. For reactions in which the loop sequence is retained (i.e. the loop and nick are on different strand), a nick on the non-looped strand anywhere 5’ to the loop site (on the nicked strand) would allow for ‘flattening’ of the loop (Figure 5-3B). A simple gap-filling reaction would then be required for completion of repair. Given the similarities results between the 3’-8C and 3’12V substrates, we do not think that two
A. Single incision model; loop and nick on the same strand

![Diagram A]

B. Single incision model; loop and nick on opposite strands

![Diagram B]

Figure 5-3. Single incision models of ILR for loop retention or loop removal. A. When the loop and nick are on the same strand, a nick created 5' to the loop will create a flap-like branched structure. These types of structures are recognized and processed by the FEN1 protein which can either excise back from the 5' end, or cut at the branch structure to remove the entire ‘branch’ of DNA. If a nick were made 3' to the loop, an analogous activity with a preference for 3' ends would be required. DNA polymerase activity will be dependent on where the nick is made relative to the loop. B. When the loop and nick are on different strands, an incision on the non-looped, pre-nicked strand anywhere opposite the loop site will allow the loop to ‘flatten’, creating a gapped DNA molecule. The 5' end could then be used by any of the DNA polymerases to restore the DNA to normal.
radically different mechanisms are acting on them. Unfortunately, the results do not readily support any of the models proposed, making the 3’ nicked ILR mechanism unclear.

IV. Nick independent loop removal mechanism

Nick independent loop removal appears to occur by a fundamentally different mechanism from any of the known nick-directed pathways. The characteristics of repair (loop removal) are intriguing, since it is not immediately clear why loop removal would desirable over loop retention. Several potential models can be envisioned for this type of repair. First, similar to the model described above (Figure 5-3A), a single incision on the 5’ side of the loop would create a long flap structure. Whether a flap of 30 nt (and potentially longer) could be processed by FEN1 is unknown (Bambara et al., 1997). Also, why some of the reactions would abort after the incision step (giving the longer band) and why some continue on with excision (giving the smaller band) needs to be explained (see Figure 4-6B). One potential explanation is that the loop is recognized as a ‘lesion’ and is processed by an unknown repair pathway, similar to NER. Uncoupled incision on either side of the loop would explain the dual bands observed. Depending on the sites of cleavage, a very small repair patch could be required. In fact, no DNA resynthesis would be required if the nicks were made precisely at the junctions of dsDNA and ssDNA. The drop in repair observed when DNA synthesis is limited may reflect the repair events that occurred after the endogenous dNTP pool was exhausted. A very intriguing possibility in this area is whether the NER 5’ incision complex XPF/ERCC1 is active on these substrates. This complex has also been implicated in the processing of DNA interstrand cross links and has recently been shown to cut on both sides of the adducted base (Kuraoka et al., 2000; Kumaresan et al., 2002). It is not hard to imagine a similar type of processing occurring with DNA loops. Loop structures, especially for larger loops, can very easily mimic a dsDNA:ssDNA junction that is the classical substrate for the XPF/ERCC1 complex. Unfortunately, we were not able to directly test this, due to complications with the XPF−/− cell line. Clearly, though, XPG is not involved in the 3’ nick, as ni-LLR was competent in a cell line that lacks XPG.

V. Last words

The results presented here indicate the importance of DNA stability. This work has identified at least 3 separate mechanisms for DNA processing separate from the three major
excision repair pathways. All three pathways can process DNA loops that will presumably arise
during errors in replication and during recombination between non-homologous sequences.
Whether recombination is a critical step in any of these pathways is an especially important
question to be answered. The analysis of reaction intermediates was performed using cell lines
that contained both hMutSα and hMutSβ. Both MSH2 and MSH3 (the components of hMutSβ)
have been implicated with XPF/ERCC1 containing pathways by genetic screens. hMutSβ is also
known to bind to recombination intermediates (Evans et al., 2000), so it may be that hMutSβ
mediated recombination events are the cause of both the ni-LLR intermediates (Figure 4-6) and
the 3’-ILR intermediates (Figure 3-5C and Figure 3-6C). Clearly, this work is only scratching
the surface of the DNA loop repair pathways active in human cells.
APPENDIX A

Commonly used abbreviations

A adenine
AP apurinic/apyrimidinic
ATP adenosine triphosphate
BER base excision repair
bp base pair(s)
β-ME β-mercaptoethanol
C Complementary (when used in conjunction with ‘strand’); cytosine
CS Cockayne’s Syndrome
CPD cyclobutane pyrimidine dimer
CRC colorectal cancer
DDB damaged DNA binding (protein)
ddNTP dideoxy nucleotide triphosphate
dNTP deoxynucleotide triphosphate
DTT dithiothreitol
dsDNA double stranded DNA
DSB double strand break
ERCC excision repair cross complementing
EtBr ethidium bromide
FBS fetal bovine serum
FEN1 flap endonuclease 1
G guanine
guan-HCl guanidine hydrochloride
GGR global genome repair
HNPCC hereditary non-polyposis colorectal cancer

Homolog proteins and/or complexes using a single letter species designation:
  h human
  y yeast
ILR intermediate loop repair
kbp kilobasepairs
LLR large loop repair
  ni-LLR nick independent large loop repair
MLH MutL homolog
MR mismatch repair
MSH MutS homolog
MSI microsatellite instability
MutLα complex of MLH1 and PMS1 (yeast) or PMS2 (human)
MutSα complex of MSH2 and MSH6
MutSβ complex of MSH2 and MSH3
NER nucleotide excision repair
nt nucleotide
PAGE polyacrylamide gel electrophoresis
<table>
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PMS</td>
<td>post meiotic segregation</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>Pol</td>
<td>polymerase</td>
</tr>
<tr>
<td>RFC</td>
<td>replication factor C</td>
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<td>RPA</td>
<td>replication protein A</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single stranded DNA</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate (also known as sodium lauryl sulfate)</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TCR</td>
<td>transcription coupled repair</td>
</tr>
<tr>
<td>TFIIH</td>
<td>transcription factor IIH</td>
</tr>
<tr>
<td>TTD</td>
<td>trichothiodystrophy</td>
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<tr>
<td>U</td>
<td>uracil; unit</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Viral (when used in conjunction with ‘strand’); Volts</td>
</tr>
<tr>
<td>WRN</td>
<td>Werner Syndrome helicase/exonuclease</td>
</tr>
<tr>
<td>WS</td>
<td>Werner Syndrome</td>
</tr>
<tr>
<td>XRCC</td>
<td>x-ray repair cross complementing</td>
</tr>
<tr>
<td>XP</td>
<td>Xeroderma pigmentosum</td>
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