IN VIVO ANALYSIS OF CRUCIFORM EXTRUSION AND RESOLUTION OF DNA PALINDROMES IN EUKARYOTES

by

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ABSTRACT

DNA palindromes are implicated in several examples of gross chromosomal aberrations in the human genome, however, the molecular mechanism(s) that govern palindrome instability are largely under-investigated. Because of their propensity for intrastrand base pairing, it is suspected that the acquisition of a secondary structure, such as a hairpin or cruciform, instigates the rearrangement process. A significant hurdle in defining palindrome-provoked instability lies in the fact that reliable methods for examining in vivo cruciform extrusion remain underdeveloped. A challenge is to provide straightforward evidence for cruciform extrusion in eukaryotic cells. Here, I present a plasmid system for use in Saccharomyces cerevisiae that enables for the detection of cruciforms in vivo. Cruciform extrusion, of either an in vitro-prepared palindrome or a near-palindrome from the human genome, is monitored by scoring for the product of cruciform resolution, being a dually hairpin-capped linear DNA molecule. These results not only provide evidence for the occurrence of cruciform extrusion in eukaryotic
chromatin, they also identify a novel source of endogenous double strand break formation.

A screen for candidate genes that are required for resolution revealed that the Mus81 Endonuclease, a candidate Holliday junction resolvase, provides the majority of cruciform resolution activity in mitotic cells, validating the notion that cellular HJ resolvases can misrecognize a cruciform for a Holliday junction. A second screen identified a requirement for the Sgs1-Top3-Rmi1 complex in the prevention of double strand break formation, including cruciform resolution, of DNA palindromes. These results uncover a new role for the RecQ helicase in prevention of palindrome-provoked instability, possibly through the intrusion of cruciform structures. Together, this work contributes significantly to our understanding of cruciform metabolism in eukaryotes and supports suggestions that cruciform extrusion instigates instability in the human genome.
ACKNOWLEDGEMENTS

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<td>$\sigma$</td>
<td>superhelical density</td>
</tr>
<tr>
<td>$\Delta$</td>
<td>gene deletion</td>
</tr>
<tr>
<td>$\mu g$</td>
<td>microgram</td>
</tr>
<tr>
<td>$\mu l$</td>
<td>microliter</td>
</tr>
<tr>
<td>ATLD</td>
<td>ataxia telangiectasia-like disorder</td>
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<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>BIR</td>
<td>break induced replication</td>
</tr>
<tr>
<td>BFB</td>
<td>Breakage Fusion Bridge</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>Can</td>
<td>canavanine</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>Chx</td>
<td>cycloheximide</td>
</tr>
<tr>
<td>DC</td>
<td>digestion-circularization</td>
</tr>
<tr>
<td>der</td>
<td>derivative chromosome</td>
</tr>
<tr>
<td>DM</td>
<td>Double Minute chromosome</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>ds</td>
<td>double strand</td>
</tr>
<tr>
<td>DSB</td>
<td>double strand break</td>
</tr>
<tr>
<td>Exo</td>
<td>Exonuclease V</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>$\Delta G$</td>
<td>free energy</td>
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<tr>
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<tr>
<td>GAPF</td>
<td>genomic analysis of palindrome formation</td>
</tr>
<tr>
<td>HJ</td>
<td>Holliday junction</td>
</tr>
<tr>
<td>HSR</td>
<td>homogeneous staining region</td>
</tr>
<tr>
<td>hp</td>
<td>hairpin</td>
</tr>
<tr>
<td>IR</td>
<td>inverted repeat</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LCR</td>
<td>low copy repeat</td>
</tr>
<tr>
<td>Lk</td>
<td>linking number</td>
</tr>
<tr>
<td>L₀</td>
<td>linking number of relaxed DNA</td>
</tr>
<tr>
<td>MATα</td>
<td>mating type α</td>
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<tr>
<td>MATα</td>
<td>mating type α</td>
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<tr>
<td>ml</td>
<td>milliliter</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<td>MRN</td>
<td>Mre11-Rad50-Nbs1</td>
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<tr>
<td>MRX</td>
<td>Mre11-Rad50-Xrs2</td>
</tr>
<tr>
<td>NBS</td>
<td>Nijmegen breakage syndrome</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibromatosis-1</td>
</tr>
<tr>
<td>N</td>
<td>NheI</td>
</tr>
<tr>
<td>NHEJ</td>
<td>non-homologous end joining</td>
</tr>
<tr>
<td>oc</td>
<td>open circle</td>
</tr>
<tr>
<td>PATRR</td>
<td>palindromic AT-rich repeat</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>P</td>
<td>PvuII</td>
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sc  supercoiled circle
ss  single strand
Ura  uracil
wt  wild-type
X  XbaI
Chapter 1

Introduction
The human genome is constantly bombarded by numerous agents that inflict damage, including reactive oxygen species, chemical mutagens, gamma-irradiation, and ultra-violet radiation (for a review, see (Gupta and Lutz, 1999)). These have been long-since identified as having significant effects on the integrity of DNA. Not only can exogenous factors inflict harm, genomic elements have the potential to instigate chromosomal aberrations. The human genome is littered with various types of sequences that provoke instability. These consist of segments of DNA that are repeated in a direct or indirect orientation. With the ongoing identification of diseases and neoplasias that are associated with repeat instability, effort is now focused on characterizing the molecular mechanisms of various types of repeat-associated rearrangements.

One class of repeat, the DNA palindrome, is associated with several human diseases and cancers. There is evidence suggesting that chromosomal rearrangements instigated at palindromes and near-palindromes are implicated in the development of Emanuel syndrome (Shaikh et al., 1999), some cases of Neurofibromatosis-1 (Kurahashi et al., 2003), and thalassemia (reviewed in (Lewis and Cote, 2006)). Gene amplifications were shown to be arranged as suspected palindromic arrays (Ford and Fried, 1986). They are "suspected" palindromes because to date, no amplification array has been shown, at the sequence level, to be palindromic. Recent evidence suggests that, prior to gene amplification, large suspected palindromes arise de novo in tumor cells in disease-specific chromosomal positions (Tanaka et al., 2005).

Although palindromes and suspected palindromes have been implicated in gross chromosomal aberrations, their biological behavior is not well characterized. The
mechanism(s) of palindrome formation and palindrome instability in the human genome requires further investigation.

This chapter will focus on the following: 1) describing structural transitions in DNA palindromes, 2) outlining the known examples of palindrome-provoked genome rearrangements as well as examples of palindrome formation in humans, and 3) reporting on the mechanisms of palindrome instability, with emphasis on model system discrepancies.
1.1 Structural transitions in DNA palindromes

A palindrome is a segment of DNA that is followed directly by a copy positioned in the reverse complementary orientation (Figure 1-1A). This arrangement permits the palindrome to adopt two secondary DNA structures: the single strand hairpin and the double strand cruciform. A palindrome's propensity to cause havoc in the genome is associated with the acquisition of a secondary structure (Leach, 1994). When in lineform (i.e. in the absence of a secondary structure), a palindrome may therefore be quiescent in terms of damage potential. Using a combination of in vitro and in silico techniques, researchers are characterizing the sequence and symmetry requirements of secondary structure formation. Although the significance of DNA palindromes was discussed as early as the 1960's, few in vivo models were developed in the ensuing years. Such models were highly manipulated, hence there was doubt for some in the field that palindromes assumed cruciform structures to any significant extent but in vitro (for an example, see (Gellert et al., 1983)).

1.1.1 Energetics of cruciform formation

Palindromic DNA sequences can convert from lineform to cruciform in double strand DNA via an extrusion process thought to initiate with the melting of a small number of central base pairs (reviewed in (Sinden, 1994)). The dissolved bonds are quickly replaced by intrastrand base pairing after which the self-paired ‘arms’ extend through branch migration (Figure 1-1).

The extrusion of a cruciform is an energy-driven process. The free energy required for the melting of central base pairs and cruciform extrusion is provided by negative supercoiling; when a DNA molecule is linear (and untethered) or circular
Cruciform extrusion is forced by negative supercoiling. A. Sequence example of a perfect palindrome symmetry axis. B. Negative supercoiling is represented for convenience in this as reduced twist (counterclockwise arrows). The lineform to cruciform transition does not require extensive strand separation. The process initiates by melting about 10bp; after this, hairpins form and elongate through branch migration.
but relaxed, a cruciform will not extrude (Lilley, 1980; Mizuuchi et al., 1982b; Panayotatos and Wells, 1981).

How is free energy ($\Delta G$) provided by negative supercoiling? Richard Sinden provides a thorough discussion of this in (Sinden 1994), a summary of which is outlined below. When DNA is negatively supercoiled, there is enough free energy in the molecule to permit extrusion. Negative supercoiling is a topological property of DNA where the linking number (Lk) of the molecule in question is smaller than the linking number in the relaxed state ($L_{k0}$). The linking number is the number of times that a double helix is rotated by 360° in DNA. Linking number can be written as:

$$Lk = T + W$$

where $T$ is twist (the number of times the individual strands coil around one another) and $W$ is writhe (the number of coilings of the duplex in space). In relaxed DNA, the molecule is in its lowest energy state and the linking number is the total number of base pairs divided by the number of bases in one 360° helical turn (10.5bp). There is no writhe in relaxed DNA, therefore:

$$L_{k0} = T$$

The free energy of supercoiling is proportional to the difference in Lk (between relaxed and supercoiled DNA) and the larger the difference in Lk (usually through the induction of writhe) the larger the available energy in the molecule. Free energy can be written as:

$$\Delta G = (1100 \text{ RT/N}) \cdot (Lk - L_{k0})^2$$

where $R$ is the gas constant, $T$ is the temperature, and $N$ is the number of bases in the DNA molecule. The superhelical density ($\sigma$) is the degree of supercoiling of a particular molecule, as follows:
\( \sigma = (L_k - L_{k_0}) / L_{k_0} \)

The extrusion process relieves negative superhelical stress (Mizuuchi et al., 1982b). An extruded cruciform is a stable structure because extrusion results in the reduction in writhe, reducing the free energy so that the molecule is closer to its relaxed state (Benham, 1982). Negative supercoiling provides both the energy needed to initiate cruciform extrusion and to stabilize the four-way branch.

Extrusion is energetically unfavorable because it requires melting of central base pairs through the loss of hydrogen bonds and base stacking interactions and formation of new intrastrand base pairing (the proto-cruciform). Therefore, central symmetry interruptions will dramatically affect the formation of the proto-cruciform. Central symmetry “spacers” increase the number of unpaired nucleotides in the loops at the ends of the arms and will also inhibit formation of the initial proto-cruciform, with a spacer of 10bps completely abolishing extrusion under physiological conditions (Courey and Wang, 1988; Murchie and Lilley, 1987; Zheng and Sinden, 1988). Using computer simulation technology and in vitro methods, it was shown that sequence interruptions distal to the symmetry center also affect branch migration of the cruciform arms, requiring increased energy to drive past the mismatch (Benham et al., 2002).

1.1.2 Nomenclature

It is important to differentiate between palindromes and imperfect inverted repeats with central spacers (Figure 1-2). Spaced inverted repeats and palindromes are not interchangeable with respect to cruciform potential. Motifs termed palindromes range from small, perfectly or imperfectly matched inverted repeats to very large inverted repeats with kilobase-sized spacers. Because these various sequence arrangements can
have quite different biophysical and biological properties, it is important to state how “palindrome” is used in the following thesis. Here, palindrome is defined as a DNA sequence that is immediately juxtaposed to an exact inverted (that is, reverse complementary) copy of itself. A palindrome has no central spacer and no mismatches between arms.

There are sequences that deviate subtly from the strict definition of a palindrome. They are inverted repeats with very small spacers and/or small discrepancies between the two arms (Figure 1-2). The biophysical behavior of these “near palindromes” can be similar to real DNA palindromes (Benham et al., 2002), but this is not easily predicted by sequence gazing. The term “near-palindrome” will be used to define these arrangements. Near-palindromes are functionally defined as sequences that are like palindromes in terms of biological and biophysical character, with some quantitative differences only.

1.1.3 Hairpin formation

A hairpin can form when naked single strand DNA is available to allow for random intrastrand collision. Such an opportunity can occur during replication, because each strand is forcibly separated to allow for DNA synthesis, provided that the strands are not coated by single strand binding protein, for example, eukaryotic RPA (Fanning et al., 2006)). The length of the central spacer, if any, will determine whether the hairpin possesses a tight turnaround or if it is in the form of a stem-loop. Spacer size will also influence hairpin formation. The spacing of Okazaki fragment primers dictates the maximum length of available single strand DNA on the lagging strand that may be free to transiently self-pair. Because Okazaki fragments vary in length between organisms,
Table 1-2

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**Figure 1-2**
Intrastrand annealing of inverted repeat DNA sequence creates two separable categories of hairpin (stem-loop) structure: single strand hairpins (second row) and double strand cruciforms (bottom row). Top two rows: Single strand hairpins can form when the lagging strand template is forcibly separated from its partner by replication, as shown. This involves loop closure by random collision in the unpaired strand. Inverted repeats with spacers that exceed the length of an Okazaki initiation zone (several hundred to several thousand base pairs depending on the organism) will not have an opportunity to self anneal during replication; see right-most diagram. **Bottom**: Cruciform extrusion is forced by negative supercoiling. Spaced inverted repeats, for which limited torsionally induced melting cannot expose the self-complementary repeats, are more durably lineform under strain.
hairpin formation may also vary accordingly. Okazaki fragment spacing varies from 100-200bp in eukaryotes to 1-2kb in prokaryotes (reviewed in (Ogawa and Okazaki, 1980)). There exists evidence that the formation of hairpins during replication in \textit{E. coli} and in eukaryotes has an \textit{in vivo} impact (for examples, see (Bzymek and Lovett, 2001; Voineagu et al., 2008)). Voineagu \textit{et al.} demonstrate that inverted repeats are sites of replication fork stalling and they speculate that hairpin formation blocks fork progression. Inverted repeats are also deleted from the lagging strand through a replication bypass mechanism (more in section 1.3.1).

1.1.4 Cruciform extrusion

1.1.4.1 Cruciform extrusion: A biological reality?

Since the first suspicion of the occurrence of cruciform extrusion, there has been much debate as to its occurrence \textit{in vivo}; being that extrusion of a cruciform is an energetically-driven process, it is largely believed that they cannot form \textit{in vivo} (Courey and Wang, 1983; Gellert et al., 1983). The genomes of prokaryotes are negatively supercoiled, with an average superhelical density of $\sigma=-0.05$ (Sinden et al., 1980). Even though the prokaryotic genome has a superhelical density that favors extrusion, there exists limited direct evidence for \textit{in vivo} cruciform extrusion in bacteria. Replicons containing long palindromes are inviable in \textit{E. coli}, as shown by the inability of palindrome-containing phage to make plaques on bacterial lawns. When very small (only a few base pairs) central spacers that are introduced into palindromes in phage genomes increase plaque formation significantly (Chalker et al., 1993; Davison and Leach, 1994). Such small changes in symmetry are not predicted to affect hairpin formation in single strand DNA, providing indirect evidence for cruciform formation \textit{in vivo}.
The genomes of eukaryotes are structurally more complex, organized by the wrapping of DNA around nucleosomes (Finch et al., 1977). Thus, the negative supercoils in eukaryotic DNA are taken up by writhe around the nucleosomes resulting in functional relaxation (for a thorough discussion, see (Bates and Maxwell, 2005)). Moreover, unlike bacteria, eukaryotes do not possess topoisomerases that introduce negative supercoiling (Champoux, 2001). The topological restraint of eukaryotic DNA as well as the lack of enzymes capable of introducing negative supercoiling in the genome are factors that would disfavor cruciform extrusion. More recently however, using a psoralen probing technique, it was found that discreet domains of unconstrained underwound DNA were detected in the fly genome (Matsumoto and Hirose, 2004). Therefore, at least transiently, domains of negative supercoiling are present that could theoretically provide sufficient free energy to drive extrusion.

The domains of unconstrained supercoiling were dependent on active transcription (Matsumoto and Hirose, 2004). Years earlier using bacterial models, it was shown that discreet regions of supercoiling are formed by the action of an advancing RNA polymerase (Liu and Wang, 1987). In the "Twin Domain Model" of transcription-generated supercoiling, an RNA polymerase will generate waves of positive supercoils ahead of it and equal waves of negative supercoiling behind it (Wu et al., 1988). The newly generated waves of supercoiling would be readily lost through dissipation, however, the presence of a physical barrier such as nuclear membrane anchorage or a DNA binding protein would create a chromatin domain that could retain winding (Albert et al., 1996). Albeit transient in that the actions of topoisomerases would rapidly remove overly wound DNA, this process could potentially provide conditions for extrusion in
mammalian cells. Transcription-induced supercoiling has been experimentally demonstrated to influence cruciform formation in bacteria (Bowater et al., 1994). Other possible sources of transient superhelical tension include, but are not limited to, replication and nucleosome displacement, which are also eliminated by the actions of DNA topoisomerases (Wang, 2002).

1.1.4.2 Detecting cruciform extrusion in vitro

To date, the majority of methods for quantifying cruciform extrusion are applied to DNA in vitro. Although the use of these procedures has provided evidence for in vivo cruciform extrusion, there are significant limitations. These methods measure cruciform extrusion of DNA present in a hypernegatively supercoiled molecule. Through the action of topoisomerases, bacterial plasmids are maintained as negatively supercoiled molecules (Zechiedrich et al., 2000). A plasmid from bacteria is hypernegatively supercoiled and does not reflect upon the in vivo topology of eukaryotic DNA, which is constrained by nucleosome wrapping. As mentioned earlier, cruciform extrusion is largely dependent on superhelical density, therefore, the topology of the DNA in question should be considered. The discrepancy in superhelical density is surely to result in extrusion-positive scoring of sequences that otherwise do not extrude in eukaryotic chromatin in vivo.

A number of techniques have been developed over the years for the purpose of demonstrating cruciform formation. One commonly used method relies on analyzing plasmid-borne palindromes after gel electrophoresis in one or two dimensions. It is possible to measure cruciform extrusion as a reduction in linking number (Lk) that is observed as a decrease in electrophoretic mobility (for an example, see (Vologodskai...
Another method is to probe cruciforms using secondary structure-recognition endonucleases. P1 and S1 Endonucleases cleave single strand regions, including unpaired regions on hairpin tips (Desai and Shankar, 2003), while T4 Endonuclease VII and T7 Endonuclease I cleave four-way junctions (Declais and Lilley, 2008). Some studies employ antibodies raised against four-way junctions that bind to cruciforms \textit{in vitro} (Frappier et al., 1987). Direct visualization of cruciforms is also possible with the use of transmission electron microscopy (Mizuuchi et al., 1982b) or atomic force microscopy (Shlyakhtenko et al., 1998).

Utilization of a combination of techniques is ideal; for example, using several methods, including electron microscopy, S1 Nuclease and T7 Endonuclease I sensitivity, gel electrophoresis, and electrophoretic mobility shift with anti-cruciform antibodies, Kurahashi \textit{et al.} showed that the human 11q23 and 22q11 PATRRs, when cloned and isolated from bacteria, extrude cruciforms \textit{in vitro} (Kurahashi et al., 2004) (Kogo et al., 2007a). Whether these same sequences extrude \textit{in vivo} in either bacteria or humans is difficult to assert.

\textbf{1.1.4.3 Detecting cruciform extrusion \textit{in vivo}}

In order to bypass the caveats associated with \textit{in vitro} methods, a number of techniques are available for the purpose of detecting cruciforms within the cell. There have been three types of \textit{in vivo} extrusion detection methods: chemical modification, nuclease sensitivity testing, and antibody binding affinity. A widely utilized technique relies on the use of chemicals that modify bases when present in an unpaired form. Bacteria are grown in the presence of the compound and DNA is then harvested for analysis. Chemical-based modification of bases using osmium tetroxide and
chloroacetaldehyde is employed for detecting secondary structure formation in *E. coli* (Dayn et al., 1992; del Olmo and Perez-Ortin, 1993; Rahmouni and Wells, 1992). A second common chemical probe, psoralen, can successfully capture extruded hairpins through DNA crosslinking (Zheng et al., 1991). Nuclease sensitivity assessment includes the use of a number of enzymes, some of which take advantage of the presence of enzyme recognition sequences at the symmetry axis for their utilization. If a restriction endonuclease or methylase site is present at the symmetry axis of the palindrome, the resistance to modification upon the expression of the appropriate enzyme provides evidence for extrusion (Allers and Leach, 1995). When such sequences are not available, four-way junction cleaving enzymes and single-strand DNA endonucleases are used to probe for secondary structure formation in bacteria and in *Xenopus* extracts (Leonard and Patient, 1991; Panayotatos and Fontaine, 1987). Although not an *in vivo* method, cruciforms can be detected in whole nuclei spreads (in which some protein-DNA interactions are preserved) using an anti-cruciform antibody (Frappier et al., 1987).

While the *in vivo* techniques are consistent with the possibility of cruciform extrusion, all have caveats. Chemical modification of bases may induce radical physiological changes within the cell that could, in turn, affect DNA topology. Moreover, these chemicals can directly induce such topological transitions. It was shown through the analysis of psoralen-DNA adduct crystal structures that cross linking of DNA with psoralen can induce the formation of cruciforms in palindromic arrangements (Eichman et al., 2001). The binding of expressed nucleases or antibodies to the palindromic sequence may also induce conformational changes in DNA structure. An ideal *in vivo* assay would identify cruciform formation with minimal manipulation through the
detection of a biological event that results as a consequence of their extrusion. I developed one such method. It is described in Chapter 2.

1.1.4.4 Cruciforms vs. Holliday junctions

The Holliday junction is a DNA structure intermediate arising during homologous recombination. Four-way junctions between two DNA duplexes are formed after invasion, extension, and second end capture of a 3’ single strand DNA end into the other homologous sequence. In order to disjoin synaptonemal complexes and form crossover products during meiosis, cells have acquired nucleases that specifically cleave or "resolve" Holliday junctions (reviewed in (Lilley and White, 2001)). All resolvases introduce nicks into two of the four strands at the interconnecting four-way Holliday junction and, in vitro, are able to cut the highly similar four-way junction of an extruded cruciform. For example T4 DNA Resolvase and RuvC both act by making cross-diagonal single strand cleavages at a cruciform base in vitro, breaking apart the 4-stranded structure into two linear, hairpin-capped cleavage products (Iwasaki et al., 1991; Mizuuchi et al., 1982a). The appearance of a T4 resolvase-sensitive structure is considered one of the most rigorous proofs of cruciform extrusion (Mizuuchi et al., 1982a). As shown in Figure 1-3, the Holliday junction is a four-way branched DNA and may be structurally identical to the base of an extruded cruciform. Therefore, it is conceivable that in the cell, proteins whose role is to act upon one substrate could mistakenly recognize the other. This will be described further in Chapter 3.

In thinking about how DNA palindromes could contribute to genome instability, one possibility is that aberrant breaks are introduced by HJ resolvases acting upon the four-way junction of a cruciform. Cruciform resolution is not a
Junction resolving enzymes can fracture DNA at cruciform structures. **Left:** Resolution of a Holliday junction. (i) Two homologous duplex molecules. (ii) A recombination intermediate arising after strand exchange. (iii) The junction can be equivalently diagrammed in an open square configuration as shown. A resolvase will introduce a pair of single strand nicks at diagonal positions across the junction. (iv) The strand breaks physically disconnect the recombined duplexes. **Right:** Resolution of an extruded cruciform. (i) A lineform DNA sequence containing a palindrome is depicted. (ii) Torsional strain induces a cruciform structure. (iii) A junction-resolving enzyme introduces correlated diagonal nicks at the base of the cruciform. (iv) The resolvase-fractured duplex has hairpin DNA ends with internal nicks. (The single strand nicks occur at a distance from the hairpin termini and are ready substrates for re-sealing by ligation).
constructive process like HJ resolution. Resolution of a Holliday junction separates two duplex molecules while resolution of a cruciform introduces a potentially detrimental hairpin-terminated double strand break into a single, formerly intact DNA molecule (Figure 1-3). However, this threat is only conceivable if the human genome contains extrusion prone palindromes. With the discovery of human diseases whose signature chromosomal rearrangements are associated with palindromes and near palindromes, cruciform resolution in the human genome is plausible. The challenge nonetheless is to demonstrate that the human genome contains palindromes that extrude in vivo.
1.2 Palindromes in human disease

The precise palindromic content of the human genome is currently unknown. Long palindromes and near-palindromes (150-200bp in length) cannot be propagated in wild-type or mutant strains of *E. coli* (reviewed in (Leach, 1994)). Palindromes are either corrupted during replication, cut by structure-specific nucleases, or eliminated by otherwise preventing the maintenance of the replicon in which they are situated ((Cromie et al., 2000; Leach, 1994; Leach, 1996; Lovett, 2004) and cited therein). The difficulties are fundamental and to date there is no demonstrated way to circumvent the problem, even through the use of an alternative *E. coli* cloning vector (Leach, 1996; Lewis et al., 2005; Williams and Muller, 1987). Some mutant *E. coli* strains have an improved capacity to maintain repetitive DNA, but even these strains are unable to accurately and reliably maintain long DNA palindromes (Inagaki et al., 2005; Leach, 1996; Lewis et al., 2005).

The cloning barrier described above means that sequences that fail to propagate in *E. coli* have been excluded from reference genomes. An example of exclusion is provided by a near-palindrome at the human NF1 locus. The full length near-palindrome was shown to reproducibly decay to a shorter deleted version (Lewis et al., 2005). In other cases, even such corrupted versions have failed to be captured. It is only recently that, after numerous attempts, the sequence of one particular palindrome on human chromosome 22 was successfully identified (Kurahashi et al., 2007).

1.2.1 Palindrome formation in the human genome

As highlighted below, numerous palindromes and near-palindromes are associated with instability in the human genome. If the presence of a palindrome has the
potential to cause such detrimental effects on genome integrity, why do they persist in our genomes? Do they resist expulsion throughout evolution? One potential explanation is that they are not long-lived elements but that errors in genome metabolism result in the formation of palindromes. There are several proposed mechanisms for the formation of palindromes in the human genome. First, palindromes can arise as a consequence of errors by replicative polymerases. A palindrome will form if a polymerase traverses the replication fork junction, templating from the Watson to Crick strands (Aladjem and Lavi, 1992). If template switching occurs in a near-palindrome, it may result in "correction", where the degree of symmetry of the once near-palindrome is perfected (Dutra and Lovett, 2006; van Noort et al., 2003). In mammalian and yeast systems, palindromes can form if a DSB is introduced near a small invert repeat (Rattray et al., 2005; Tanaka et al., 2002) (more in sections 1.2.4.2 and 1.3.2.2). There is evidence that suspected palindromes arise through Breakage-Fusion-Bridge cycles (as for example (Ciullo et al., 2002a)), a proposed mechanism for gene amplification in cancer (more in sections 1.2.4.1) or through the integration of mobile DNA elements or viral genomes in a head-to-head or tail-to-tail orientation (Lemoine et al., 2005).

1.2.2 Palindromic AT-rich repeats

Work from the Emanuel and Kurahashi groups was pivotal in identifying palindromes and near-palindromes in the human genome that have a significant impact on health. The laboratories are responsible for the discovery and characterization of human palindromes shown to instigate reciprocal translocations (Kurahashi et al., 2006b). They are termed Palindromic AT-Rich Repeats (PATRRs) because of their relatively high AT content near the symmetry center. The first translocation studied, t(11;22), is
associated with Emanuel syndrome and is the most common recurrent balanced translocation identified to date (Figure 1-4). Balanced carriers of the translocation present with no clinical symptoms with the exception that they suffer from reproductive problems. Children of carriers can suffer from Emanuel syndrome, caused by a supernumerary der(22) t(11;22) (Shaikh et al., 1999). Patients suffer from severe mental delay and numerous facial and organ malformations. Upon sequencing der(11) and der(22) junctions and reconstructing the chromosomal breakpoints, it was found that the translocation was instigated within a few base pairs from the symmetry centers of palindromes in regions 11q23 and 22q11 (Kurahashi et al., 2000). The chromosome 22 PATRR is also involved in a second recurrent translocation with a PATRR situated within an intron of the Neurofibromin-1 gene in region 17q11 (Figure 1-4). Two patients presenting with Neurofibromatosis were found to harbor t(17;22) translocations (Kehrer-Sawatzki et al., 1997; Ledbetter et al., 1989). There are three additional PATRRs on chromosomes 1, 4, and 8 that all exchange with the chromosome 22 palindrome (Gotter et al., 2007; Gotter et al., 2003; Nimmakayalu et al., 2003).

The barrier to cloning palindromes is exemplified here. The elusive PATRR-22 resides within a series of low copy repeats (LCRs) situated in region 22q, in one of the remaining physical sequence “gaps” in the human genome consortium sequence. The PATRR-11 and PATRR-17 sequences are represented in the human genome sequence as corrupted versions that are likely to be deletion products formed upon cloning of BACs using standard bacterial techniques. Originally, it was through constructing the pre-translocation sequences from derivative junctions that the PATRR sequences could be identified.
Figure 1-4
Recurrent reciprocal translocations mediated by palindromic AT-rich repeats. Above: Diagram of t(11;22). The breakpoints of the translocation map to the symmetry centers of PATRRs on 11q23 and 22q11. Below: Diagram of t(17;22). The breakpoints of the translocation map to the symmetry centers of PATRRs on 17q11 and 22q11. The 17q11 palindrome is in the 31st intron of the Neurofibromin-1 gene. The palindrome on chromosome 22 is involved in all identified PATRR-mediated translocations to date.
A common feature in each translocation is the location of the breakpoints. All reside within a few base pairs of the palindromic symmetry centers. The breakpoints can also contain regions of microhomology, however, the two palindromes overall are not homologous and relatively long stretches of homology are not observed at the junctions. These observations are consistent with the translocations being instigated through the formation and re-sealing of double strand breaks via end joining. Emanuel and Kurahashi suggested that such DSB formation could occur through resolution of an extruded cruciform (Kurahashi et al., 2006a). In support of this, they demonstrated that both PATRR-11 and PATRR-17 form cruciforms \textit{in vitro} using electrophoretic mobility assays, nuclease sensitivity assays, and electron microscopy (Kogo et al., 2007b; Kurahashi et al., 2004). A considerable caveat in their \textit{in vitro} characterization is that the tested palindromes were derivatives rather than exact clones of the true human sequences, therefore, the existence of \textit{in vivo} cruciform extrusion in each PATRR is still questionable. Employing alternative cloning techniques such as the use of budding yeast as a cloning vehicle are ideal for capturing palindromes that would otherwise be corrupted in bacteria.

\subsection{1.2.3 The β-globin gene palindrome}

Not only are human palindromes implicated in illegitimate translocations, they can also associate with large intrachromosomal deletions that result in disease. Three different inherited hemoglobinopathies involve large deletions that terminate 3’ to the β-globin gene. The hemoglobinopathies arose in three separate racial lineages and represent independent events. Although the three deletions range from 45 to 150kb, with unrelated 5’ breakpoints, the 3’ breakpoints occur within a few bps of the symmetry
center of a 160bp perfect palindrome (Figure 1-5: (Fodde et al., 1990; Game et al., 2003; Henthorn et al., 1986)). The 160bp palindrome on 11p15 has been successfully sequenced (Henthorn et al., 1986). The ability to successfully clone the β-globin palindrome may be influenced in its relatively small size; the 160bp palindrome is within the length limit that could permit relatively stable propagation in bacteria. As shown in Figure 1-5, it is striking that, though the sizes of the deletions vary by over 100kb, all three involve a non-homologous joining event centrally located within the palindrome. No regions of homology are present at the joints. The discovery of the β-globin palindrome-mediated deletions illustrates that human palindromes that instigate genomic aberrations do not require a high AT content, as is observed for the palindromic AT-rich repeats. Moreover, the deletions also demonstrate that a palindrome will not only form illegitimate joining events with other palindromes, but will do so with what appear to be random, palindrome-independent double strand breaks.

1.2.4 Palindrome formation and gene amplification in cancer

Gene amplification is observed as a dramatic increase in the copy number of an oncogene that can be associated with poor prognosis for the patient. Amplifications are often observed as inverted repeats that can take several forms: 1) as extrachromosomal Double Minutes (DMs) arranged as inverted dimer circles (Fakharzadeh et al., 1993; Nonet et al., 1993), and 2) as Homogeneous Staining Regions (HSRs), which are often suspected palindromic tandem arrays (for an example, see (Ford and Fried, 1986)). They are only suspected palindromes or spaced inverted repeats; to date, no palindrome symmetry axis originating from a gene amplification event has been fully sequenced and verified for the presence of perfect or near perfect symmetry.
Figure 1-5
Palindrome-mediated deletions in the globin locus. A. Diagram of deletions at the globin locus (not to scale). B. DNA sequences of the 3’ breakpoints. Only the 66 central bases of the 160bp palindrome are given in the top line. The three deletions are shown below. All three inherited deletions terminate near the center of the 160bp palindrome. A small insertion 10bp to the side of the symmetry center is present in the Chilean allele as shown in lower case.
1.2.4.1 Breakage-Fusion-Bridge cycles

Examples of gene amplification events can be explained by one or a combination of two molecular mechanisms: recombination-dependent amplification and Breakage-Fusion-Bridge (BFB) cycles. Recombination-based mechanisms are usually invoked to explain an incremental increase in copy number of DNA arranged in a direct repeat orientation (Amler and Schwab, 1989; Kuwahara et al., 2004), while BFB cycles may explain the formation of repeat arrays in an inverted orientation (a review of the BFB cycle is presented in (Murnane, 2006)). Gene amplification events can often involve numerous complex genome rearrangement events; a basic description of a Breakage-Fusion-Bridge cycle is described in the following and is diagrammed in Figure 1-6: i) BFB is first instigated by the formation of a double strand break. There is evidence supporting both fragile sites and telomere erosion in break formation (Ciullo et al., 2002b; Hellman et al., 2002; Lo et al., 2002). ii) Replication of the broken chromosome followed by (iii) illegitimate end joining of the two broken sister chromatids results in the formation of a dicentric inverted dimer chromosome. (iv) During mitosis, the dicentric chromosome is pulled to each spindle pole, resulting in unequal breakage (anaphase bridges are observed in (Shimizu et al., 2005)). Resulting daughter cells now each contain broken chromosomes, one with a terminal palindrome. They can once again replicate, fuse to their sisters, and become unequally broken. If the terminal amplified region of DNA harbors gene(s) that provide the cell a growth advantage, cells with BFB events will be positively selected. Although Breakage-Fusion-Bridge does explain many examples of gene amplification events, other mechanisms possibly contribute. For example, complex rearrangement events exist that cannot be
Figure 1-6
Diagram of the initiation of Breakage Fusion Bridge cycle. (i) A double strand break is formed (yellow lightning bolt) in the chromosome. (ii) If the break is not repaired, replication of the broken chromosome results in a second broken sister chromatid. (iii) End joining of the sister chromatids creates a dicentric chromosome (centromeres in dark blue) that (iv) will be unequally broken during anaphase. (v) One cell will acquire a chromosome carrying a duplication arranged as an inverted repeat.
explained by such a mechanism as seen in "jumping DNA", where amplified regions move from one chromosome to another while telomeric DNA remains intact (Van Roy et al., 2006).

1.2.4.2 Initiating gene amplification events

A challenge in studying gene amplification mechanisms is not only in defining the event(s) that instigate the amplifications but also in determining subsequent steps that lead up to the visualization of rampant HSRs and/or DMs. Tanaka et al. (2005) have provided clues into the events leading up to amplification. Through the use of a technique for isolating snapback DNA called Genomic Analysis of Palindrome Formation (GAPF), they found that numerous cancer genomes contain long suspected palindromes and each analyzed cancer type had a reproducible signature palindrome profile (Tanaka et al., 2005; Tanaka et al., 2006). If it is established that these tandem inverted repeats are truly palindromic, this will be a significant finding. To date there has been no direct link made between palindromes and any somatic disease, a certainty in the case of genetic syndromes mentioned earlier. These suspected palindromes are not present in the normal human genome; they arise de novo in neoplastic cells. Interestingly, when the same cells were analyzed for gene amplification events, they found that copy number increases were significantly more likely to occur in a region that first acquired a suspected palindrome (Tanaka et al., 2005). This study not only supports to the notion that palindrome formation precedes gene amplification, it exemplifies the detrimental effects that palindromes can inflict on the genome.

The above findings lead to an obvious question: how are these palindromes formed? Findings published in Tanaka et al. (2002) have provided a possible
explanation. They found that the introduction of a double strand break (introduced using an ISceI site) near a short inverted repeat (129bp arms and a 29bp spacer), results in the formation of a large suspected palindrome (Tanaka et al., 2002). Further structural characterization of the chromosome revealed that the original short inverted repeat was located at the center of the suspected palindrome. A diagram of the events that result in palindrome formation is shown in Figure 1-7. Such a palindrome could form if, after DSB formation followed by resection, intramolecular recombination occurs between the repeat arms forming a hairpin. Bidirectional replication of the hairpin-ended linear chromosome creates a palindrome. Since the human genome contains inverted repeats, this mechanism could account for any number of gene amplification events.

Studies in budding yeast from Kirill Lobachev and Alison Rattray have also contributed to our understanding of the events that instigate gene amplification. Their work in a yeast model is covered in section 1.3.2.

1.2.5 Validation of palindromes in the human genome

The largest hurdle in palindrome research is the bacterial cloning barrier. It makes both determining the palindromic content of the human genome and characterizing the mechanistic relationship between palindromes and instability a challenge. Therefore, methodologies have been developed in order to clone human palindromes that bypass the bacterial cloning barrier.

First, alternative methods for cloning of palindromic and near-palindromic arrangements from the human genome have been developed where, instead of using *E. coli*, budding yeast is used as a cloning vehicle. The yeast cloning technique was utilized for the successful sequence validation of the NF1 palindrome that is currently represented
Figure 1-7
Diagram of palindrome formation from a small inverted repeat. (i) A double stand break (lightning bolt) is generated near an inverted repeat. (ii) Initiation of repair results in resection of the 5’ end. (iii) The single strand inverted repeat forms a hairpin by intramolecular recombination. (iv) Replication of the hairpin-terminated DNA forms a large palindrome. The symmetry center of the palindrome is the inverted repeat.
by a smaller corrupted version in the human genome consortium sequence (Lewis et al., 2005). A second challenge in validating palindromic sequences is in their successful sequencing. Their tendency to self-pair while single stranded largely inhibits automated cycle sequencing although some palindromes can be sequenced "by hand" using standard Sanger sequencing. Through the use of sodium bisulfite, palindromic symmetry centers can be successfully sequenced (Rattray, 2004). Sodium bisulfite treatment of DNA results in the conversion of cytosine to uracil. Treatment of a palindrome with sodium bisulfite therefore, results in the loss of symmetry, inhibiting intrastrand base pairing. The treated DNA can therefore be used for both PCR amplification and sequencing of perfect palindromes. Lastly, in order to characterize the unclonable palindromes in the human genome, large-scale methods that avoid bacterial cloning are required. Recently, novel techniques have been developed for high quality automated sequencing that completely bypass bacterial cloning (Kim et al., 2007; Shendure et al., 2005). If these methods are applied to the problem, then it may be possible to identify unclonable regions of the human genome.
1.3 Mechanisms of palindrome-provoked instability: Lessons from model organisms

Much of our understanding of palindrome-mediated instability comes from studies using model organisms. From early work in *E. coli* to the use of transgenic mice, all have contributed significantly to our understanding of the biological consequences of palindromy in the genome. Additionally, in employing such diverse models, it has become clear that while some common features exist, bacteria, yeast, and mammals also possess significant differences in the manner with which palindromes are metabolized.

1.3.1 Instability in bacteria

Experiments performed with prokaryotic models have been instrumental in characterizing several pathways of palindrome and inverted repeat-mediated genome instability.

1.3.1.1 SbcCD-dependent palindrome instability

DNA palindromes are largely unstable in bacterial hosts. When inserted in a bacteriophage genome, the phage are unable to form plaques on bacterial lawns (reviewed in (Leach, 1994)). SbcC and SbcD, homologs of eukaryotic Rad50 and Mre11, respectively, were shown to play a central role in palindrome metabolism. Mutations in SbcC or SbcD result in a stabilizing effect, observed as an increase in the number and size of plaques (Chalker et al., 1988; Gibson et al., 1992). Through biochemical analyses, the Leach group demonstrated that SbcCD possesses double strand exonuclease as well as hairpin endonuclease activity (Connelly et al., 1999; Connelly et al., 1997; Connelly et al., 1998; Connelly and Leach, 1996). This led to the speculation that SbcCD may open hairpin structures that arise during replication or may be targeting
cruciform tips. There is evidence supporting the occurrence of both hairpin-instigated (Cromie et al., 2000; Eykelenboom et al., 2008) and cruciform-provoked breakage (Bzymek and Lovett, 2001; Chalker et al., 1988; Chalker et al., 1993; Davison and Leach, 1994). More recently, direct evidence of double strand break formation at an inverted repeat was shown to be dependent on SbcCD and also demonstrated that the breaks were introduced after passage of the replication fork (Eykelenboom et al., 2008). The two-sided breaks could be produced by cruciform cleavage, however, the authors suspect that breaks are formed at a hairpin structure on the lagging strand because, in this system, DSBs are replication-dependent. After break formation by SbcCD, the break can be repaired through RecBCD-dependent or independent pathways (Cromie et al., 2000) or may be deleted through RecA-independent single strand annealing (Bzymek and Lovett, 2001). Together, this work highlights the central importance of SbcCD in hairpin processing.

1.3.1.2 Replication bypass

Palindromes and inverted repeats are deletion hotspots in E. coli. Mapping of deletion junctions shows that they always remove the center of the repeat and the joints occur at the site of a direct repeat (Sinden et al., 1991). Because 1) the deletions are SbcCD-independent, and 2) they occur more readily on the lagging strand, it is suspected that they are replication-dependent (Bzymek and Lovett, 2001; Pinder et al., 1998; Trinh and Sinden, 1991). The replication bypass mechanism of palindrome and inverted repeat deletion is in Figure 1-8. Single strand DNA, more readily liberated on the lagging strand, allows for the formation of a hairpin structure. Once the advancing polymerase encounters the obstruction, it disengages from the template and then re-anneals at a site
Figure 1-8
Replication bypass can delete palindromes and related inverted repeats. Replication bypass, also called slipped misalignment or slipped strand misalignment, is an intramolecular template switching event that can be provoked by hairpin structures on the lagging strand template. (i)-(ii) A replication fork proceeding leftward toward a palindrome. (ii)-(iii) Lagging strand synthesis is obstructed by the stem-loop structure, leading to bypass replication. Synthesized lagging strand melts off its template and re-anneals at a site of fortuitous microhomology past the obstruction. (iv) After a second round of replication templated by the bypass product, the palindrome is almost fully deleted. Alternatively replication bypass can cause a more limited internal central deletion in a palindrome if only the middle portion has formed a stem-loop structure. All bypass type deletions include the symmetry axis.
of fortuitous homology on the other side of the hairpin, permitting continuation of replication. The resulting newly synthesized strand is devoid of the palindrome or inverted repeat.

1.3.1.3 Template switching

Errors in polymerization not only result in deletion but can also provoke a number of other mutational events. There are at least two types palindrome and IR-stimulated template switching events (reviewed in (Lovett, 2004)). Intramolecular template switching (Figure 1-9B) occurs when an advancing polymerase turns and begins to template synthesis from the newly synthesized strand. An advancing polymerase that melts from the Watson strand and synthesizes DNA from the Crick strand is called intermolecular template switching (Figure 1-9A). These processes can result in quasipalindrome correction, where a near palindrome (containing mismatches or spacers) becomes more palindromic (Dutra and Lovett, 2006; Rosche et al., 1997; van Noort et al., 2003; Viswanathan et al., 2000).

1.3.2 Instability in yeast

Much of our understanding of palindrome and inverted repeat instability comes from studies in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Not only have the yeast models allowed for the characterization of IR- and palindrome-provoked breaks and rearrangement events, they have also been crucial for the identification of eukaryotic proteins that participate in DNA hairpin repair.

1.3.2.1 Double strand break formation

Initial evidence suggesting that palindromes and inverted repeats are prone to double strand break formation was obtained from work with yeast. By measuring gene
Figure 1-9
Template switching events in near palindromes can lead to their "correction". Left: Intermolecular template switching. (i) An advancing polymerase dissociates from the Crick strand and templates synthesis from the Watson strand. (ii) A second template switch results in the continuation of polymerization from the Crick strand. Right: Intramolecular template switching. (i) A DNA strand under synthesis folds back on itself and templates its own polymerization. (ii) A second template switching event results in unfolding of the turn around and templating of synthesis from the "old" strand. Palindrome arms are represented as blue arrows, with mismatches depicted as orange stars.
conversion frequencies at the locus harboring the palindrome or IR, it was found that these sequences are highly recombinogenic. DNA double strand breaks have been detected at both inverted repeats and palindromes in fission and budding yeasts and occur in both meiotic and mitotic cells (Farah et al., 2005; Farah et al., 2002; Lobachev et al., 2002; Nag and Kurst, 1997; Nasar et al., 2000). The DSBs can be so abundant that they can be directly visualized by Southern blot (Farah et al., 2005; Lobachev et al., 2002). Work by Kirill Lobachev has aided significantly in our understanding of IR-mediated genome instability. Using inverted human Alu elements separated by a 12bp spacer inserted in chromosome II, it was not only found that it became a recombination hotspot in wild-type *S. cerevisiae*, it produced enough DSBs as to visualize them by Southern blot. Using two dimensional gel electrophoresis in which one dimension was electrophoresed under denaturing conditions, it was found that the DSB ends were capped by hairpins (Lobachev et al., 2002). While breaks were observed in all strains tested, breaks capped by a hairpin were only observed in *sae2* or *mre11* cells. The formation of DSBs did not depend of the presence of the *MUS81* gene, whose product is a candidate mitotic Holliday junction nuclease. The observation of hairpins in *sae2* or *mre11* cells suggested that Sae2 and Mre11 were required for hairpin processing. Lobachev *et al.* proposed two possible mechanisms of hairpin-capped break formation: 1) hairpin formation through the generation of a DSB in proximity to the IR followed by resection and intrastrand hybridization of the single strand IR, and 2) resolution of an extruded cruciform at the inverted Alus (Lobachev et al., 2002). However, work by several groups has shown that a spacer of 10bp or longer completely abolishes cruciform extrusion (Courey and Wang, 1988; Murchie and Lilley, 1987; Zheng and Sinden, 1988).
Therefore, it is more likely that the double strand breaks formed as a consequence of hairpin-mediated replisome stalling. In support of this, Sergei Mirkin’s group found that the inverted Alus stall replication in bacteria, yeast, and Cos7 cells. The insertion of a 52bp spacer reduced the frequency of fork stalling while complete removal of the spacer (providing the highest opportunity for extrusion) did not result in an increase in fork stalling (Voineagu et al., 2008).

The Lobachev work was important in a number of respects: it provided in vivo evidence that Mre11 is a hairpin endonuclease and also exemplified the detrimental effects of inverted repeat sequences on genome integrity. What remained unclear was the mechanism of double strand break formation.

1.3.2.2 Gene amplification

Work in budding yeast has been instrumental in characterizing events that lead up to the formation of homogeneous staining regions and extrachromosomal elements observed in cancer cells. Using the same inverted Alu inverted repeat system for generating hairpin-capped double strand breaks, the Lobachev lab illustrated the consequences of failing to process hairpin DNA ends (Narayanan et al., 2006). Some of their findings are illustrated in Figure 1-10. Depending on the location of the hairpin-capped break relative to the centromere and the selectable marker, they observed the formation of extrachromosomal elements and dicentric chromosomes containing a large inverted duplication (Figure 1-10). Extrachromosomal elements were observed when the hairpin break was between the centromere and the selectable marker while dicentrics were isolated when the selectable marker was between the hairpin end and the
Figure 1-10
Diagram of chromosomal rearrangements instigated by hairpin-capped double strand DNA ends in yeast. i) Formation of hairpin-capped DNA ends (via cruciform resolution or intrastrand hybridization of a resected end) at an inverted repeat. ii) Replication of the chromosome generates a dicentric duplicated chromosome while replication of the acentric end creates a linear inverted dimer. iii) Unequal breakage during anaphase followed by end healing via telomere addition or BIR generates a chromosome with a terminal inverted duplication.
centromere. They suspected that extrachromosomal DNA, arranged as a linear inverted dimer, is formed after replication of a chromosome end that is capped by a hairpin. The formation of the chromosome with an inverted duplication could stem from the following process: i) replication of a hairpin-capped chromosome, creating a dicentric, ii) unequal breakage of the dicentric during anaphase, and iii) end "healing" through BIR or telomere addition. Multiple inverted ladders (resembling HSRs) were also detected as alternating stained regions in combed chromosomes. It is suspected that inverted ladder formation occurs by a BIR-dependent mechanism that primes synthesis from delta elements that seek homology to other delta elements on the chromosome. A second possibility is that the accumulation of inverted ladders results from multiple BFB cycles that are suspected to require end joining to form fused dicentric chromosomes (Narayanan et al., 2006). In support of the former, they found that the observed intrachromosomal copy number increases depend on RAD52, while elimination of non-homologous end joining (via deletion of DLN4) had no effect on amplification (Narayanan and Lobachev, 2007).

These observations have implications in mammalian gene amplification events, as it was observed that gene amplification is still observed in DNA-PKcs-deficient mammalian cell lines (Mondello et al., 2001; Rebuzzini et al., 2004; Salzano et al., 2008). It is possible that recombination-based amplification may be occurring in this instance instead of Breakage-Fusion-Bridge cycles, however, since chromosome end-to-end fusions are also observed in NHEJ-deficient cells, BFB may occur by an alternative end joining pathway (Rebuzzini et al., 2004).

Work by Alison Rattray demonstrated that short inverted repeats (with arm lengths between 4-6bps) with a spacer equal or shorter than 9bps can instigate the
formation of large 2.4 to 3.4 kb near-palindromes in yeast (Rattray et al., 2001; Rattray et al., 2005). In Rattray's system, a large near-palindrome (sometimes a true palindrome) is observed when a double strand break is introduced near a very short inverted repeat. Using sodium bisulfite to enable for PCR, cloning, and sequencing of the near-palindromic symmetry axis, it was found that the symmetry center contained the short inverted repeat (Rattray, 2004). It is suspected that the mechanism of palindrome formation includes a combination of break-induced replication and DNA synthesis primed by foldback of the ssDNA inverted repeat. Detectable palindrome formation is suppressed by \textit{SAE2} and \textit{MRE11} and requires genes for homologous recombination (Rattray et al., 2005). This mechanism of near-palindrome formation could potentially occur in human cells as the human genome contains numerous inverted repeats. Because the short inverted repeat required for hairpin formation only has 4-6bp arms, intrastrand hybridization could potentially occur throughout the human genome.

IRs instigating the formation of larger palindromes has also been observed in other yeast systems (Albrecht et al., 2000; Butler et al., 2002; Butler et al., 1996), in mammalian cells (covered in section 1.2.4.2) and in protozoa (Butler et al., 1995). The models outlined herein are vastly different and demonstrate that DNA capped by a hairpin structure may arise by numerous mechanisms. Each pathway highlights the detrimental effects of hairpin-capped DNA ends on genome integrity.

\subsection*{1.3.2.3 Sae2 and the Mre11-Rad50-Xrs2 complex}

All of the yeast studies to date illustrate the important roles of Mre11 in palindrome and inverted repeat metabolism. Mre11, part of the Mre11-Rad50-Xrs2 complex, is conserved from bacteria to humans (there is no known Xrs2 homolog in \textit{E.}}
coli and the functional homolog of Xrs2 in mammals is Nbs1). This complex is an instrumental caretaker of the genome; it is involved in many DNA repair and genome maintenance processes, including replication, homologous recombination, non-homologous end joining, DNA damage checkpoint signaling, and telomere maintenance (reviewed in (D'Amours and Jackson, 2002)). Molecular genetic analyses in yeast led to the supposition that the role of Mre11 in palindrome and IR processing is to cleave hairpins and the ends of a DSB (Lobachev et al., 2002). In support of this, biochemical evidence demonstrated that Mre11 possesses 3' to 5' exonuclease and hairpin endonuclease activities, however, hairpin endonuclease activity was weak and did not occur at physiological conditions (Paull and Gellert, 1998; Trujillo and Sung, 2001; Trujillo et al., 1998). It was suspected that Sae2, also known for its role in palindrome and IR processing, may be responsible for regulating the nuclease activity of Mre11. This could possibly explain the weak endonuclease activity of Mre11 in vitro and was supported by genetic data demonstrating that null mutations in \textit{SAE2} were indistinguishable from hypomorphic alleles of \textit{MRE11} (McKee and Kleckner, 1997a; McKee and Kleckner, 1997b; Neale et al., 2002; Prinz et al., 1997; Rattray et al., 2001). New biochemical data from Tanya Paull's laboratory has further defined the role of Sae2 in genome maintenance. They found that scSae2 has hairpin-proximal ssDNA endonuclease activity that is stimulated by scMre11-scRad50 (Lengsfeld et al., 2007). Their data is consistent with Sae2 and Mre11 acting cooperatively in the removal of hairpins and not in their opening at the tip (Figure 1-11). \textit{In vitro}, MR is though to widen a nick close to the hairpin, thus forming ssDNA for Sae2 to then remove the hairpin.
Figure 1-11

Hairpin removal by Sae2 and Mre11. (i) A nick proximal to a hairpin-capped DNA end is widened into a single strand gap by the 3'-5' exonuclease activity of Mre11 (red circle). The single strand region is cleaved by Sae2's ssDNA endonuclease activity (scissors). (iii) Removal of the hairpin creates an open DSB that can be further processed for repair.
With the identification of Sae2 homologs in higher eukaryotes (Penkner et al., 2007; Sartori et al., 2007; Uanschou et al., 2007), it is possible that hairpin removal is a conserved mechanism in human cells. It should be noted that although these data do support complete hairpin removal as a mechanism of hairpin processing, it does not eliminate the possibility that Mre11 may also function in hairpin tip cleavage \textit{in vivo}.

1.3.3 Instability in mammals

The advancement of our understanding of palindrome metabolism in higher eukaryotes has often been challenged by the difficulty in manipulating long palindromic arrangements. However, through the analysis of a palindrome embedded in the mouse genome as well as through the introduction of extrachromosomal palindromic substrates in cultured cell lines, the fate of palindromes in mammalian cells can be successfully characterized.

1.3.3.1 Line 78

An unique opportunity to study palindrome instability in mammalian cells arose when the Jasin lab constructed a transgenic mouse line in which two copies of an injected fragment fortuitously integrated in a tail-to-tail orientation (Figure 1-12). The 15.6kb palindrome in the Line 78 transgenic mouse has been instrumental in understanding the underlying basis for palindrome instability (Akgün et al., 1997; Cunningham et al., 2003; Lewis et al., 1999; Zhou et al., 2001). As described in Chapter 4, I made the crucial observation that the center of the Line 78 transgene is indeed a perfect palindrome (Cunningham et al., 2003).
Figure 1-12
Diagram of the Line 78 palindromic transgene. **Above:** The transgene arose when two injected PstI fragments integrated in a tail to tail orientation. The outside PstI sites were lost upon integration. Blue boxes represent LacZ repeats; the inner repeats are truncated. **Below:** The majority of revision events in the line 78 palindrome result in variable sized deletions that may or may not include the central PstI site.
Through the use of Southern blot, it is possible to map rearrangement events that occur in the palindromic arrangement. Unlike in prokaryotes, the long palindrome does not affect viability of mice nor is the transgene associated with illness or disease. Through breeding experiments, it was found that the transgene is passed on to progeny at a Mendelian ratio. However, the transgene does undergo high frequency rearrangement events in the form of small, asymmetric deletions at the palindrome's axis of symmetry (Figure 1-12). In a given litter, 15-56% of the transgenic animals harbor a rearranged transgene (Akgün et al., 1997). The events are not specific to Line 78 alone as other less-characterized examples of suspected palindrome symmetry center modifications were observed in other transgenic mouse lines (Collick et al., 1996; Honchel et al., 2001; Cote, A.G. and S.M. Lewis, unpublished).

Rearrangement rates were measured in immortalized cell lines. The Line 78 palindrome rearranges at a rate of 0.5% per population doubling (Cunningham et al., 2003). In almost two hundred analyzed subclones, the majority of rearrangement events only modified the symmetry center of the palindrome. Large scale rearrangements such as translocations, chromosome end loss, large deletions, or gene amplifications were never observed (Cunningham et al., 2003). There were occasional inversions, gene conversion, or repeat expansion events but none were ever observed to be explosive amplification events (Akgün et al., 1997; Lewis et al., 1999; Zhou et al., 2001). Moreover, the central modification events stabilize the palindrome, preventing further rearrangements. Cell lines with the rearranged palindrome could be cultured indefinitely without acquiring further rearrangements (Cunningham et al., 2003).
An obvious explanation for the observed near-center deletion events in the Line 78 palindrome would be a replication slippage event (depicted in Figure 1-8). However, upon detailed sequence analysis of rearrangement events, it became clear that many deletions could not be caused by replication bypass. Replication bypass produces deletions that remove the symmetry center of the palindrome, however, a significant fraction of the deletions did not even remove the central PstI site (Figure 1-12) (Cunningham et al., 2003). Sequence analysis of the deletion junctions suggested that the deletion ends were joined by non-homologous end joining. Large regions of homology were not observed as is a requisite for bypass or single strand annealing. An alternative mechanism of formation must account for these rearrangement events (more on this in section 1.3.3.3).

1.3.3.2 Mammalian extrachromosomal palindrome assay

Introduction of extrachromosomal palindromic plasmids into mammalian cells is an alternative method utilized for examining palindrome instability in higher eukaryotes. Transient transfection of a completely palindromic dimer circle plasmid results in the formation of circular monomer products (Lewis, 1999). The dimer contains two perfect symmetry axes and is prepared entirely in vitro. Unlike the palindromic dimer, monomer rearrangement products are able to transform E. coli, thus enabling for their harvest and amplification in bacteria. Through fine mapping of the monomers, it was apparent that the products are formed by an axis-specific rearrangement process, where each symmetry axis is thought to be cleaved and the resulting DNA ends are then ligated by end joining. A fraction of joints also contained inserts, usually vector-derived, suggesting that a 3’ liberated DNA end can participate in invasion and extension reactions before ligation.
Lewis, 1999). The similarities between the joints formed in Line 78 rearrangement events and in the extrachromosomal assay suggest that the latter reflects physiological events.

1.3.3.3 Center-Break palindrome revision

By what mechanism does the palindrome acquire relatively small, centrally-localized modifications that do not compromise the rest of the genome? Why doesn't the Line 78 palindrome cause gross DNA rearrangements in mouse cells? It is possible that the 15.6kb palindrome forms secondary structures that do not instigate gross chromosomal aberrations. The central deletions may be a deliberate means to modify an extrusion prone DNA arrangement, thereby preventing potentially serious consequences. Lineform DNA that is interrupted by the extrusion of a cruciform can interfere with cellular processes such as transcription and replication (Bagga et al., 1990). Long palindromes extrude to create a cruciform structure and it is postulated that this structure, rather than the palindrome sequence, that is what undermines DNA function. In the Center-Break model of palindrome revision, an extruded cruciform will be recognized, centrally-modified, and repaired for the purpose of introducing enough asymmetry into the palindrome to prevent further extrusion events without compromising nearby sequences. The proposed mechanism of Center-Break revision is depicted in Figure 1-13. First, a palindrome that extrudes a cruciform is recognized and cleaved at each of the hairpin tips by a hairpin endonuclease. After resorption of the cruciform, the resulting double strand break is repaired by non-homologous end joining. Because NHEJ is an imprecise mode of repair, the palindrome is converted into an inverted repeat with a central spacer. Such
Figure 1-13

Center-Break palindrome revision. Center-Break revision is proposed to remove sequences that can extrude as cruciforms. i) Torsional strain induces extrusion. ii) The cruciform junction is shown in an open square configuration. (iii) A single strand nick is introduced at each hairpin terminus; these nicks need not occur exactly at the tips. iv) Branch migration resorbs the cruciform and reveals the double strand break created by the nicks in step iii). (v) Deletion of sequence and other end processing steps might take place before or after the cruciform is resorbed. (vi) Ligation reconnects ends. End joining does not require microhomology.
modifications create an energetic barrier to further cruciform extrusion events. The Center-Break pathway can be regarded as a genome-maintenance mechanism that has evolved to deal with pathogenic lesions by first recognizing secondary structure formation and then subtly modifying the locus in order to eliminate the structure-prone feature. Examples of human palindrome-provoked gross-chromosomal aberrations as described in sections 1.2.2 and 1.2.3 can be regarded as failed attempts to properly stabilize regions of palindromy or may have simply failed to be recognized by the Center-Break machinery altogether, instead instigating large genome rearrangement events that would otherwise be prevented.

1.3.4 Summary

Through the investigation of palindrome instability in a variety of organisms, palindrome-provoked mechanisms of genome instability are beginning to be deciphered. Significant parallels exist between models, notably the importance of the Mre11 nuclease in hairpin processing, however, there also exist many distinctions. Mre11 (or SbcD in *E. coli*) is thought to cleave cruciform and hairpin tips in bacteria (Bzymek and Lovett, 2001; Eykelenboom et al., 2008) and fission yeast (Farah et al., 2005) while in budding yeast, Mre11 (with Sae2) is suggested to process hairpins at the end of double strand breaks (Lobachev et al., 2002). Most importantly, palindromes cannot be maintained in bacteria while evidence suggests that they can be propagated in both yeast and mammalian cells. In higher eukaryotes, there is evidence of a palindrome stabilizing mechanism while no such pathway has been observed in single cell organisms. Mechanisms of palindrome-provoked rearrangement and cruciform "repair" may be highly divergent, evolving relatively rapidly in order to accommodate different genome environments and for the general requirement of such mechanisms in each organism.
1.4 Thesis rationale

It is now clear, thanks to the thorough characterization of chromosomal perturbations in certain genetic diseases, that DNA palindromes cause significant havoc to the genome. Even though the notion that extrusion of a cruciform structure instigating rearrangements has gained some acceptance (for example, see (Kato et al., 2008; Lobachev et al., 2002)), until the work described in this thesis, there existed no definitive proof for their formation in vivo in eukaryotic cells. Most models for palindrome-instigated instability assume the formation of cruciform structures without empirical evidence supporting their occurrence. Several groups have provided evidence for in vivo cruciform extrusion in bacteria, however, the methodologies used for their detection have significant caveats. Moreover, because of the differences between prokaryotic and eukaryotic nuclear organization, evidence for cruciform extrusion in E. coli cannot be extrapolated to yeast, mice, and humans. Therefore, it is of central importance that cruciform extrusion be addressed in a eukaryotic model.

Investigations into the biology of DNA secondary structures have begun to reveal the molecular genetic dependencies of palindrome instability. Mre11 and Sae2 have been shown by several groups to play a pivotal role in the processing of hairpin intermediates, however, prior to my work, no other proteins had been identified that participate in palindrome-mediated DNA damage.

The aim of my work was to further characterize palindrome instability through the following: 1) establishing in vivo cruciform extrusion in eukaryotes, and 2) by identifying and characterizing the role(s) of protein(s) involved in palindrome instability.
In Chapter 2, I provide the most conclusive evidence to date that palindromes and near-palindromes can extrude cruciforms in yeast chromatin in vivo. The human NF1 near-palindrome, associated with the recurrent t(17;22) translocation, extrudes in vivo, providing evidence for a cruciform-mediated mechanism of translocation in humans. I show that cruciforms are cross-diagonally cleaved by four-way junction resolvases resulting in the formation of a doubly hairpin-capped double strand break. These findings highlight the potentially detrimental effects of nucleases on genome integrity and identify a novel mode of endogenous DNA damage.

In Chapter 3, I identify the Mus81 endonuclease as being the primary four-way junction cleaving enzyme responsible for cruciform cleavage in vegetative budding yeast. In the absence of Mus81, cleavage can be replaced by RusA, a bona fide bacterial Holliday junction resolvase. I also show that the sole yeast RecQ helicase, Sgs1, plays a significant role in palindrome stability. I provide evidence that Sgs1 is responsible for preventing multiple pathways of DSB formation, including cruciform resolution. This work provides in vivo evidence that Mus81 is indeed a HJ resolvase and illustrates the central role of RecQ helicases in the prevention of palindrome-provoked double strand breaks and rearrangement.

In Chapter 4, I use the mammalian extrachromosomal palindrome assay to determine if hypomorphic mutations in murine Mre11 and Nbs1 affect palindrome revision. From these analyses, I provide evidence that the $Mre11^{ATLD1}$ and $Nbs1^{AB}$ mutations do not affect palindrome revision. I also provide irrefutable evidence that the Line 78 transgene is a perfect palindrome. Using digestion-circularization PCR, I developed a method to separately amplify and sequence the left and right sides of the
symmetry center for sequence analysis. This work demonstrates that long perfect palindromes can be stably maintained in mammalian genomes.
Chapter 2

In vivo cruciform extrusion of a human palindrome in Saccharomyces cerevisiae

The work outlined in sections 2.3.1 to 2.3.6 and 2.3.8 is published in Molecular Cell, Vol.30, Cote, A.G. and S.M. Lewis, Mus81-dependent double strand DNA breaks at in vivo-generated cruciform structures in S. cerevisiae, 800-812.

I performed all experiments in this chapter except for the construction of NF1 plasmids, which was performed by Susanna M. Lewis. Section 2.3.11 was performed with help from Constence Zhu.

Section 2.3.11 was performed in collaboration with Catherine Freudenreich (Tufts University). C.F. provided the Flex1 sequences.
2.1 Abstract

Long DNA palindromes in the human genome are implicated in gross chromosomal rearrangements in humans, but the molecular events underlying the association remain a matter of conjecture. One proposal is that palindromes lead to double strand breaks when such sequences self-pair and create a cruciform structure. The four-way DNA junction at the cruciform’s base, which is structurally like a Holliday junction (HJ), is thought to be susceptible to aberrant cleavage \textit{in vivo} by HJ-specific resolvases. Though compelling, none of the components of the ‘cruciform resolution’ proposal has been experimentally established. Here I describe a system by which to examine intermediates and genetic dependencies in palindrome metabolism. A test was designed using a plasmid-based system in \textit{S. cerevisiae}. These studies provide new evidence that palindromic sequences embedded in eukaryotic chromatin will indeed undergo cruciform extrusion \textit{in vivo}. Moreover, synthetic as well as naturally occurring human palindromes acquire spontaneous, site-specific breaks. Characterization of break products validates cruciform resolution as the mechanism. Stereotypical rearrangements caused by cruciform resolution \textit{in vivo} are suppressed by Mre11 and Sae2. These studies establish key features of palindrome DNA metabolism in eukaryotes, raising the possibility that mitotic resolvases can promote radical genome destabilizing damage in the human palindrome-bearing genome when Mre11 and associated proteins are inactivated.
2.2 Introduction

One largely untested hypothesis is that the extrusion of a cruciform in a palindrome is related to the observed instances of palindrome-provoked genome rearrangements in humans. Palindromic DNA sequences can convert from lineform to cruciform through an energy-driven extrusion process that is thought to initiate with the melting of central base pairs (Sinden, 1994). Although cruciform extrusion has been shown to occur in negatively supercoiled molecules \textit{in vitro}, much skepticism exists as to whether these structures do in fact form \textit{in vivo}. A hurdle in cruciform research is in detecting the actual occurrence of cruciform extrusion \textit{in vivo}. To date, means for identifying extrusion rely largely on \textit{in vitro} methodologies. However, when isolated free of protein, a major change in the availability of energy in the isolated DNA positively influences extrusion, thus \textit{in vitro} techniques may not accurately depict its occurrence \textit{in vivo}. Tools or techniques utilized for the detection of cruciform extrusion \textit{in vivo} in eukaryotic chromatin also remain under-developed. While the \textit{in vivo} results are consistent with the possibility of cruciform extrusion, all have considerable caveats. An ideal assay would identify cruciform formation through the detection of a biological event that results as a consequence of their extrusion or metabolism.

Structure-specific nucleolytic cleavage of cruciform DNA, to yield a double strand break \textit{in vivo} was years ago postulated as a possible outcome of Holliday Junction (HJ) resolvase activity (Leach and Stahl, 1983). The notion of "cruciform resolution", first proposed according to observations in \textit{E. coli}, has been revisited with interest because it forges a straightforward, logical link between palindromes, double strand breaks, and pathogenic rearrangement in higher eukaryotes (reviewed in (Kurahashi et
al., 2006b; Lewis and Cote, 2006; Lobachev et al., 2007)). Until now, positive evidence that experimentally distinguishes this mechanism from other possibilities has not been forthcoming.

According to *in vitro* studies, when an HJ resolvase acts upon an extruded cruciform, a break is produced in which both of the DNA ends have hairpin termini (Mizuuchi et al., 1982a). The dually hairpinned break is a diagnostic feature of cruciform resolution. Other patterns of strand breakage imposed upon an inverted repeat, whether structured or lineform, yield only one hairpin end at most (Figure 2-1C,D vs. A, B).

Here I report the occurrence of spontaneous (un-selected) breaks at plasmid-born palindromes maintained in *S. cerevisiae*. Both long artificial palindromes as well as a PATRR from human chromosome 17 are cleaved. The palindrome-specific breaks are detected in *mre11* and *sae2* mutant cells and the presence of hairpin termini at both ends of the linear cleavage product provides the sought-for evidence of cruciform resolution. Breaks are observed whether an intact palindrome-bearing plasmid is introduced as naked DNA or, through karyogamy, as a chromatinized molecule, indicating that the structural transition preceding cleavage can be achieved *in vivo*.

I also describe methodologies for utilizing the extrachromosomal palindrome system for the semi-quantitative assessment of *in vivo* cruciform extrusion of any sequence of interest. Using this system, I evaluate the cruciform extrusion capacities of variants of the Neurofibromatosis-1 palindrome. The NF1 palindrome, present in an intron of the Neurofibromin-1 (NF1) gene and implicated in the recurrent t(17;22) translocation, was introduced episomally into *S. cerevisiae* and monitored by Southern
Figure 2-1
Dual strand breaks at a palindromic sequence with and without a cruciform structure.  

A. Single strand nicks made on opposite sides of the symmetry center of a lineform palindrome will not directly result in a double strand break.  

B. Breaks on the same side of the palindrome can give a hairpin terminus only if one strand is resected.  At most, only one of two DNA ends can contain a hairpin.  

C. Cross-junction cuts in a palindrome that has extruded into a cruciform directly produces a double strand break.  Two hairpin capped ends with a nick distal to the tip are the initial cleaved products.  Known Holliday Junction (HJ) resolvases introduce a pair of positionally-correlated cross-junction nicks as diagrammed (Lilley and White, 2001).  

D. Cross-junction nicks of an extruded cruciform introduced at uncorrelated positions across the four-way junction should also give a pair of hairpin termini for which one terminus contains an internal single strand gap, and the other, a corresponding single strand flap.  

E. Cruciforms cut on one side rather than diagonally across the four way junction after the fashion of an HJ resolvase, will not give two hairpin-capped cleavage products.  Presumably, single strand interruptions in the products diagrammed in parts C and D will readily be converted to covalently closed form in vivo.  Direct production of fully covalently-closed resolution products is a theoretical possibility, but has not been seen for any HJ resolvase studied to date.
blot. I found that, for the polymorphic NF1 palindrome, there exists significant variation in the degree of cruciform extrusion, where some alleles are completely inert while others show high levels of spontaneous extrusion and double strand break formation.
2.3 Results

2.3.1 A fully palindromic plasmid is maintained in yeast and can be site-specifically cleaved in vivo

To investigate palindrome processing, a fully palindromic plasmid, pPX (Figure 2-2A) was produced by preparative head-head/tail-tail ligation of a PvuII plus XbaI-linearized yeast/E. coli shuttle vector (pYES2.1/V5-His-TOPO Invitrogen; see Experimental Procedures). As is well established for dimeric plasmids with an inverted repeat arrangement, pPX does not transform bacteria (Table 2-1).

Gel-purified pPX DNA was transformed directly into wild-type and mutant S. cerevisiae. Initially the choice of recipient strains was guided by previous work demonstrating that MRE11 and SAE2 had significant effects upon the metabolism of both palindromes and inverted repeats in yeast (Farah et al., 2005; Farah et al., 2002; Lobachev et al., 2002; Rattray, 2004; Rattray et al., 2001; Rattray et al., 2005). Quite unlike the situation in E. coli, the fully palindromic plasmid was able to transform S. cerevisiae, and not only sae2-null or mre11Δ strains but also wild-type cells expressed the plasmid-specific marker (Table 2-1). To examine the DNA isolated from the transformants, Southern blots were performed with the plasmid-specific probe diagrammed in Figure 2-2A. Uncut samples from all three strains gave the same bands, typical of most plasmid DNA, representing supercoiled, linear, and relaxed forms (Figure 2-2C; lanes 1, 3, and 5, respectively). The only unusual feature was the presence of a fourth band at a size consistent with a 5.8 kb linear. This was seen to have originated during a denaturation step in the DNA isolation protocol which (with nicked or broken molecules) facilitated spontaneous self-annealing (snap-back) of individual pPX strands.
Table 2-1. Efficiency of transformation of pPX and pSH1 DNA in bacteria and yeast.

### E. coli

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate DNA</th>
<th>Transformation efficiency (cfu/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top10F’</td>
<td>pSH1</td>
<td>1.8 x 10⁶</td>
</tr>
<tr>
<td></td>
<td>pPX</td>
<td>6.2 x 10³</td>
</tr>
<tr>
<td>SURE</td>
<td>pSH1</td>
<td>2.6 x 10⁶</td>
</tr>
<tr>
<td></td>
<td>pPX</td>
<td>1.6 x 10³</td>
</tr>
</tbody>
</table>

### S. cerevisiae

<table>
<thead>
<tr>
<th>Relevant genotype (Strain)</th>
<th>Substrate DNA</th>
<th>Transformation efficiency (cfu/µg)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (GRY2565)</td>
<td>pSH1</td>
<td>7.6 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>pPX</td>
<td>9.3 x 10³</td>
</tr>
<tr>
<td>sae2</td>
<td>pSH1</td>
<td>2.4 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>pPX</td>
<td>3.4 x 10³</td>
</tr>
</tbody>
</table>

*I noted that the transformation efficiency of pPX is 10-fold lower than it is for pSH1 in yeast. Although not formally tested, this may be due to a decreased ability to maintain the palindromic dimer and/or due to the topology of incoming DNA. pSH1 was a mixture of relaxed and supercoiled circles while the pPX preparation was entirely relaxed.*
Figure 2-2

**Fully palindromic dimers are propagated in yeast.**

A. Diagram of in vitro-prepared palindromic dimer pPX. The source plasmid was the yeast vector pYes2.1V5/His-Topo with a small insert. The in vitro prepared pPX was made in quantity without transformation of E. coli by ligation of an XbaI to PvuII fragment as described (see Experimental Procedures). The hybridization probe is indicated by the black bar. The probe-positive NheI fragment in pPX is indicated by a dashed line, along with the expected size. B. Map of possible cruciform resolution products. If extrusion and cleavage occurs at the XbaI-defined symmetry center, an “X” type hairpin linear is formed. Cruciform resolution at the PvuII-defined pole would produce a “P” type linear. There is no specific prediction as to the ratio of the two products. Probe-positive NheI fragments are indicated as in part A. C. Southern blots of DNA isolated from pPX transformants. Left panel: A palindromic dimer can be propagated in the three tested strains. Right panel: The 11.5kb linear is sensitive to Exonuclease V in wild-type pPX transformants, but resistant in mre11Δ (hollow arrow). A half-sized 5.8 kb unit linear band is an artifact as described in Figure 2-3. Supercoiled palindromic dimer circles (sc) are present in some but not all samples due to nicking during DNA isolation. N, NheI; ExoV, *Micrococcus luteus* Exonuclease V; P, PvuII; and X, XbaI. All sizes are in kilobases.
A. Unit length (5.8kb) linears are generated by denaturation of pPX. Panels show DNA prepared from pPX-transformed mre11Δ. In panels (i) and (ii), DNA was isolated by the method of Hoffman and Winston (Hoffman, C. S. and Winston, F., 1987) then split in two. The DNA was further purified by the standard method, which includes denaturation (Experimental Procedures) in panel (i). The DNA was not further treated in panel (ii). DNA in panels (iii) and (iv) were from a single culture that had been split into two. In panel (iii), DNA was isolated by the standard method. In panel (iv) DNA was isolated from spheroplasts, without denaturation (Holm, C., 1986). Although the 11.5 kb dimer linear signal is present in each DNA preparation, the 5.8 kb unit linear is almost undetectable in (ii) and (iv). Panels (i) and (ii) are from a single gel image, as are panels (iii) and (iv). Slower migrating species in the first lane of sample (i) are often seen in samples prepared with denaturation. Their absence in sample (ii) indicates that at least some of these bands are likely to be improperly renatured DNA rather than higher mw DNA. B. Origin of the denaturation-dependent artifact. The two DNA strands in an incompletely replicated or nicked pPX molecule can fully separate under denaturing conditions (because they are not physically interlinked). Individual strands are then free to self-anneal in neutral buffer to become unit length (5.8kb) hairpin-terminated linears seen in panels i and iii. A very faint 5.8kb linear is present in the left lanes of panels ii and iv. This may conceivably be an in vivo bi-polar cleavage product, or the result of a low residual amount of strand separation.
To more fully examine the structure of the DNA isolated from pPX transformants, aliquots were digested with NheI. NheI was predicted to cleave pPX at two sites to give a probe-positive band of 6.5 kb, as was observed (Figure 2-2C; lanes 2, 4, and 6). Unexpectedly, an additional band of ~3.25 kb was also revealed. This fragment persisted even when samples were prepared without denaturation (Figure 2-3). The 3.25kb NheI digestion product indicated a site-specific break was already present in some of the pPX DNA in vivo, and that the break mapped near the XbaI site-defined symmetry axis of pPX; one of two positions in the palindromic plasmid at which a cruciform might theoretically arise. This observation raised the possibility that the linear 11.5 kb band appearing in untreated DNA was not simply the result of random breakage during isolation, but was instead the product of cruciform resolution. Cruciform resolution might logically be expected to also occur at the PvuII site-defined symmetry axis, creating the “P” isoform (Figure 2-2B). The alternative resolution product may well be formed here, but cannot be established because the probe-positive fragment predicted upon NheI digestion is indistinguishable from the 6.5kb band derived by NheI cleavage of intact pPX (Figure 2-2A).

### 2.3.2 Cleavage of a large artificial palindrome by “cruciform resolution”

If pPX is indeed linearized through cruciform resolution, an opportunity is provided to verify the mechanism by examining the termini of the resulting linear molecule. Cleavage of a circle creates a product in which both ends of the break are physically linked, allowing one to test for dually-formed hairpin structures as predicted by cruciform resolution (Figure 2-1). Micrococcus luteus Exonuclease V is a useful enzymatic probe for hairpin termini (Roth et al., 1992) because it will completely degrade
DNA with “open” ends while sparing DNA capped by hairpin structures (as well as sparing intact and internally-nicked circular DNA, Figure 2-4). Here, incubation with ExoV degraded the 11.5 kb linear signal from a wild-type DNA sample (Figure 2-2C; lane 9). In contrast the 11.5 kb pPX linear isolated from \textit{mre11Δ} survived ExoV treatment (Figure 2-2C; lane 13). DNA from either wild-type or mutant transformants was sensitive to ExoV if first digested with NheI (Figure 2-2C; lanes 10 and 14). The artifactual 5.8 kb linear from both sources was ExoV-resistant, as expected (Figure 2-3B). ExoV treatment of DNA isolated from cells transformed with a circular direct dimer, pDD, did not result in the observation of a resistant linear species (Figure 2-5).

In brief, according to these tests, the 11.5 kb linear pPX DNA present in samples isolated from \textit{mre11Δ} (or \textit{sae2} nulls; not shown) was blocked at both termini. The linear in wild-type DNA samples, in contrast, was sensitive to degradation by ExoV. Thus, if the 11.5kb linear signal in wild-type cells is also a cruciform resolution product, its hairpin termini must be rapidly removed. The possibility is consistent with \textit{in vitro} and \textit{in vivo} evident that Mre11 and Sae2 have hairpin endonuclease activity (Lengsfeld et al., 2007; Lobachev et al., 2002; Paull and Gellert, 1998; Trujillo and Sung, 2001). Collectively these results provide strong experimental support that cruciform resolution (Figure 2-6) is an endogenous mechanism by which DNA double strand breaks can form \textit{in vivo}.

2.3.3 Plasmids bearing human genome-derived palindromes are susceptible to a stereotypic, amplifying rearrangement

In order to examine cruciform resolution in a more physiological model, I investigated a ~200bp near-palindrome found within the Neurofibromin 1 (NF1) locus.
Micrococcal luteus Exonuclease V degrades dephosphorylated ends and ends with or without overhangs. Upper panel. To confirm the specificity of the enzyme, pSH1 plasmid that had been linearized with either PvuII (blunt ends) or XbaI (5’ overhangs) and that either did or did not possess 5’ phosphates was tested for resistance to ExoV. Dephosphorylation was confirmed by failure to ligate with T4 DNA ligase. In multiply-treated samples, the order of treatment was restriction enzyme, phosphatase, ligase, exonuclease. Lower panel. Ethidium-stained gel of hairpin linear DNA (hpPX) prepared as described in Experimental Procedures. This is resistant to Micrococcus luteus Exonuclease V (lanes 1, 2). Xbal-digested pSH1 (lanes 3, 4) is fully sensitive, and uncut pSH1 plasmid (lanes 5, 6) is fully resistant to treatment with ExoV. Open circular DNA (arrow marked oc) was in a form that could not be supercoiled by DNA Gyrase (i.e. it was nicked; not shown), and this DNA is not degraded by ExoV. Black and white have been reversed in the image. oc; relaxed circle, sc; supercoiled plasmid, M; 1kb ladder (Fermentas).
Figure 2-5
Structure of direct dimer circle pDD. A. Map of 11.8kb pDD circle. It was constructed by self ligation of XbaI-digested pYES2.1 (see Experimental Procedures). B. Southern blot of DNA isolated from wild-type, sae2Δ, and mre11Δ pDD transformants. In ExoV-treated samples (lanes 3, 7, and 11), the linear 11.8kb species is fully degraded. Sizes are in kilobases. N; NheI, Exo; Exonuclease V.
Figure 2-6
Diagram of cruciform resolution possibilities in pPX. Depending upon the extrusion site, the linear is either a P or X linear with two hairpin ends.
This is one of the six identified PATRRs in humans (Gotter et al., 2007; Kurahashi et al., 2006). A previous report described methodologies used for cloning two alleles of the NF1 PATRR as well as a third in vitro-derived variant into the pYES2.1 shuttle vector (Figure 2-7; (Lewis et al., 2005)). The clones had to be isolated and propagated in S. cerevisiae; if introduced into E. coli at any point, the inserts immediately began to accumulate variable internal deletions (Lewis et al., 2005). Three sequence-verified DNA minipreparations of the test plasmids, each isolated from wild-type S. cerevisiae, were used throughout these experiments.

I initially focused on the clone bearing the most palindromic of the three inserts, pH1#21 (Figure 2-7; (Lewis et al., 2005)). When introduced and reisolated from wild-type cells, pH1#21 gave a pronounced ladder pattern on Southern blots (Figure 2-8D; lane 1; the ladder is clearer in subsequent figures). The ladder was generated by unrearranged topological isomers of pH1#21, because Nhe1 digestion yielded only a single 6.2 kb band (Figure 2-8D; lane 2). In contrast, when introduced into sae2, reisolated DNA samples, though still exhibiting an input plasmid ladder, also evidently contained a rearranged species. Some sae2 transformants apparently lacked any pH1#21 whatsoever, exhibiting only rearranged DNA. The latter transformants were used to further investigate the nature of the reproducible rearrangement, and results indicated that they harbored a dimeric, pPX-like plasmid (compare Figure 2-8D lanes 3-6 to Figure 2-2C lanes 11-14). Briefly, the topmost band in uncut DNA co-migrated with a circular marker (not shown) twice the size of the original plasmid. Below this, a band migrated at the position of a dimer-sized linear. A unit-sized monomeric linear was also present. This smaller (1X) linear was a denaturation-dependent species (data not shown).
Figure 2-7

**Palindrome-bearing plasmids.** The NF1 PATRR palindromes are diagrammed as if extruded; vertical branches are continuous with the rest of the plasmid and one self-paired strand is shown. None of the three plasmids carries a perfect palindrome because a 3bp spacer is present and arms have mismatches as indicated by increased spacing between the non-complementary bps. Stretches of (AT)\textsubscript{n} sequence that are not incorporated into the main arm of a maximally self-paired structure are diagrammed as variably-located side branches. The three NF1 inserts also include a common 110bp of non-palindromic sequence flanking the palindromic sequences that are not given in the Figure. The sequence of the non-palindromic pSH1 plasmid insert, in its entirety is: GTCTAGTGCTTGCTCAGAGATGTCGAAAGGTGTAGTAGAGATGT. The insert in pH1#23 originated as a PCR stutter-generated variant of a palindrome from the genome of individual “H1”. The insert of pΔ95 arose as a possible replication by-pass product from pH1#21. The two plasmids, pH1#21 and pH4#4 are the sequence-validated clones of the NF1 palindrome from individuals H1 and H4 respectively. pΔ95 and pSH1 can be propagated without rearrangement in *E. coli*, the others are maintained in wild-type yeast.
Figure 2-8
Plasmids with a palindromic insert undergo axis-specific “Escape”. A. Diagram of palindrome-bearing plasmids in the study. The hybridization probe, restriction sites, and expected fragment sizes are labeled as in Figure 2-2. B. Escape product. The original plasmid is duplicated in head-head and tail-tail form, reflected about a point defined by symmetry center of the original palindrome. C. Linear species. The “1X” linear is the initial cruciform resolution product. Dimeric “2X” linear forms are produced by cruciform resolution of the escaped palindromic dimer circle. D. Southern blot of pH1#21 vs. an escaped sample. Lanes 1 and 2 are DNA from a wild-type (BY4741) pH1#21 transformant. NheI digestion gives a 6.2 kb band. Lanes 3 to 6 contain DNA from an example of a rearranged sae2 pH1#21 transformant. NheI digestion gives a 7.1 kb band and a 3.5 kb band. The 7.1 kb fragment is released by digestion from the 12.4kb palindromic dimer circle and also from any P-type 2X linears. The 3.5 kb fragment is released from X-type linears and from the 1X linear which in this experiment is a snap-back artifact and from the product formed by resolution of the NF1 palindrome (see Figure 2-3). The supercoiled dimer form that might have been expected to migrate faster than the 12.4kb linear was undetected due to nicking during DNA isolation.
Together, the rearranged samples closely approximated a pPX banding pattern (Figure 2-8D; lanes 3, 5 and Figure 2-2C; lanes 11, 13, respectively). Further, NheI cleavage of the fully rearranged DNA samples gave two bands, one at ~7.1 kb, and a second at ~3.5 kb, again consistent with a conversion of pH1#21 to a completely palindromic pPX-like form (compare 2-8D; lane 4 to Figure 2-2C; lane 12). Both the unit and dimer-sized linear species were ExoV resistant, just as when pPX is isolated from sae2 cells (Figure 2-8D; lane 5 and Figure 2-2C; lane 13).

For convenience, the reproducible rearrangement of the PATRR-bearing plasmid pH1#21, observed upon transformation of sae2 as well as mre11 mutants (below), is here termed "escape". Escape is meant to denote a specific type of gross rearrangement that achieves both a copy number increase as well as an expansion of palindromy. As diagrammed in Figure 2-9, escape can be explained by a multi-step process that requires the presence of an extrusion-prone sequence and involves cruciform extrusion, resolution, and replication in turn. Escape is inherently amplifying, and demonstrates a facet of palindromic DNA metabolism that, apart from double strand break formation itself, would be expected to threaten genome integrity. Given the significant pathogenic implications of both cruciform resolution and escape, I undertook further experiments to establish key steps in each.

### 2.3.4 Lack of detectable escape in wild-type S. cerevisiae

Implicit in the scheme in Figure 2-9 is that covalently closed hairpin linears created by cruciform extrusion persist long enough to be replicated. To test genetic requirements for escape, multiple transformants were analyzed after introducing pH1#21 DNA into wild-type and isogenic mre11Δ cells in parallel. No escape was detected in ten
NF1 plasmid cruciform resolution and Escape. Extrusion, resolution, and replication can account for all the species as described in Figure 2-8B-D. “Cruciform resolution” is the cleavage of an extruded cruciform. “Escape” is cruciform resolution combined with replication. In theory, escape is not self-limiting, and if unrestrained could be imagined to initiate rampant amplification.
of ten wild-type transformants. In contrast, three of the six recovered \textit{mre11}\Delta transformants contained only escape products and the other three bore escape species mixed with unrearranged pH1#21 (Figure 2-10). Qualitatively consistent results were obtained with the additional palindrome-bearing plasmids, pH1#23 and pH4#4 (Figure 2-11). Over all, escape was detected only in \textit{mre11}\Delta or \textit{sae2} strains, \textit{never} in wild-type transformations. It would appear therefore, that escape is actively contravened by the action of Sae2 and Mre11. One possibility is that escape is a consequence when a linear cruciform resolution product is not exposed to hairpin-endonuclease(s).

2.3.5 A hairpin linear DNA replicates to become a palindromic dimer

The major rearrangement in escape is achieved through replication of a hairpin linear cruciform resolution product. A covalently closed hairpin linear, hpPX, was prepared in order to demonstrate its replication potential. As for pPX, it was necessary to produce the required amounts of hpPX DNA \textit{in vitro} (see Experimental Procedures). In general, any hairpin-terminated linear DNA has a close intrinsic relationship to a palindromic dimer circle, because the former is, in effect, a single-stranded and self-annealed version of the latter. Here, hpPX is the single-stranded form of pPX (Figure 2-12A, B) so that, if it is indeed replicated \textit{in vivo}, transformants will be seen to contain pPX (compare Figure 2-12D; lanes 1-4 to Figure 2-2C; lanes 11-14). In fact, \textit{mre11}\Delta cells transformed with the hairpin linear hpPX uniformly exhibited the pPX pattern (Figure 2-13A). (The only difference was the presence of a supercoiled plasmid signal, which is variable due to nicking during DNA preparation).

Wild-type cells transformed with hpPX gave the same result as \textit{mre11}\Delta cells (not shown). In the wild-type strain however, the linear was instead ExoV-sensitive
**Figure 2-10**

**pH1#21, carrying a cloned allele of the polymorphic NF1 palindrome, escapes in mre11Δ cells.** Southern blots show DNA samples from each of 16 Ura+ colonies isolated after transformation with pH1#21. The transforming pH1#21 DNA was itself a minipreparation from BY4741. All Ura+ colonies were picked and are shown in the figure. The bands that denote escape are indicated with red lines. In discriminating between samples with and without Escape the most useful diagnostic is the 7.1 kb NheI digestion product (position labeled in red and underlined). Thus, although the 3.5 kb fragment is released by NheI digestion from escape dimers, we have seen that in some strain backgrounds, as here, a similar sized palindrome-specific signal is present (without being accompanied by a 7.1kb signal). A 12.4kb linear and circular species in uncut DNA indicate Escape but do not represent a stringent test due to the possibility that these could be generated if non-palindromic dimers are present in a particular sample. Dimer bands can be shown to include palindromic Escape species by further tests such as visualization of the 7.1 kb NheI digestion product.

Samples were escaped if the 7.1 NheI digestion product could be detected. Supporting evidence in the form of 3.5 and 12.4kb bands was often present. Samples were scored as input (unrearranged) if no 7.1kb band was seen. Confirming the designation was the absence of prominent dimer bands. Samples with bands corresponding to both input and rearranged plasmid were scored as mixed. The difference between wild-type and mutant transformants with respect to Escape is significant (two tailed P value is 0.036 if samples 13,15, and 16 are scored as input rather than mixed, and P value is 0.0001 if these samples are scored as mixed rather than input; Fisher’s exact test).

**Escape species only:** samples 11,12,14.
**Mixed escape and input:** samples 13, 15,16.
**No escape (input only):** samples 1-10
**Figure 2-11**

**Escape of pH1#23 and pH4#4 is observed in mre11Δ cells.** Plasmid DNA for transformations was prepared from wild-type BY4741. All Ura+ colonies were picked. **A.** pH1#23-transformed wild-type and mre11Δ strains are as indicated. Samples are numbered and run uncut as well as after NheI digestion. **B.** pH4#4, in wild-type and mre11Δ strains. Gaps in the images indicate panels where lanes from two gels are grouped. **C.** Escape, scored according to the 7.1 NheI digestion product (see legend to Figure 2-10). Palindromes in the plasmids in A and B are less perfect than pH1#21. Sequences are given in text Figure 2-7. pH4#4 contains a sequence-verified human variant. pH1#23 is an in vitro variation that arose during a cloning attempt.
A hairpin-ended linear is replicated in vivo to give a palindromic dimer circle. A diagram of in vitro prepared hpPX. B. Double stranded form of hpPX (identical to pPX, Figure 2-A). C. Map of hairpin-capped 2X linear molecules. D. Southern blot of DNA isolated from a representative mre11Δ hpPX transformant. Bands in an hpPX transformant fully reproduce those observed with pPX. The dimer-sized linear (black triangle) is ExoV resistant. A supernumerary band due to probe contamination with 2-micron sequences is marked by the bullet. Diagrams are labeled as in Figure 2-2. E. Replication of a transformed hairpin linear followed by resolution of the resulting palindromic dimer reproduces Escape.
Figure 2-13
Transformation activity of hairpin linear and palindromic dimer circle in wild-type and mre11 mutants. The mutant strains, along with the corresponding wild-type strains were transformed in parallel with 25ng of pSH1 (control circle), pPX (palindromic dimer circle), and hpPX (hairpin-ended linear). Transformation details are given in Experimental Procedures. Bars show transformation activity relative to pSH1. A. The wild-type strain was BY4741 and the colony count with pSH1 was 1085. The pSH1 colony count in corresponding mre11Δ strain was 1040. B. The wild-type strain was W303-1A and gave a pSH1 colony count of 370. The mre11-H125N strain gave 217 colonies with pSH1. C. Results shown in A and B are tabulated for comparison. Black; hpPX, grey; pPX.
(not shown). No palindromic dimer circles were generated when either wild-type or mutant cells were transformed with an “open” linear fragment (Figure 2-13B). Thus all of the proposed steps in escape subsequent to cruciform resolution were reconstructed by transformation of cells with a covalently closed hairpin linear (Figure 2-12E).

Transformation requires uptake, establishment and maintenance of introduced DNA. The transforming ability of a particular DNA in wild-type vs. mutant strains can give a broad overview of differential metabolism. To this end, the control plasmid pSH1 (from which the hairpin linear and palindromic dimer forms were made) hpPX and pPX were transformed into two mre11 mutants as well as into the corresponding wild-type strains in parallel. The lowest transformation activity was with hpPX DNA, but only when it was introduced into wild-type cells. Notably, hpPX exhibited the highest transforming activity when introduced into mre11 strains (Figure 2-13). Thus even though escape is mimicked in wild-type cells if a pre-formed hairpin linear is exogenously supplied (suggesting that Mre11 or Sae2 may block cruciform resolution), it would also appear that escape can be suppressed in MRE11 and SAE2 strains subsequent to the cruciform resolution step.

2.3.6 Cruciform extrusion of the NF1 palindrome in vivo

The data presented thus far do not establish that the PATRR insert in pH1#21 was able to extrude to a cruciform structure in vivo. Plasmids bearing palindromes can spontaneously acquire cruciform structures if the energy associated with supercoiling becomes available to drive extrusion, which can occur when DNA is isolated from cells and stripped of associated proteins (DeLange et al., 1984; Kurahashi et al., 2004; Mizuuchi et al., 1982b; Sinden, 1994). In one instance an E.coli-
Figure 2-14
A. Transformation with a hairpin linear regenerates an escape pattern. Left panel. Uncut DNA from 15 wild-type transformants. Right panel. Uncut DNA from 16 \textit{mre11\Delta} transformants. All but two show an escape pattern. Transformant #5 contains a monomer circle that is unavoidably present as a low level contaminant in hpPX DNA preparations and transformant #10 is a mix of a deleted and non-deleted escape form that was not further analyzed. B. Transformation with an open linear does not regenerate an escape pattern. Open linear (XbaI-digested pSH1) DNA was transformed into yeast cells. Left panel: uncut DNA from 9 wild-type transformants. Right panel: uncut DNA from 9 \textit{mre11\Delta} transformants. All but one of the transformants contain a monomer circle. The exception, transformant #2 in the right hand panel, was a direct dimer, as confirmed by Nhel digestion (not shown).
propagated plasmid bearing a PATRR insert was demonstrated to extrude in vitro, and it, like pH1#21, exhibited a ladder pattern on one-dimensional agarose gels (Kurahashi et al., 2004). It appeared likely therefore that the pH1#21 DNA used in our transformation experiments already contained pre-existing cruciforms.

The possibility of prior acquisition of a cruciform structure in vitro can be eliminated by conveying DNA from a wild-type cell to a mutant recipient cell using an abortive mating procedure called “plasmoduction” (Georgieva and Rothstein, 2002). A kar (karyogamy) mutation in one haploid parent prevents nuclear fusion, but because heterokaryons still form, small DNA molecules such as plasmids are efficiently transduced between nuclei (diagrammed in Figure 2-15). The desired mononuclear plasmoductant can be selected with the use of appropriately marked donor and recipient strains (Georgieva and Rothstein, 2002).

Donor strains were derived first by transformation of either pH1#21 or non-palindromic control plasmid pSH1 into the kar1Δ15 strain W2108-14C (R. Rothstein Columbia University). Upon confirmation by Southern Blot that the plasmids were monomeric in these transformants (wild-type for MRE11 and SAE2), matings of plasmid-bearing donor strains (e.g. MATa CYH2 CAN1 kar1Δ15 ura3-1 p{H1#21 URA3}) to either wild-type or mutant recipients (e.g. MATa cyh2 can1 ura3-1 mre11-H125N) were performed. Thereafter, Ura+ ChxR CanR plasmoductants (e.g. MATa cyh2 can1 ura3-1 mre11-H125N p{H1#21 URA3}) were isolated. One informative modification in these experiments was the use of a ‘nuclease-dead’ allele of MRE11 (mre11-H125N; (Lewis et al., 2004; Moreau et al., 1999; Zhang and Paull, 2005)). For analysis, DNA was
Figure 2-15

**Plasmduction.** Plasmid DNA from one nucleus is efficiently incorporated into the plasmid-minus nucleus in a heterokaryon. Plasmductants can be isolated by the indicated selection.
isolated from plasmoductants without denaturation (see Experimental Procedures) in order to eliminate generation of any artifactual species.

Eight or more independent plasmoductants from each mating were examined by Southern blot. Whereas before individual \textit{mre}11 or \textit{sae}2 pH1#21 transformants had exhibited a range of full, partial, or barely detected escape (Figure 2-10; right hand panel), plasmoductants were all the same as one another. The single isolates shown in each of the panels of Figure 2-16 are representative of identical independent plasmoductants. Virtually identical results were obtained with a \textit{sae}2 null mutant (data not shown).

A ladder pattern was seen in uncut DNA from plasmoductants receiving palindrome-bearing plasmids regardless of genotype (a tighter supercoiled band was seen for pSH1; Figure 2-16; first and third lanes). No indication of either site-specific cleavage or escape was observed in wild-type plasmoductants, as confirmed by the single band generated by NheI digestion (Figure 2-16; second lanes). In contrast, uncut pH#21, pH1#23, or pH4#4 DNA isolated from \textit{mre}11-H125N plasmoductants contained additional species (Figure 2-16; third lanes). Here, NheI digestion confirmed the presence of additional 3.5 kb and 7.1 kb bands diagnostic of cruciform resolution and escape (Figure 2-16; fourth lanes).

The fact that escape can be observed with the plasmoduction procedure indicates that the palindromic insert must have extruded at a point in time after the DNA had been stably propagated in the wild-type donor, ruling out prior acquisition of a cruciform structure \textit{in vitro}.

\textbf{2.3.7 \textit{In vitro} extrusion is not a true indicator of \textit{in vivo} behavior}
Figure 2-16

Escape and cruciform resolution in vivo. DNA was isolated without denaturation. Escape does not require the formation of cruciforms acquired in vitro. Lanes 1 (uncut) and 2 (NheI cut) contain DNA from individual wild-type colonies plasmoducted with the indicated plasmid. Lanes 3 and 4 are similarly treated DNA from mre11-H125N colonies. Open arrowheads (\(\downarrow\)) indicate dimeric circular and dimeric linear bands in uncut DNA. Even-numbered lanes contain NheI-digested samples (lane header, “N”). The bands marked \(\downarrow 7.1\) and \(\downarrow 3.5\) are both released from escape forms by NheI (Figure 2-8). The label for the 7.1kb band is underlined to indicate that it is specifically diagnostic for escape, whereas the 3.5kb signal may be generated from the initial cruciform resolution product as well (Figure 2-8). Wild-type W303-1A cells do not exhibit the 7.1 kb or 3.5kb bands (lanes 2 versus lanes 4). Evidence of escape is also absent in DNA from either strain when plasmoducted with the control plasmid, pSH1 (right hand panel). DNA samples from the mre11-H125N cells contain escape forms after any of the three palindrome-bearing plasmids is introduced by karyogamy. The panels are from a single membrane.
I wanted to illustrate the importance of utilizing the yeast cruciform extrusion/resolution system by demonstrating that \textit{in vitro} cruciform extrusion is not a true indicator of \textit{in vivo} extrusion. The palindrome pΔ95 contains a near-perfect 98bp palindrome with one mismatch within the arms (Figure 2-7). pΔ95 arose spontaneously as a putative replication-associated deletion product from the larger palindrome H1 (Figure 2-7). The one indication that the palindrome extruded \textit{in vitro} was seen when uncut samples were analyzed by Southern blot. As observed for sample pH1, pΔ95 ran in an atypical ladder pattern in the absence of ethidium bromide (for a clear example, see Figure 2-17C; lane 5). In wild-type cells, this ladder collapsed to a single band when cut with NheI, indicating that these were isomeric species (Figure 2-17C; lane 6). Such a ladder is not normally observed for non-palindromic plasmids, as was the case for the related plasmid pSH1 (Figure 2-17C; lane 9). Others have verified that a distinctive ladder pattern is the result of cruciform structures (for examples, see (Inagaki et al., 2005; Kurahashi et al., 2004)). The results with pH1 and pΔ95 concur with published examples of cruciform electrophoretic mobility properties in one dimension.

I next tested for Δ95’s sensitivity to the four-way junction-cleaving enzyme T7 Endonuclease I \textit{in vitro}. DNA isolated from wild-type yeast transformants was incubated with the enzyme and was subsequently digested to completion with NheI. A band of 3.5kb indicated that T7 Endo I cleaved within the palindromic sequence (Figure 2-17A). Results from Southern blots are shown in Figure 2-17B. When pH1 and pΔ95 were treated with T7 Endo I then digested with NheI, a distinct 3.5kb band was observed (lanes 3 and 7). When the non-palindromic control plasmid pSH1 was treated with T7 Endo I followed by NheI, no 3.5kb signal was produced (Figure 2-17B; lane 11). These
Figure 2-17
Assessment of cruciform extrusion using in vitro and in vivo methodologies. A. Map of plasmids used in the assay. After T7 Endonuclease I treatment, digestion with NheI will release a 3.5kb band if T7 cleaves an extruded cruciform. Probe is indicated as a black bar. B. Southern blot of pH1#21, pΔ95, and pSH1 plasmid DNA that were treated with T7 Endonuclease I then digested with NheI. Release of a 3.5kb band in T7 and NheI-treated samples is an indicator of in vitro cruciform extrusion. Both pH1#21 and pΔ95 extrude cruciforms in vitro. C. Southern blot of DNA isolated from plasmocclusions of pH1#21, pΔ95, and pSH1 into wild-type and mre11-H125N strains. The appearance of 7.1kb and 3.5kb bands in NheI-cut samples from mre11-H125N cells is an indicator of in vivo extrusion. Only pH1#21 extrudes a cruciform in vivo. D. Southern blot of DNA isolated from wild-type and mre11Δ cells that were directly transformed with pΔ95. As in C, 7.1kb and 3.5kb bands in NheI-cut samples indicate escape and therefore, cruciform extrusion. From these criteria, pΔ95 does not escape when introduced directly into cells. T7; T7 Endonuclease I, N; NheI, C; circular plasmid. All sizes are in kilobases.
results demonstrate that both the H1 and Δ95 palindromes form cruciform structures *in vitro*. 

Next, the Δ95 palindrome's *in vivo* extrusion potential was assessed by introducing the plasmid in *mre11-H125N* cells. pΔ95 was plasmoducted from wild-type to *mre11-H125N* mutant strain. Representative Southern blots of DNA isolates are shown in Figure 2-17C. While a robust escape signal was detected for pH1 (lanes 1 and 2), there was no evidence for escape in *mre11-H125N* plasmoductants bearing the pΔ95 plasmid (lanes 7 and 8). No dimer circles were present in uncut DNA nor were 7.1kb or 3.5kb bands observed in NheI digests. I did not observe any species indicative of escape when the plasmid was introduced into *mre11-H125N* cells by direct transformation (Figure 2-17D). The plasmoduction experiment demonstrates that when present in a plasmid, the Δ95 palindrome does not detectably extrude *in vivo*. Although plasmoduction is the most stringent method for formally demonstrating *in vivo* cruciform extrusion, I have found that direct transformation of cells reflects results obtained from plasmoduction.

### 2.3.8 Detection of the unit linear produced by cruciform cleavage

As diagrammed in Figure 2-9, the first step toward escape is taken when the palindromic insert is cut by cruciform resolution. There was no *a priori* expectation that this initial cleavage event should be detectable however I noted a band migrating at the position of a unit-sized linear in *mre11-H125N* plasmoductants that was less pronounced in wild-type cells and, additionally, was not seen for the pSH1 control (Figure 2-16; third lanes). To examine the structure of the linear, the topoisomer ladder was first collapsed to a single band by incubation with the site-specific nicking enzyme Nb.BsmI. This
treatment relaxed the obscuring circular forms to a single band that was well resolved on gels from the 6.2 kb linear signal (Figure 2-18; second and 5th lanes). The 1X linear cruciform resolution product could then be demonstrated according to its ExoV-resistance as observed in mre11-H125N but not wild-type plasmductants (Figure 2-18; black diamond, left three panels, 6th versus 3rd lanes). No ExoV-resistant linear band appeared in pSH1 plasmductants of either strain (Figure 2-18; rightmost panel, lanes 3 and 6). In addition, dimer-sized escape linears and circles (Figure 2-18; open arrowhead) were detectable in mre11 but not wild-type cells (Figure 2-18; compare the 6th to the 3rd lanes in the leftmost three panels).

The visualization of a 1X hairpin linear provided direct evidence of cruciform resolution even when palindromy is confined to an insert within an otherwise non-palindromic plasmid. I conclude that the levels of torsional strain that are achieved in biologically active eukaryotic chromatin are sufficient to support the extrusion of natural human genome-derived palindromes. Ongoing cruciform extrusion and resolution occurs in a permissive strain at such a high spontaneous frequency that it can be observed in mitotic cells in the absence of any specific selection.

The uniformity of the plasmduction results enabled me to make a semi-quantitative assessment of cruciform resolution based upon how much monomer plasmid signal is present in the form of an ExoV-resistant linear (Figure 2-18; black diamond, lanes 3 and 6). The three tested inserts had the identical 75bp sequence at the palindrome center, but then had differences further away from the center as indicated in Figure 2-7. Cruciform resolution, and escape as well, was measurably less pronounced for pH4#4 than for the other two plasmids. This is consistent with the suggestion that that arm-to-
Figure 2-18
Cruciform resolution occurs for plasmids transferred to mutant cells in vivo. Aliquots of the same DNA samples as in Figure 2-17 were treated with Nb.BsmI ("Nick"), and Micrococcus luteus Exonuclease V ("ExoV") as indicated. Cruciform resolution is confirmed by detection of the monomeric ExoV-resistant linear in \textit{mre11-H125N} (L\textbullet{}), as seen in the 6\textsuperscript{th} lane of each set. No product is observed in parallel plasmiductions into wild-type cells (3\textsuperscript{rd} lanes). For comparison, the signals representing monomeric circles (C) and those at the location of the ExoV-resistant linears were quantified for lanes 3 and 6 and the \% resolution \[\% \text{ resolved} = \frac{\text{C} + \text{L}}{\text{C} + \text{L}} \times 100\] was calculated. Open arrows indicating dimeric circles and linears provide further confirmation of escape and cruciform resolution. Dimeric forms are not included in the quantification. Two membranes are shown, separated by a gap in the image; these were processed together and exposed identically.
arm identity within a palindrome influences the probability of extrusion (Nag and Kurst, 1997) and/or the persistence of the structure.

2.3.9 Cruciform extrusion of variants of the human NF1 palindrome

The human NF1 palindrome is highly polymorphic. In the process of determining an approach for the stable cloning and propagation of palindromic sequences, our laboratory successfully cloned numerous human NF1 palindrome alleles bearing varying degrees of length and symmetry (Figure 2-19). Here, I wanted to determine extrusion potential of additional human NF1 sequences. I transformed the plasmoduction karl donor strain with plasmids pH2, pH3, and pH5 along with pH1 and pH4 and the non-palindromic control pSH1. Plasmid integrity in the donor was assessed by Southern blot (not shown). Plasmoductions into wild-type and mre11-H125N strains were performed and DNA was isolated from 4 independent plasmoductants for Southern blot. Southern blots of plasmoductant DNA are shown in Figure 2-20. As expected, pH1 gave the highest ratio of escape molecules when compared to unescaped plasmid. In uncut samples, palindromic dimer circle and dimer linear species were observed and when digested with NheI, distinct 7.1kb and 3.5kb bands, predicted NheI escape bands, were present (Figure 2-20; lanes 3 and 4). The second most palindromic allele, pH4, gave a similarly high escape signal (lanes 7 and 8). I observed faint but detectable 3.5kb bands for variants pH3 and pH5, however, the 7.1kb signal is not detectable (lanes 12 and 16). I observed no escape for the least palindromic allele, pH2, where no 7.1kb or 3.5kb signals were detected in NheI digested DNA (lane 20).

2.3.10 Quantification of NF1 variant cruciform resolution
Figure 2-19

Palindrome-bearing plasmids. The NF1 PATRR palindromes are diagrammed as if extruded and maximally self-paired; vertical branches are continuous with the rest of the plasmid and one self-paired strand is shown. The NF1 inserts also include a common 110bp of non-palindromic sequence flanking the palindromic sequences indicated by the lined and dotted boxes. All plasmids here are sequence-validated clones of the NF1 palindrome from individuals H1 to H5. pH1 and pH4 are identical to pH1#21 and pH4#4 diagrammed in Figure 2-7. Structures are diagrammed as a visual aid and do not necessarily reflect in vivo conformations.
Figure 2-20
Escape of human NF1 palindrome variants in vivo. Southern blots of DNA isolated from wild-type and mre11-H125N plasm dutants isolated without denaturation. The appearance of 7.1kb and 3.5kb species in NheI-cut DNA indicate escape and cruciform resolution (for maps see Figure 2-8). All sizes are in kilobases.
Although escape is a versatile tool for assessing cruciform extrusion, it is an indirect consequence of cruciform resolution. To visualize and quantify resolution products directly, wild-type and \textit{mre11-H125N} plasmoductant DNA from each of the 5 NF1 variants and control pSH1 was first treated with the site-specific nicking enzyme Nb.BsmI, resulting in the collapse of the topoisomer ladder and clear visualization of the linear band. Samples were then treated with \textit{Micrococal luteus} Exonuclease V. Cruciform resolution products are the fraction of exonuclease-resistant hairpin-capped linear species. Samples were analyzed by Southern blot (Figure 2-21B). For variants H1 and H4, distinct hairpin linears were present in \textit{mre11-H125N} samples (upper panel, lanes 6 and 12). pH3 and pH5 contained very faint hairpin linear species (upper panel, lane 18 and lower panel, lane 6) while the least palindromic variant, H2, did not contain a distinct hairpin linear species (lower panel, lane 12). ExoV-resistant linears were not observed in wild-type samples nor were they detected in the non-palindromic pSH1 control. The observed resolution species were quantified by measuring the fraction of ExoV-resistant linears (♦) over total monomer DNA (♦ + ☐) and are shown in Figure 2-21C. Taken together, the results demonstrate that variants of the human NF1 palindrome occupy a significant range of resolution propensities.

\textbf{2.3.11 An AT-rich segment of the FRA16D fragile site does not extrude in \textit{vivo}}

As diagrammed in Figures 2-7 and 2-19, the NF1 palindromic variants are all highly AT-rich. Segments of AT-rich DNA, which can promote bending in the double helix, are associated with instability (for an example, see (Hou and Wei, 1998)). I was
Figure 2-21
Quantification of in vivo cruciform resolution for variants of the NF1 palindrome.  

A. Diagram of plasmid and cruciform resolution species.  

B. Aliquots of the same plasmiductant DNA samples as in Figure 2-20 were treated with Nb.BsmI ("Nick"), and Micrococcus luteus Exonuclease V ("ExoV") as indicated. Cruciform resolution is confirmed by detection of the monomeric ExoV-resistant linear in mre11-H125N (◆). The signals representing monomeric circles (Ø) and those at the location of the ExoV-resistant linears were quantified for lane 6 and the proportion resolution [◆÷(Ø÷◆)] was calculated. The graph shows the mean proportion resolution for each NF1 variant and pSH1. Error bars represent the standard error for three individual experiments performed on different days. Sizes are in kilobases.
interested in determining if the propensity of extrusion in the NF1 palindrome was solely caused by its AT-richness and specifically, by \((AT)_n\). Therefore, I chose to determine the propensity of cruciform extrusion for a second unstable human sequence. A subregion of the FRA16B fragile site, called "Flex1" has been shown to cause replication fork stalling and is associated with double strand break formation in a yeast system (Zhang and Freudenreich, 2007). Flex1 contains an AT tract that varies in size between individuals. Zhang and Freudenreich successfully cloned numerous human Flex1 sequences, the one with the longest AT tract containing \(AT_{(34)}\) repeats that caused the most dramatic replication fork stalling (Zhang and Freudenreich, 2007). The authors postulated that secondary structure formation, possibly a cruciform, in the \(AT_{(34)}\) was causing fork stalling.

Here, I wanted to determine if the \(AT_{(34)}\) repeat forms a cruciform structure \textit{in vivo}. The Flex1 sequences were tested, each with varying numbers of AT repeats, and are diagrammed in Figure 2-22. Three Flex1 variants were tested: Flex1-AT(17), Flex1-AT(34), and a third containing two tandem Flex1 sequences in with AT(17) and AT(36). Each variant was cloned into pYES2.1 and plasmoducted into wild-type and \textit{mre11-H125N} cells. Southern blots of representative DNA samples are shown in Figure 2-23B. When cut with NcoI, a single band of 6.4kb was observed in both wild-type and \textit{mre11-H125N} plasmoductants for all three Flex1 plasmids tested. The absence of 8.8kb and 4.4kb bands associated with cruciform extrusion and escape (see Figure 2-23A) demonstrates that the Flex1 subregion of FRA16B does not extrude in this system \textit{in vivo}. 
Figure 2.22
Structure of the Flex1 subregions from FRA16B.  A. Diagram of Flex1 plasmids.  B. Structures of Flex1 inserts.  Solid grey boxes depict AT tracts. N; NcoI, P; PvuII, X; XbaI.
**Figure 2-23**

**No detectable escape for Flex1 sequences.**  
A. Maps of Flex1 plasmid and expected cruciform resolution and escape products.  
B. Southern blot of Flex1 plasmid and expected cruciform resolution and escape products. Lanes 1 and 2 are DNA from wild-type transformants. NcoI digestion gives a 6.4 kb band. Lanes 3 and 4 contain DNA from mre11-H125N plasmiductants. NcoI digestion gives a sole 6.4kb band. If Flex1 cruciform extrusion occurred and resulted in plasmid escape, NcoI digestion would result in the visualization of 4.4kb and 8.8kb bands. Supercoiled species in the two leftmost panels are absent due to degradation. P; PvuII, X; XbaI, N; NcoI. All sizes are in kilobases.
2.4 Discussion

Cytologically-detected genome rearrangements are a prominent feature in human genetic disease as well as in acquired malignancies, but in only a few examples have the molecular origins of rearrangement been pinpointed. It remains a challenge to identify the early abnormalities in DNA metabolism that trigger low frequency, highly selected events. Apart from aberrant rearrangements in the immune system, where errors in V(D)J recombination will at times leave a clear molecular imprint (for an example, see (Marculescu et al., 2006)), the root cause of any given genome rearrangement is rarely apparent. The investigation of certain constitutional translocations in humans has recently highlighted a connection between specific chromosomal aberrations and the presence of long palindromes (Kurahashi et al., 2006b). The reciprocity of these exchanges at the DNA sequence level strongly indicated that double strand DNA breaks were involved. I provide evidence for “cruciform resolution” in a model eukaryote, defining a new endogenous source of DNA damage in living cells.

2.4.1 In vivo cruciform resolution in eukaryotes

A plasmid-based system provides an informative approach by which to experimentally isolate and investigate cruciform resolution. As illustrated in Figure 2-1, determination of whether or not two hairpin termini arise upon cleavage of a palindromic sequence can be used to differentiate cruciform resolution from other modes of DNA break formation. Because cleavage of a circle captures both of the created ends on a single DNA molecule, the plasmid-based assessment makes it possible to ask a) whether one-or two-ended breaks arise (e.g. see (Eykelenboom et al., 2008)) and, b) whether or not both termini at the break site are hairpin-capped. I find that palindrome-bearing
plasmids are susceptible to site-specific cleavage, where, in *sae2* and *mre11* mutant strains the cleaved linear molecule bears hairpins at both ends. Hairpin structure was inferred not only according to resistance of the linearized molecule to *Micrococcus luteus* Exonuclease V but also by the spontaneous appearance of a fully palindromic dimer circle. Moreover, the outcome of this major rearrangement, called “escape” was a molecule that was itself subject to a subsequent round of site-specific cleavage. Further supporting evidence, confirming the basis for escape was the finding that a hairpin-capped linear, when transformed, was uniformly converted to a palindromic dimer circle. All effects, the initial break by cruciform resolution and subsequent escape occurred strictly *in vivo*. Because palindromes showing break formation and rearrangement (cruciform resolution and escape) were sequences that occur naturally in the human genome, the potential significance of these observations with respect to risk factors in human disease is not difficult to imagine.

2.4.2 *In vitro vs. in vivo* cruciform extrusion

In this system, the palindrome is present on a 2-micron circle. Measuring extrusion in such a context closely resembles a chromosomal environment, as 2-micron circles are entirely chromatinized and are replicated once per cell cycle. One potential caveat of this system is that by measuring extrusion of a sequence outside of its normal chromosomal environment, it is possible that a true prediction of its normal extrusion potential is not possible as other local or distant chromosomal elements/processes that influence superhelical tension (such as transcription or nucleosome placement) may be present. This could be overcome by cloning palindromes that include a large amount of flanking sequence in the hope of capturing some of these elements.
To date, the majority of tools for quantifying cruciform extrusion do so when the DNA is *in vitro*. These often require that a sequence of interest is cloned in a plasmid and is maintained in *E. coli*. This procedure poses two challenges to the accurate prediction of cruciform extrusion. Firstly, palindromes longer than ~200bp are not stably maintained in *E. coli*. Long palindromes acquire large central deletions or cause inviability of their host. Second, these measure cruciform extrusion of naked DNA present in a hypernegatively supercoiled molecule. Measurement of cruciform extrusion in a plasmid with high superhelical density could potentially result in the positive scoring of extrusion that would otherwise not occur *in vivo*.

Such a discrepancy between *in vitro* and *in vivo* behavior is verified here. Although *in vitro* analysis was performed on DNA isolated from yeast, the same principles as above apply. The stripping of nucleosomes from DNA during the isolation procedure removes the constraints on supercoiling, resulting in a negative net superhelical density. For the Δ95 palindrome, isolating plasmid DNA from yeast cells resulted in *in vitro* cruciform extrusion, as demonstrated by T7 Endo I cleavage and by the ladder in uncut DNA (Figure 2-17). However, Δ95 did not detectably extrude *in vivo*; no escape species were observed. Interestingly, I found that Δ95 was more susceptible to T7 Endo I cleavage than H1 (Figure 2-17). This was surprising because H1 was highly prone to cruciform extrusion *in vivo*, as determined by escape and quantification of resolution species. T7 Endo I does possess modest sequence specificity, where it prefers to cleave adjacent to pyrimidines (Dickie et al., 1987; Picksley et al., 1990). Sequence analysis of Δ95 showed that it contains no central spacer, while H1 is centrally-interrupted by a 3 base pair spacer (see Figure 2-19). These discrepancies in spacer
length may have measurable effects on *in vitro* extrusion, while other factors, including arm length or base composition, may have more significant effects on *in vivo* extrusion. This remains an interesting series of questions for future analysis. In any case, my results illustrate the non-conformity of *in vitro* and *in vivo* extrusion potential.

### 2.4.3 Heterogeneous cruciform extrusion in the human genome

The validity of the cruciform assay was assessed by performing semi-quantitative measurements of cruciform extrusion for various alleles of the human NF1 palindrome. Using this assay, I found that individuals carry NF1 palindrome alleles that differ significantly in their *in vivo* extrusion capabilities. Certain alleles were highly extrusion prone, others were minimally susceptible and one tested allele appeared to be inert for extrusion (Figure 2-21). Potential clinical relevance may apply to the association of the NF1 palindrome with the t(17;22) translocation. First, my work provides invaluable evidence of cruciform-mediated double strand break formation. Thus palindromes emerge as an important source of endogenous DNA damage and I define a mechanism for a subset of human translocations. Second, in the context of cruciform extrusion, I have shown that individuals are not created equal. There exists a broad range of risk associated with the acquisition of extrusion-mediated double strand breaks and genome rearrangements, with some alleles having little inherent ability to adopt a secondary structure and others showing a high risk of extrusion and breakage. Kurahashi and colleagues have evidence supporting this for PATRR-11, where they found that the degree of symmetry of the near-palindrome correlates with translocation frequency in sperm (Kato et al., 2006). My results confirm and extend their findings by providing a mechanistic rationale underlying their observations. Palindrome-provoked instability is a
conditional type of DNA damage. When in lineform, palindromes inflict no damage however, when extruded, a cruciform structure may inflict havoc to the genome. By molecular cloning of human palindromes in yeast and quantifying cruciform extrusion, one's risk of acquiring palindrome-provoked genome rearrangements can be assessed. Interestingly, when the original NF1 palindrome sequence was extrapolated from der(17) and der(22) in each of the two documented examples of t(17;22) translocation, we found that each was relatively high in symmetry and most similar in sequence to H1 and H4, the most extrusion-prone palindromes tested here (Lewis et al., 2005). This suggests that extrusion capacity correlates positively with instability. Strengthening this proposal requires the discovery and analysis of more cases of t(17;22) and/or of additional polymorphic palindromes associated with genome rearrangements.

2.4.4 Genetic requirements for escape

The gross DNA rearrangement denoted escape (Figure 2-9) provides insight into another facet of the damage potential of cruciform resolution. Unless curtailed, escape could theoretically spawn ever-larger palindromes, supporting a variety of amplifying, pathogenic processes (Figure 2-24). Escape rearrangement is fully separable from Breakage-Fusion-Bridge modes of amplification because it is observed here without involving chromosomes, telomeres, or centromeres. Whereas in yeast, highly efficient homologous recombination, by reducing higher-order multiples to a palindromic dimer likely limits copy-number increase, other interventions may be needed to suppress explosive amplification in mammalian cells.

One step, perhaps more, in the resolution and subsequent escape of plasmid-borne palindromes is opposed by SAE2 and MRE11. The most subtle mutation of Mre11 tested
Lift-Out model of gene amplification. A. The Lift-Out model can potentially explain certain gene amplification events alone, or in conjunction with other events such as BFB cycles and/or recombination-based models. This model is based on the steps in the palindrome-provoked escape process. The basic observations presented here, being: 1) formation of a hairpin-capped linear DNA molecule from the cross-diagonal cleavage of a cruciform, and 2) formation of a palindromic dimer circle from the replication of a hairpin-capped linear DNA, can be applied here. Consider two palindromes embedded in a single linear chromosome. These two palindromes can be present in an HSR, having arisen as a consequence of BFB cycles or they can be endogenous ones normally present in non-cancer cells. Cruciform extrusion followed by cross-diagonal cleavage of both palindromes would result in the formation of a hairpin-capped linear DNA molecule. Provided that the linear DNA contains an origin of replication, a fully palindromic dimer circle would result from one round of replication of the hairpin-capped DNA. The result is the formation of a circular extrachromosomal element, doubling the copy number of DNA situated between the two palindromes. B. An extrachromosomal DNA arranged as a fully palindromic dimer circle contains two perfect symmetry axes. Extrusion at one of either symmetry axes, followed by cross-diagonal cleavage, would result in the formation of a dimer-length hairpin-capped linear molecule. Subsequent replication of this molecule would result in a tetramer circle with four symmetry axes. Further rounds of extrusion, cleavage and replication would result in an exponential increase in DNA copy number from 4X to 8X, 16X, 32X, etc. An HSR could be created if a large circular array is linearized and inserted in a broken chromosome.
here, *mre11-H125N*, eliminates the nuclease function only without affecting production of the protein, its incorporation into the Mre11-Rad50-Xrs2 repair complex or the complex’s ability to promote non-homologous end-joining (Lewis et al., 2004; Moreau et al., 1999; Zhang and Paull, 2005). The nuclease-dead allele confers a phenotype indistinguishable from that of *mre11-* and *sae2-* null cells in the present system. It has also recently been discovered that Sae2 is a nuclease that, in collaboration with Mre11, can open hairpin DNA structures *in vitro* (Lengsfeld et al., 2007). A straightforward conclusion therefore is that opening of hairpin structures prevents escape.

Whether hairpin cleavage blocks escape by limiting the survival of a hairpin-terminated resolution product (Lobachev et al., 2002), the initial extrusion of cruciforms (Cunningham et al., 2003) or both is a key upcoming question (see discussion in (Lobachev et al., 2007)). It is possible that the hairpin linear is directed away from the escape process when hairpin ends are opened, thus uncapping the ends so that double strand break repair enzymes can return the DNA to its original form (Lobachev et al., 2002). However, large (30 to 200-fold) differences with respect to hairpin linear transformation activity are seen between *mre11* and wild-type cells (Figure 2-14). This suggests that under some circumstances hairpin structures are targets for cleavage followed by degradation. Moreover, no evidence excludes the possibility that a cruciform’s hairpin structures are cut prior to, or in the absence of, cruciform resolution (Cunningham et al., 2003). These and other questions will become important to explore as we gain understanding of the relationship between cruciform resolution and pathogenic DNA damage in humans.

### 2.4.5 Palindrome metabolism and genome instability
The number of long palindromes in the human genome is not yet known because palindromes are notoriously difficult to clone and characterize (Lewis et al., 2005). It is clear in any case that palindromes are not rapidly purged from the human genome (Gotter et al., 2007; Inagaki et al., 2005; Lewis et al., 2005) and thus have the potential to affect the integrity of nuclear DNA. The NF1 palindrome examined here is an example of a persistent human palindrome that is present in some individuals but not others (Lewis et al., 2005) and one that is associated with a rare reciprocal translocation in humans (Kurahashi et al., 2003).

That the NF1 palindrome is subject to cruciform resolution in yeast is an observation with several important implications. For many scientists it remains an open question whether palindromes in eukaryotic chromatin are able to extrude at any significant frequency. Here, without any exceptional manipulations, breaks bearing distinctive hallmarks of cruciform resolution (Mizuuchi et al., 1982a) can be detected at high levels (6-18%) in vivo (Figures 2-18 and 2-21). This means that biologically active eukaryotic chromatin is under sufficient torsional strain, at least transiently, to drive extrusion. Plasmid-borne sequences in yeast have long been accepted as a model for understanding chromatin structure and function; no aspect of the present system obviously limits the generality of the observations. I conclude that palindromic sequences in the human genome possess potential for cruciform extrusion under physiological conditions.

These results provide an experimental basis in support of the concept of cruciform resolution; i.e. that palindromes can give rise to double strand breaks by extrusion into a cruciform structure, thereby presenting a four-way DNA junction to
endogenous HJ resolvases (Leach and Stahl, 1983; Lobachev et al., 2002). It has been suggested that DNA ends arising from cruciform resolution may be more conducive to aberrant events such as translocation than other types of broken DNA, because the terminal hairpin structure foils efficient repair (Lobachev et al., 2002). The importance of Mre11 and Sae2 in preventing cruciform resolution and escape in the yeast system invites the speculation that opening hairpin DNA ends in order to suppress palindrome-induced rearrangement is a highly important aspect of MRN’s role in human genome maintenance.
2.5 Experimental Procedures

2.5.1 Yeast strains

All strains were haploid and cultured either in YEPD or in synthetic complete media lacking uracil. The *sae2* mutant strain GRY2566 and the wild-type strain from which it was derived, GRY2565, are described elsewhere (Lewis et al., 2005). LSY716A, the *mre11-H125N* mutant and the corresponding wild-type strain, W303-1A are described in (Moreau et al., 1999). For plasmoductions, cycloheximide (CHX)-resistant derivatives of W303-1A and LSY716A strains were isolated by plating a 0.5mL overnight cultures on five 10cm dishes containing YEPD supplemented with 1µg/mL CHX and incubating at 30˚C for 3 days. CHX-resistant mutants were confirmed by restreaking on CHX. The *kar1* strain, W2108-14C (*MATα kar1Δ15 CAN1 CYH2 lys2Δ his3-11,15 ura3-1 trp1-1 leu2-3,112 ade2-1*) was the gift of Dr. Rodney Rothstein (Columbia University). The *mre11Δ* strain is from the yeast deletion collection derived from BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*).

2.5.2 Plasmids

All plasmids are derived from pYES2.1/V5-His-TOPO (Invitrogen). The vector possesses an *E. coli* pUC-derived origin of replication and the bacterial β-lactamase gene for selection in bacteria. It also includes the yeast *URA3* gene and 2-micron circle origin, however other 2-micron circle elements, the FLP recombinase gene, and “FRT” recognition sites are not present. Our Southern blot data confirms that the vector and derived constructs do not become fused to resident 2µ circles through site-specific recombination.
The control plasmid, pSH1, is a pYES2.1 clone with a non-palindromic 44bp insert. pH1#21 and pH4#4 contain human NF1 sequences as described previously (Lewis et al., 2005).

pYES2.1-Flex1-2X was constructed through insertion of the ~400bp EcoRV/XbaI fragment of pFlex1-2X (from Catherine Freudenreich) at the PvuII/XbaI site of pYES2.1. The pYES-Flex1-AT(17) construct, containing a single Flex1-AT(17) insert, was constructed by digesting pYES-Flex1-2X with PacI followed by religation, resulting in the release of the second Flex1-AT(36) insert. The EcoRI and blunt-ended fragment from pFlex1-AT(34) (from C.F.) containing Flex1-AT(34) was cloned into pSH1 at the PvuII site.

2.5.3 Hairpin linear (hpPX) and palindromic dimer (pPX) DNA preparations

Neither hpPX nor pPX can be propagated in *E. coli*, requiring that the necessary quantities of these DNAs be assembled and purified *in vitro*. pPX was prepared from the large PvuII to XbaI fragment of pSH1 (including only pYES2.1 vector sequence). DNA was purified on an agarose gel containing 0.5µg/ml ethidium bromide. The DNA was extracted with a Geneclean DNA isolation kit, which removed residual ethidium bromide. 50µg of DNA was incubated with 2000 units T4 DNA ligase (New England Biolabs) in a final volume of 500µl for 16 hours at room temperature. The dimeric circle was again gel purified.

hpPX was prepared from a sample of pPX that was digested to completion with BglII. This gives two linear palindromic molecules of 2.9 kb and 8.6 kb which were ethanol precipitated. The fragments were denatured by resuspending the pellet in 20µl of
1.5M NaCl, 0.5M NaOH and incubating for 5 minutes. Neutralization by the addition of
980µl 5X TE pH8.0 was followed by a 2 hour incubation at 68°C (Pearson and Sinden,
1996). Successful self-annealing to give 4.3 kb and 1.45 kb snap-back molecules was
confirmed on an analytical gel. The snap-back molecules were joined at their non-hairpin
termini with T4 DNA ligase. The desired 5.75 kb ligation product was purified away
from others on gels and isolated as above.

The hpPX and pPX final preparations were almost completely free of any
contaminating source plasmid. Residual levels of pSH1 could be quite simply monitored
by measuring the transforming activity of the preparations in E. coli DH10B.

2.5.4 Yeast plasmid DNA preparation

For the experiments shown in Figures 2-16, 2-17, 2-18, 2-20, 2-21, and 2-22,
DNA was isolated from 10 ml cultures of S. cerevisiae grown in synthetic complete
media lacking uracil using the method of Hoffman and Winston (Hoffman and Winston,
1987). Samples in all other Figures were further purified after ethanol precipitation with
a Qiaprep spin DNA miniprep kit (Qiagen) as per the manufacturer’s protocol. This
includes a denaturation step.

2.5.5 Yeast transformations

DNA was transformed into yeast using the EZ transformation kit (Zymogen).
When transforming with in vitro-constructed molecules, hpPX and pPX, approximately
25-50ng DNA was used. Palindrome-bearing plasmids in these studies were unstable in
E. coli, and so were never introduced into that organism. Therefore, in order to introduce
NF1 plasmids into yeast, they were transformed with a miniprep from a wild-type yeast
strain. Quantification of the plasmid in yeast miniprep DNA samples was not attempted,
instead volumes up to 5µl were used. Transformants were selected on synthetic complete medium lacking uracil.

2.5.6 Plasmuductions

Plasmuductions were performed as described in Georgieva and Rothstein (Georgieva and Rothstein, 2002). Donor W2108-14C strains were first created by transformation with a given test plasmid, confirmed, and then mated to each of the recipient strains. Mating mixtures were incubated on YEPD for 6 hours then replica plated on synthetic complete media lacking uracil and arginine and supplemented with 1µg/mL cycloheximide and 60µg/mL canavanine. Replicas were incubated at 30°C for 3-4 days, after which papillae representing independent plasmuductants were patched a second time on selective media before analysis. Plasmuductions of RusA plasmid recipient strains were performed as outlined above except that matings were replica plated on media also lacking leucine.

2.5.7 Nb.BsmI treatment

10µl aliquots of yeast miniprep DNA were incubated for 3 hours at 65°C in 1X reaction buffer and 10U Nb.BsmI in a final reaction volume of 20µl (New England Biolabs).

2.5.8 ExoV treatment

10µl aliquots of yeast miniprep DNA or the Nb.BsmI-treated samples were incubated for 30 min at 37°C in 66.7mM glycine-NaOH pH 9.4, 30mM MgCl₂, 8.3mM β-mercaptoethanol, 0.5 mM ATP and 1U Exonuclease V (US Biologicals) in a final reaction volume of 40µl. Reactions were added to sample buffer and immediately run on 0.8% agarose gels without ethidium bromide for Southern blot analysis.
2.5.9 Southern blot analysis

10-20µl uncut or NheI-digested yeast miniprep DNA samples were run on 0.8% agarose gels lacking ethidium and transferred onto Genescreen Plus nylon membrane using upward capillary transfer in alkaline transfer buffer. A hybridization protocol provided by Dr. Matthew Lorincz (Life Sciences Centre, University of British Columbia, personal communication) was followed with some adaptations. Membranes were probed overnight at 42°C in Stark's buffer containing 0.2mg/mL Torula RNA, 10µg/mL SDS, 5µg/mL powdered milk, and 25ng of a purified 1.25 kb ApaLI fragment from pSH1 that was $^{32}$P labeled to specific activity of $\sim 1-2 \times 10^{9}$ cpm/µg. The next day, membranes were first washed in 2X SSC 0.1%SDS at 42 °C for 15 minutes then in 0.2X SSC 0.1%SDS for another 15 minutes.
Chapter 3

Genetic analysis of factors that affect palindrome instability


I performed all experiments in this chapter except for the construction of NF1 plasmids, which was performed by Susanna M. Lewis.
3.1 Abstract

Cleavage of a cruciform by a Holliday junction resolvase has been proposed to be a mechanism of palindrome-provoked double strand breakage. Using the system described in Chapter 2, I demonstrate that cruciform resolution is largely dependent on the Mus81 endonuclease. Resolution can be restored by expression of the *bona fide* bacterial HJ resolvase, RusA. Having developed a specific method for identifying cruciform-instigated DSBs, I next wanted to apply the approach to define the genetic barriers that normally prevent break formation at palindromes. A candidate screen identified the RecQ helicase Sgs1 as being required for the prevention of palindrome-provoked double strand break formation. Through genetic analysis, I provide evidence that Sgs1 prevents multiple double strand break pathways, including cruciform resolution by Mus81.
3.2 INTRODUCTION

Using the plasmid-based system described in Chapter 2, I demonstrated that cruciform resolution occurs spontaneously in both a perfect *in vitro*-constructed palindrome and a 200bp near-palindrome from the human genome. As was demonstrated for *bona fide* Holliday junction resolvases, four-way junction cleavage yields a double strand break that is capped on each end by a hairpin. Resolution of a cruciform embedded in a circular plasmid permits the capture of each side of a double strand break on a single molecule. Indeed, I found that double strand breaks at cruciforms yielded a linear molecule that was dually-hairpin capped. These hairpin-capped molecules could be observed in *sae2* or *mre11* mutant cells. Taken together, such observations provided undisputable evidence that: 1) cruciform extrusion takes place in eukaryotic chromatin *in vivo*, and 2) that cruciforms are resolved by cellular machinery, confirming a long-presumed, but never proven pathway for the formation of endogenous double strand breaks. Although the findings thus far proved to significantly advance our understanding of the cellular machinery responsible for processing palindromes and inverted repeats, there still remains much to uncover. Besides Sae2 and the MRX complex, no other proteins have been identified to play a role in palindrome processing.

With the development of a system for monitoring cruciform extrusion and resolution in a genetically tractable eukaryote, I was interested in identifying additional factors that participate in palindrome metabolism. First I performed a candidate screen for genes that, when mutated, suppressed cruciform resolution. I show that the *MUS81* gene product is responsible for roughly 90% of the observed cruciform resolution. In the
absence of Mus81, cruciform resolution is restored by expression of a *bona fide* HJ resolvase from *E. coli*.

I performed a second candidate screen for genes that are required to prevent palindrome breaks. I found that the sole RecQ helicase in budding yeast, Sgs1, played a role in maintaining the stability of DNA palindromes. Cells deleted for *SGS1* as well as *TOP3* or *RMI1*, whose gene products form the Sgs1-Top3-Rmi1 complex, display an altered palindrome maintenance phenotype. I show that the Sgs1-Top3-Rmi1 complex is required to prevent rampant double strand break formation in both an artificial and a natural human palindrome. Through molecular genetic analysis, I propose that the Sgs1-Top3-Rmi1 complex prevents multiple pathways of double-strand break formation, including Mus81-dependent cruciform resolution.
3.3 Results

3.3.1 Mus81 is required for cruciform resolution

In Chapter 2, I showed that, when present in a plasmid, the human NF1 palindrome extrudes a cruciform that is resolved, instigating a DNA rearrangement event called escape. What was unknown was the protein(s) involved in the actual resolution process. The observation of escape provided a straightforward assay with which to identify the relevant cruciform resolvase: the responsible nuclease ought to be among gene products that, when mutated, suppress the formation of ExoV resistant hairpin linears in an *mre11* or *sae2* strain background. *MUS81* was first in a small group of genes investigated because it is thought to be important in the recombinational repair of blocked replication forks and is a meiotic HJ resolvase in *S. pombe* ((Boddy et al., 2001) and reviewed in (Osman and Whitby, 2007). In addition to *MUS81*, a number of genes were tested, including other genes required for survival in the absence of *SGS1*. The complete list of genes screened for escape suppression (or any kind of phenotype differing from that in *sae2* cells) is listed in Table 3-1.

To test for a role of Mus81 in cruciform resolution, pH1#21 was plasmoducted into *mus81Δ mre11-H125N*, into each single mutant and into the corresponding wild-type strain. Figure 3-1 shows a Southern blot of two independent plasmoductants for each mating. The escape-specific 7.1 and 3.5 kb bands detected in the *mre11-H125N* single mutant were almost completely eliminated in the absence of a functional *MUS81* gene (Figure 3-1A; 2nd lanes). Likewise the initial unit-length cruciform resolution product all but disappeared (Figure 3-1B; 3rd lane). Quantification of the hairpin linear indicated that
Table 3-1. List of genes surveyed for suppression of cruciform resolution in a *sae2* background.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CST9</td>
<td>SUMO E3 ligase; involved in synaptonemal complex formation</td>
</tr>
<tr>
<td>MUS81</td>
<td>Structure-specific endonuclease</td>
</tr>
<tr>
<td>RAD51</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>RAD52</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>SLX1</td>
<td>Structure-specific endonuclease</td>
</tr>
<tr>
<td>SLX4</td>
<td>Structure-specific endonuclease</td>
</tr>
<tr>
<td>SLX5</td>
<td>Ubiquitin ligase</td>
</tr>
<tr>
<td>SLX8</td>
<td>Ubiquitin ligase</td>
</tr>
<tr>
<td>TOP1</td>
<td>Topoisomerase</td>
</tr>
<tr>
<td>TOP3</td>
<td>Topoisomerase</td>
</tr>
<tr>
<td></td>
<td>wt</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>N</td>
<td>1</td>
</tr>
</tbody>
</table>

DNA samples were isolated without denaturation. **A.** Analysis of escape in mutant and wild-type strains plasmoded with pH1#21. A pair of independent isolates is shown for each experiment. The 7.1 kb and 3.5 kb bands indicative of escape are reduced in the **mus81**Δ**mre11-H125N** double mutant. **B.** Quantification of cruciform resolution in mutant and wild-type strains. After relaxation of DNA, samples were treated with M. luteus Exonuclease V. The remaining ExoV-resistant fraction is the cruciform resolution product (1X hairpin linear species). The fraction resolution is calculated as the intensity of the 1X linear over the total intensities of the 1X linear and the open circle, or written as ♦/(*)(♦+♦). The unit-length resolution product seen in **mre11-H125N** (♦L) is not observed in the absence of **MUS81** (lanes 3).
> 90% of the observed cruciform resolution was attributable to Mus81 (Figure 3-1B; 3rd lanes).

To determine whether a known HJ resolvase could restore the escape and cleavage signals in a mus81Δ mre11-H125N mutant, the experiment was repeated after a vector expressing the bacterial RusA resolvase had been transformed into recipient strains (Figure 3-2A). The 7.1 and 3.5 kb NheI digestion products indicative of escape reappeared with RusA complementation (Figure 3-2A). In addition, the initial cruciform resolution linear (denoted by a black diamond) was again observed in the complemented mus81Δ mre11-H125N double mutant (Figure 3-2B; rightmost panel, compare 3rd to 6th lanes). Cruciform resolution by RusA, measured as the percent of monomer plasmid in an ExoV-resistant linear form (mre11-H125N background), was at about 40% of the level seen with the endogenous resolvase Mus81 (Figure 3-2B; 3rd panel, lane 3; 4th panel, lane 6). I have found that plasmoduction of RusA complemented cells gives a more variable outcome than for other plasmoduction experiments (Figure 3-3). One possibility is that there may be incidental side-effects of the interaction of the bacterial resolvase with its DNA target in yeast that influence the relative rates of cruciform cleavage (production) or replication (removal) of the hairpin linear. Nonetheless, the results clearly showed that cruciform resolution and escape were re-established with complementation, and there were no additional or unexplained bands in the experiment. I conclude that an established HJ resolvase, RusA, compensates for the absence of Mus81 with respect to cruciform resolution and escape.

3.3.2 A system for studying in vivo cruciform resolution in eukaryotes
Figure 3-2

RusA can substitute for Mus81 in promoting escape and cruciform resolution. DNA samples were isolated without denaturation. A and B: Complementation of escape (A) and cruciform resolution (B) signals in mutant cells with bacterial RusA. Bands were visualized with a probe that hybridized to URA3 in order to avoid detection of the RusA expression vector. The probe gives background hybridization to genomic sequences (because the recipient strains carry the ura3-1 allele). In the RusA (-) controls, the plasmiducted cells contained an empty expression vector (see Experimental Procedures). A. RusA restores escape in the double mus81Δ mre11-H125N mutant. The diagnostic 7.1 and 3.5 kb bands in NheI-digested DNA (**, lanes 2, 4) and the dimeric linear signal in uncut DNA (<lanes 1, 3) are detected in doubly mutant cells if transformed with the RusA expression vector. B. RusA restores cruciform resolution. The same samples as shown in A were treated as indicated to test for the presence of an ExoV-resistant monomeric linear. This is seen if mus81Δ mre11-H125N cells contain the RusA expression vector (rightmost lane). The escape-specific dimer linear is also present in the complemented recipient. In part B, two membranes were prepared, hybridized, and exposed in parallel. % resolution was calculated as in Figure 3-1.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>AVG.</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>mre11-H125N</td>
<td>19.2</td>
<td>±1.0</td>
</tr>
<tr>
<td>+RusA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mre11-H125N, mus81Δ +RusA</td>
<td>10.6</td>
<td>±5.3</td>
</tr>
</tbody>
</table>

**Figure 3-3**

**Variability of Escape in RusA-complemented cells.** To investigate reproducibility, the intensity of the signal at 3.5kb relative to the sum of the signal intensities at 6.2kb and 3.5kb together was determined, after NheI digestion, for independent plasmoductants. This determination is different from that used to quantify cruciform resolution (as in Figure 3-1). The above measurement includes a signal produced by cruciform resolution of the escape product in addition to cleavage of the input plasmid form so that it is less directly interpretable. Nonetheless the measurement can readily be applied to a large number of samples and is suitable in an assessment of reproducibility. Values in the table combine both escape and cleavage because the 3.5kb band represents NheI fragments that are released not only from the unit linear formed by cruciform resolution of pH1#21 (before escape), but also from “X” type dimer linears, an escape product. Maps and diagrams are in Figure 3-6.

Individual experiments are distinguished by the labels “a”, “b”, “c”, or ‘d’. Plasmoductants with the same letter are “siblings” from the same mating. The bolded and underlined samples labeled “b” appear in text Figure 3-2 A 2nd lanes. The same DNA preparations have been evaluated for cruciform resolution in Figure 3-2 B.

As can be seen in the above table, there is good reproducibility for values obtained for mre11-H125N, even as determined on separate experiments (20.3% +/- 1.8). When RusA is added into this strain or to an mre11 mus81 cell, the results are more varied, even when DNA is derived from sibling plasmoductants and processed in parallel. Overall, my experience is that reproducibility using plasmoduction is high and, to date, variability has been encountered only in the case of the RusA complementation experiments.
In Chapter 2, I described a second system for detecting palindrome-instigated double strand breaks. A perfectly palindromic dimer circle containing two perfect symmetry axes was constructed by self-ligation of two copies of the large PvuII and XbaI-digested fragment from the pSH1 E.coli/yeast shuttle vector (Figure 3-4A). This molecule cannot transform bacteria and therefore must be prepared in quantity entirely in \textit{vitro}. I found that in strains deficient in Sae2 or Mre11, axis-specific hairpin-capped linear products were detected as would result from the cross-diagonal cleavage of a cruciform structure.

With this system, two genes previously implicated in both palindrome and inverted repeat metabolism, \textit{MRE11} and \textit{SAE2}, were shown to be instrumental in preventing the accumulation of the linear resolution products. To eliminate a technical artifact caused by random DNA breakage, a more gentle DNA isolation technique was used. With this approach, I retested \textit{MRE11} and \textit{SAE2}, and extended the analysis to the two other genes in the MRX complex, \textit{RAD50} and \textit{XRS2}.

DNA isolated from the transformants was analyzed by Southern blot using the same plasmid-specific probe as previously described in Chapter 2 and diagrammed in Figure 3-4B. Wild-type DNA samples gave results typical of an uncut circular plasmid DNA, with bands representing supercoiled, linear, and relaxed forms (Figure 3-4C; lane 1). Samples were digested with NheI to establish whether or not the linear form was a site-specific product, as predicted for cruciform resolution, which can occur at the PvuII or XbaI symmetry axes of pPX. As expected, intact pPX was cut by NheI at two sites, giving a probe-positive band of 6.5kb (Figure 3-4C; lane 2). A second band migrating
Figure 3-4
A plasmid system for monitoring cruciform extrusion and resolution in *Saccharomyces cerevisiae*.  

**A.** Diagram of possible cruciform resolution products generated in the pPX dimer circle. A resolution product, generated by the cross-diagonal cleavage at the four-way junction of an extruded cruciform, yields a linear product that is capped on each side with a hairpin. When the PvuII symmetry axis extrudes a cruciform and is cross-diagonally cleaved, the P-type hairpin-capped linear is created. The X-type hairpin-capped linear is generated when cruciform resolution occurs at the XbaI-defined symmetry axis.  

**B.** Top: Diagram of *in vitro*-prepared palindromic dimer pPX. The hybridization probe is indicated by the black bar. The probe-positive NheI fragment in pPX is indicated by a dashed line, along with the expected size. Bottom: Map of cruciform resolution products. Probe-positive NheI fragments are indicated.  

**C.** Southern blots of DNA isolated from pPX transformants. Wild-type and MRX(S) strains stably maintain pPX and exhibit axis specific cleavage of the palindromes. Unlabeled lanes contain uncut DNA. Open circle (OC), linear, and supercoiled circle (SC) are indicated. The supercoiled signal is variable due to nicking during the DNA isolation process. NheI-digested DNA samples (lanes labeled "N") give the expected 6.5 and 3.25 kb bands.  

**D.** Southern blots of DNA treated with *Micrococcal luteus* Exonuclease V. In wild-type cells, the 11.5 kb linear is completely degraded by ExoV (lane 3). In *mre11Δ* cells, the linear species is resistant to exonuclease treatment (solid triangle; lane 7). All markers are in kilobases.
Figure 3-5
Axis-specific double strand breaks accumulate in sgs1Δ cells transformed with a palindromic dimer plasmid. A. Southern blot of DNA isolated from pPX transformants. sgs1Δ, top3Δ, and rmi1Δ cells contain relatively large amounts of 11.5kb linear species in uncut samples. The linear species are axis-specific products, as indicated by the intense 3.25kb signal in NheI digested samples. B. Southern blot of DNA treated with M. luteus Exonuclease V. As for wild-type cells, the 11.5kb linear species in sgs1Δ, top3Δ, and rmi1Δ cells is sensitive to ExoV (solid triangle). Diagrams are labeled as in Figure 3-4. C. Diagnostic digests of pPX transformants. P; PvuII, X; XbaI, N; NheI. All sizes are in kilobases.
more slowly than a 3.25 kb marker indicated the presence of double strand breaks located near the XbaI symmetry axis. In order to determine if the reduction in migration was due to the presence of additional DNA, an NheI/XbaI was performed. Double digest of the samples caused the >3.25kb species to migrate with a 3.25kb marker, suggesting that breaks in wild-type cells were occurring past the XbaI symmetry axis (see Figure 3-5C). As described in Chapter 2, the combination of probe and digest used here does not monitor breakage at the PvuII axis. The PvuII axis may also be a second site of double strand break formation however, NheI digestion of this linear would create a 6.5kb band, indistinguishable from that generated by NheI digestion of intact pPX (Figure 3-4B). I conclude that in the wild-type background, at least a proportion of the linear 11.5 kb band in untreated DNA is the product of site-specific double strand breakage.

Uncut DNA from sae2Δ, mre11Δ, rad50Δ, and xrs2Δ cells also gave similar bands as wild-type cells, with two notable exceptions. One was an enhancement in the abundance of intact circular pPX (Figure 3-4C, lanes 3, 5, 7, and 9). The other is the location of termini. When digested with NheI, mutant DNA samples revealed both the 6.5kb and 3.25kb bands. Unlike wild-type samples, the species resulting from digestion of the X-type linear migrated with the 3.25kb marker (Figure 3-4C, lanes 4, 6, 8, and 10). Where in wild-type cells, the break band signal was disperse, the 3.25kb band in the mutants was sharp.

The definitive test for cruciform resolution is through the detection of dually-hairpin-capped linears, as illustrated for the mre11 mutant in Figure 3-4D. Samples were treated with Exonuclease V to determine whether the linear form was resistant, as predicted if the circular pPX was opened by cruciform resolution. In wild-type samples,
incubation with ExoV resulted in the disappearance of the 11.5 kb linear signal (Figure 3-4D; lane 3), however, the 11.5 kb pPX linear from mre11Δ cells resisted ExoV degradation (Figure 3-4D; lane 7). Similarly, sae2Δ, rad50Δ, and xrs2Δ DNA isolates were also resistant (not shown).

These results agree with and extend previous analyses with Sae2 and Mre11. It is evident that the MRX complex as well as the nuclease functions of Sae2 and Mre11 are necessary to intercept cruciform resolution. Whether this interference occurs at the stage of cruciform extrusion and/or at the stage of the accumulation of resolved forms is not addressed. The species migrating above the 3.25kb marker that is present in wt cells but absent in the mutants may indicate the presence of a second mechanism for palindrome-induced breakage in addition to cruciform resolution, that is dependent on Sae2 and MRX.

3.3.3 sgs1 helicase mutants accumulate axis-specific double-strand breaks

To broaden our understanding of the circumstances leading specifically to cruciform resolution, the pPX circle was used to transform a group of candidate strains mutated for genes involved in various areas of genome maintenance and repair, including replication, DNA damage-induced checkpoints, homologous recombination, and non-homologous end joining. Several structure-specific DNA nucleases and DNA helicases were also included in the survey. The complete list is given in Table 3-2.

Each strain was transformed with pPX as usual. Strains were transformed in parallel with the non-palindromic control plasmid pSH1, in order to assess their ability to maintain pPX relative to a standard plasmid. Transformation efficiencies are presented in Table 3-3. While transformation efficiencies ranged about 2-fold above and below wild
Table 3-2. List of surveyed genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA4</td>
<td>Non-homologous end joining(^a)</td>
<td></td>
</tr>
<tr>
<td>DUN1</td>
<td>Cell cycle checkpoint</td>
<td></td>
</tr>
<tr>
<td>EXO1</td>
<td>Exonuclease involved in DNA end resection(^d)</td>
<td>Additional species migrating above dimer linear</td>
</tr>
<tr>
<td>LIF1</td>
<td>Non-homologous end joining(^a)</td>
<td></td>
</tr>
<tr>
<td>MEC1</td>
<td>DNA damage-induced checkpoint</td>
<td>Increased abundance of open circle dimer species</td>
</tr>
<tr>
<td>MRE11</td>
<td>DSB repair(^bd)</td>
<td>Hairpin-capped linear species</td>
</tr>
<tr>
<td>MUS81</td>
<td>Structure-specific endonuclease(^bd)</td>
<td></td>
</tr>
<tr>
<td>NEJ1</td>
<td>Non-homologous end joining(^a)</td>
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<tr>
<td>PIF1</td>
<td>DNA helicase(^d)</td>
<td></td>
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<tr>
<td>POL4</td>
<td>DNA polymerase(^a)</td>
<td></td>
</tr>
<tr>
<td>PSO2</td>
<td>Structure-specific endonuclease(^d)</td>
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<td>RAD1</td>
<td>Structure-specific endonuclease(^d)</td>
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<td>RAD10</td>
<td>Structure-specific endonuclease(^d)</td>
<td></td>
</tr>
<tr>
<td>RAD27</td>
<td>Flap endonuclease; replication(^d)</td>
<td>Very low transformation efficiency</td>
</tr>
<tr>
<td>RAD50</td>
<td>DSB repair(^d)</td>
<td>Hairpin-capped linear species</td>
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<td>RAD51</td>
<td>Homologous recombination(^b)</td>
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<tr>
<td>RAD52</td>
<td>Homologous recombination(^b)</td>
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<td>RAD53</td>
<td>DNA damage-induced checkpoint</td>
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<tr>
<td>RMI1</td>
<td>DNA helicase(^bd)</td>
<td>Increased abundance of dimer linear species</td>
</tr>
<tr>
<td>RRM3</td>
<td>DNA helicase(^d)</td>
<td></td>
</tr>
<tr>
<td>SAE2</td>
<td>DSB repair(^bd)</td>
<td>Hairpin-capped linear species</td>
</tr>
<tr>
<td>SGS1</td>
<td>DNA helicase(^bd)</td>
<td>Increased abundance of dimer linear species</td>
</tr>
<tr>
<td>SLX1</td>
<td>Structure-specific endonuclease(^bd)</td>
<td></td>
</tr>
<tr>
<td>SLX4</td>
<td>Structure-specific endonuclease(^bd)</td>
<td></td>
</tr>
<tr>
<td>SLX5</td>
<td>Ubiquitin ligase(^bd)</td>
<td></td>
</tr>
<tr>
<td>SLX8</td>
<td>Ubiquitin ligase(^bd)</td>
<td></td>
</tr>
<tr>
<td>SML1</td>
<td>Regulation of dNTP production</td>
<td></td>
</tr>
<tr>
<td>SRS2</td>
<td>DNA helicase(^bd)</td>
<td></td>
</tr>
<tr>
<td>TEL1</td>
<td>DNA damage-induced checkpoint</td>
<td></td>
</tr>
<tr>
<td>TOP1</td>
<td>Topoisomerase(^a)</td>
<td></td>
</tr>
<tr>
<td>TOP3</td>
<td>Topoisomerase(^bd)</td>
<td>Increased abundance of dimer linear species</td>
</tr>
<tr>
<td>XRS2</td>
<td>DSB repair(^d)</td>
<td>Hairpin-capped linear species</td>
</tr>
<tr>
<td>yKU70</td>
<td>Non-homologous end joining(^a)</td>
<td></td>
</tr>
<tr>
<td>yKU80</td>
<td>Non-homologous end joining(^a)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Indicates genes that were screened by Southern blot in which DNA was isolating by glass bead disruption.
\(^b\)Indicates genes that were also tested for escape by transformation with pH1\#21.
\(^c\)If left blank, the observed phenotype matches that of wild-type.
\(^d\)Transformation efficiencies are presented in Table 3-3.
type, the only mutant from which I was unable to recover pPX transformants was rad27Δ. The few transformants that were recovered from rad27Δ transformation were not carrying intact pPX but were instead maintaining pSH1 or other molecules that appear to be pSH1 or pPX with large deletions or rearrangements. It is currently unknown if these arose in rad27Δ cells or are simply low-level contaminants in the pPX preparation.

To examine details of palindrome maintenance and metabolism, DNA was isolated from individual transformants and eight independent transformants per mutant were analyzed. Uncut and NheI-digested DNA samples were analyzed by Southern blot. No striking difference in pPX maintenance relative to wild-type was detected in most mutants. However, when DNA from pPX-transformed sgs1Δ cells was analyzed by Southern blot, there was a dramatic change in both the abundance and types of species as compared to wild-type cells (Figure 3-5A). In uncut samples, sgs1Δ cells contain a significantly large amount of the linear 11.5kb species while retaining a similar amount of intact open circle as wild-type cells (Figure 3-5A; compare lane 5 to lane 1). I also observed the presence of additional bands that migrated between the circular and linear pPX species. The linear species were generated by axis-specific breaks as demonstrated by digestion with NheI. The >3.25kb band was obviously more pronounced in the sgs1Δ mutant (Figure 3-5A; compare lane 6 to lane 2). As observed in wild-type cells, an NheI/XbaI double digest caused the >3.25kb species to co-migrate with a 3.25kb marker (Figure 3-5C; lane 17). pPX maintenance in top3Δ and rmi1Δ cells phenocopies that in sgs1Δ (Figure 3-5A, lanes 7-10). This pPX maintenance phenotype was specific for the Sgs1-Top3-Rmi1. Transformation of other helicase mutants, including srs2Δ, pif1Δ, and
RRM3, did not give the high breakage phenotype. PIF1 and RRM3 cells maintained pPX as wild-type cells did, however, the SRS2 mutant displayed a relatively low pPX transformation efficiency (Table 3-3). Southern blot of Ura+ transformants showed that, similar to SGS1 cells, there were additional species migrating between circular and linear pPX, however unlike SGS1 cells, these were also observed in pDD transformants and were inconsistently present (not shown). These results demonstrated the increase in double strand breaks near the XbaI palindromic symmetry axis was unique to SGS1 cells among the tested strains.

The >3.25kb break band from SGS1 cells was disperse and migrated more slowly than a 3.25kb marker as was observed in wild-type. In order to further characterize the breaks in SGS1 cells, DNA samples were treated with Exonuclease V (Figure 3-5B). The dimer linear species from SGS1, Top3, and RMI1 cells were fully degraded by ExoV (Figure 3-5B). This suggests that palindrome-provoked DSBs are greatly enhanced in SGS1 cells.

3.3.4 Increased double strand breaks at a human palindrome in SGS1 cells

The above results indicate that Sgs1 will normally prevent double strand breaks near the symmetry axis of an artificial palindrome. In the absence of Sgs1, a fully palindromic circle is site specifically broken. By eye, one can see that the ratio of uncut to linear pPX is skewed. Paradoxically, elevated break formation does not appear to have any gross impact on the maintenance of pPX. In fact both the transformation efficiency and the levels of plasmid detected on blots is elevated in SGS1.

As I have shown in Chapter 2, cruciform resolution leads directly to gross rearrangements and copy number increase monitored in an assay using a human
Table 3-3. pPX transformation efficiencies.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Transformation efficiency(^$)</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.411 ±0.105</td>
<td>0.411 ±0.105</td>
</tr>
<tr>
<td>EXO1</td>
<td>1.699 ±1.277</td>
<td>1.699 ±1.277</td>
</tr>
<tr>
<td>MRE11</td>
<td>0.811 ±0.142</td>
<td>0.811 ±0.142</td>
</tr>
<tr>
<td>MUS81</td>
<td>0.412 ±0.279</td>
<td>0.412 ±0.279</td>
</tr>
<tr>
<td>PIF1</td>
<td>0.483 ±0.073</td>
<td>0.483 ±0.073</td>
</tr>
<tr>
<td>RAD1</td>
<td>0.576 ±0.204</td>
<td>0.576 ±0.204</td>
</tr>
<tr>
<td>RAD10</td>
<td>0.959 ±0.899</td>
<td>0.959 ±0.899</td>
</tr>
<tr>
<td>RAD27</td>
<td>0.158 ±0.062</td>
<td>0.158 ±0.062</td>
</tr>
<tr>
<td>RAD50</td>
<td>2.475 ±1.560</td>
<td>2.475 ±1.560</td>
</tr>
<tr>
<td>RMI1</td>
<td>0.334 ±0.049</td>
<td>0.334 ±0.049</td>
</tr>
<tr>
<td>RRM3</td>
<td>0.660 ±0.482</td>
<td>0.660 ±0.482</td>
</tr>
<tr>
<td>SAE2</td>
<td>0.858 ±0.217</td>
<td>0.858 ±0.217</td>
</tr>
<tr>
<td>SGS1</td>
<td>0.598 ±0.080</td>
<td>0.598 ±0.080</td>
</tr>
<tr>
<td>SLX1</td>
<td>0.411 ±0.078</td>
<td>0.411 ±0.078</td>
</tr>
<tr>
<td>SLX4</td>
<td>0.248 ±0.076</td>
<td>0.248 ±0.076</td>
</tr>
<tr>
<td>SLX5</td>
<td>0.128 ±0.015</td>
<td>0.128 ±0.015</td>
</tr>
<tr>
<td>SLX8</td>
<td>0.172 ±0.046</td>
<td>0.172 ±0.046</td>
</tr>
<tr>
<td>SRS2</td>
<td>0.243 ±0.096</td>
<td>0.243 ±0.096</td>
</tr>
<tr>
<td>TOP3</td>
<td>0.509 ±0.089</td>
<td>0.509 ±0.089</td>
</tr>
<tr>
<td>XRS2</td>
<td>4.203 ±2.825</td>
<td>4.203 ±2.825</td>
</tr>
</tbody>
</table>

\(^\$\) Transformation efficiencies with pPX and pSH1 were performed in parallel and are expressed as the ratio of total pPX colonies to total pSH1 colonies. Given is the mean transformation efficiency for three independent experiments.
palindrome. In brief, this second test uses a human palindrome that naturally occurs within an intron of the Neurofibromin-1 gene (for the sequence, see Chapter 2). When propagated in \textit{sae2} or \textit{mre11} strains, I observed the products of \textit{in vivo} cruciform extrusion and resolution in the form of a unit length hairpin-capped linear DNA molecule. Cruciform resolution was shown to lead to a rearrangement process termed "escape" (described in detail in Chapter 2).

In order to determine if Sgs1 had a role in preserving the integrity of a natural human palindrome, \textit{sgs1}Δ cells were transformed with pH1#21, a 2µ plasmid containing the most palindromic NF1 allele (for a sequence diagram, see Figure 2-7 or 2-19). Ura+ transformants were selected and DNA was isolated for Southern blot analysis. In uncut samples from wild-type cells, input NF1 plasmid was present as a ladder of topoisomers migrating between fully relaxed and fully supercoiled species (Figure 3-6C; lane 1). This was confirmed by digestion with NheI, where a prominent 6.2kb band indicated the presence of the input monomer circle (lane 2). An additional band of 3.5kb was also observed in NheI-cut wild-type DNA, indicating the presence of site-specific breaks positioned at the NF1 palindrome. The 3.5kb band in wild-type cells is strain-specific, where it is only observed in S288C and not in W303. No other bands were visible in wild-type samples. Escape is observed in \textit{sae2Δ} cells, as indicated by the presence of input monomer plasmid along with additional bands co-migrating with a 12.4kb dimer circle and 12.4kb dimer linear (lane 3). When cut with NheI, in addition to the 6.2kb input plasmid signal, digestion released bands of 7.1kb, demonstrating the formation of a palindrome (lane 4). A 3.5kb band was also present, indicating the presence of site-specific breaks. Introduction of pH1#21 into \textit{sgs1}Δ cells clearly indicated escape. The
circular pH1#21 plasmid was accompanied by additional bands migrating at the positions of the dimer circle and dimer linear (lane 5). This was confirmed with Nhel digestion. 7.1kb and 3.5kb bands indicated that they are palindromic dimer circle and dimer linear species (lane 6). Samples from sgs1Δ cells largely resemble those from sae2Δ cells except that the 3.5kb band was tailed by smaller species. Unlike in pPX transformants, a species migrating slightly more slowly than the 3.5kb break band, indicating a break past the NF1 palindrome, was not observed in wild-type or sgs1Δ cells. The escape and breakage phenotype was also observed in top3Δ and rmi1Δ cells (lanes 7-10). For comparison, pH1#21 was also introduced into srs2Δ cells, however, escape was not observed (lane 11-12).

3.3.5 Sgs1-Top3-Rmi1 prevents cruciform resolution

DNA palindromes can adopt both hairpin and cruciform structures that, when processed by cellular repair machinery, can result in the formation of double strand breaks. The two classes of breaks are difficult to differentiate experimentally and the most unambiguous tests hinge upon the demonstration of co-formed hairpin termini. In cells that are wild-type for SAE2 and genes of the MRX complex, such termini, if formed, are rapidly opened so that this is difficult to demonstrate. However, as shown in Chapter 2, cruciform resolution of pH1#21 leads to escape. The occurrence of escape in sgs1Δ cells therefore provides evidence for cruciform resolution. I have shown that escape occurs when a dually hairpin-capped linear is replicated. Therefore, my results strongly imply that Sgs1 is required for intercepting DNA rearrangements that initiate with cruciform resolution of a human palindrome.
Figure 3-6
Double strand breaks and escape in sgs1Δ cells containing a human palindrome. A. Scheme of cruciform resolution and escape of the NF1 plasmid. The plasmid, containing the ~200bp human Neurofibromin-1 palindrome, extrudes a cruciform that is diagonally cleaved across the four-way junction, creating a linear, hairpin-capped molecule. Replication of this molecule generates a fully palindromic dimer circle. Either symmetry axis of the dimer can undergo further extrusion and resolution events. B. Maps of input NF1 plasmid and escape species. The hybridization probe, restriction sites, and expected fragment sizes are labeled as in Figure 3-4. The NF1 cruciform resolution product is labeled at 1X linear. Dimer 2X linear forms are produced by cruciform resolution of the palindromic dimer circle. C. Southern blot of DNA isolated from NF1 plasmid transformants. As for sae2Δ cells, sgs1Δ, top3Δ, and rmi1Δ cells contain resolution and escape species, as indicated by the presence of 12.4kb circles and 12.4kb linears in uncut DNA as well as 3.5kb and 7.1kb products in NheI-cut samples. The 3.5kb signal in wild-type DNA cut with NheI indicates that double strand breaks are forming at the NF1 palindrome. Breaks are also observed in srs2Δ cells. All sizes are in kilobases.
Recent work in budding yeast showed that Sgs1 is involved in the resection of double strand breaks (Mimitou and Symington, 2008; Zhu et al., 2008). Thus far, it was uncertain if the role of Sgs1 in palindrome processing was in the repair of breaks that arise from resolution or in a step earlier to processing of resolution products. Such steps may include i) preventing the action of the resolvase(s) or ii) preventing/removing the cruciform. If the role of Sgs1 is limited to processing breaks rather than a role at an earlier step in the pathway, then elimination of Sgs1 in cells that lack hairpin opening activity should not result in an enhanced escape phenotype. If its role is to prevent/remove cruciforms or prevent resolution rather than processing breaks, elimination of hairpin opening activity in sgs1 mutant cells would increase both escape and the formation of hairpin-capped resolution species. To determine if Sgs1 is involved in a step prior to hairpin-capped break processing, the pH1#21 plasmid was introduced into top3Δ sae2Δ cells (because of the lethality of sgs1Δ sae2Δ cells) and isolated DNA was analyzed by Southern blot. I found that in the top3Δ sae2Δ double mutant, the intensity of the 7.1kb and 3.5kb species in NheI-digested samples was more intense than those of either single mutant (Figure 3-7A; compare lanes 8 to 4 and 6). In NheI digested samples, a crisp 3.5kb band is visible in the double mutant and not the tailed 3.5kb band observed in top3Δ cells. This indicates that Sgs1-Top3-Rmi1 plays a role in the cruciform resolution pathway prior to processing of hairpin-capped DNA ends.

I also examined the formation of the unit length hairpin-capped linear product resulting from cruciform resolution of the NF1 palindrome. DNA samples were first treated with the site-specific nicking enzyme Nb.BsmI, resulting in the collapse of topoisomer ladders, making visualization of the unit length linear more clear. The
Figure 3-7

**Increased escape and cruciform resolution in sae2Δ top3Δ cells.** A. Southern blot of DNA isolated from NF1 plasmid transformants for the detection of escape. In uncut samples, the intensity of the 12.4kb circle and 12.4kb linear in sae2Δ top3Δ cells is more intense than either linear as is the intensity of the 7.1kb and 3.5kb signals in NheI-digested samples. Diagrams are labeled as in Figure 3-6. B. Southern blot of DNA isolated from NF1 plasmid transformants for the direct detection of NF1 cruciform resolution. After relaxation of DNA, samples were treated with M. luteus Exonuclease V. The remaining ExoV-resistant fraction is the cruciform resolution product (1X hairpin linear species). The fraction resolution is calculated as the intensity of the 1X linear over the total intensities of the 1X linear and the open circle, or written as ♦/(♦♦♦♦ + ♦♦♦♦). The ExoV-resistant linear in sae2Δ top3Δ cells is more intense than either single mutant. All sizes are in kilobases.
samples were then treated with ExoV; any ExoV-resistant linears represent cruciform resolution products. The abundance of resolution products (quantified as the ratio of hairpin-capped linear DNA to nicked monomer DNA) in top3Δ sae2Δ cells was higher than in sae2Δ cells (Figure 3-7B; compare lanes 9 to 12). Wild-type and top3Δ cells did not contain detectable amounts of resolution product (Figure 3-7B; lanes 3 and 6). The enhancement of escape and resolution in the double mutant suggests that Sgs1-Top3-Rmi1 contributes to the prevention of cruciform resolution and not only in the repair of resolution breaks as is suspected to be the case for Sae2 and the MRX complex.

3.3.6 Sgs1 prevents resolution in variants of the NF1 palindrome

The H1#21 allele is the most symmetric cloned NF1 variant. To determine if Sgs1 also prevented double strand break formation in variants with lesser symmetry, plasmids with several variants of the NF1 palindrome were introduced into sgs1Δ cells. pH1#21 in addition to pH1#23, pH4#4, and pΔ95 are described in Chapter 2. Each has varying degrees of symmetry and escape propensity, with pΔ95 being inert for escape. Southern blots of DNA isolates are shown in Figure 3-8. In sgs1Δ cells, pH1#21 and pH1#23, the most symmetric alleles, showed the highest amount of escape. pH4#4 had a significantly lower amount of escape than the more symmetric variants. pΔ95 did not show any species consistent with escape. These results suggest that Sgs1 counteracts resolution of palindrome-provoked structures that can arise in near-palindromes of varying symmetry. Moreover, the absence of breakage or escape of pΔ95 in sgs1Δ cells demonstrates that palindromes that are not prone to structure formation in wild-type, mre11, or sae2 cells do not become unstable in the absence of Sgs1. These results suggest that, in sgs1Δ cells,
Figure 3-8
DNA breaks and escape in sgs1Δ cells transformed with variants of the NF1 palindrome. Wild-type and sgs1Δ cells were transformed with four variants of the NF1 palindrome, including one (pΔ95) that arose by a central deletion event in yeast. Southern blots show two transformants per plasmid except for pSH1, where only one is shown. Escape is observed as 12.4kb circular and 12.4kb linear species in uncut DNA and 7.1kb and 3.5kb species in NheI-digested lanes. In wild-type cells, a 3.5kb band is observed in NheI-cut DNA, indicating the presence of NF1 palindrome-specific breaks. In the bottom blot, the third and fourth lanes from the right contain, in addition to monomer pΔ95, an unidentified species that migrates in the position of a dimer.
DNA topology is not affected in a manner that would encourage cruciform extrusion; the role of Sgs1 may not be to directly regulate DNA topology.

3.3.7 Sgs1 prevents multiple types of palindrome-provoked DSBs

Because Mus81 was shown to be required for cruciform resolution of the NF1 palindrome, I wanted to determine if Sgs1 was preventing double strand breaks caused by resolution by Mus81. The synthetic lethality of mus81 sgs1 double mutants is suppressed by eliminating homologous recombination, therefore, I constructed a mus81Δ sgs1Δ rad51Δ triple mutant. It and relevant double and single mutants were transformed with the NF1 palindrome and DNA was isolated for Southern blot analysis. Results are shown in Figure 3-9. Deletion of rad51Δ alone had no effect on NF1 palindrome maintenance (lanes 3 and 4). In mus81Δ and mus81Δ rad51Δ cells, I observed that the 3.5kb band in NheI-digested samples was absent, indicating that double strand break formation was reduced, as expected (lanes 8 and 12). Interestingly, the formation of the 12.4 kb palindromic dimer was suppressed in sgs1 rad51 cells (lane 9). This was confirmed by the absence of a 7.1kb signal in NheI-cut DNA (lane 10). Breakage of the NF1 palindrome is still occurring, as the 3.5kb species is still visible. I suspect that Rad51 is required for the formation of the palindromic dimer from the hairpin-capped linear resolution product. Surprisingly, I found that breakage of the NF1 palindrome was still prominent in mus81Δ sgs1Δ rad51Δ cells (lane 14). Upon closer inspection of the 3.5kb tailed species, it was apparent that there was a reduction in its intensity and a shift in its migration in the triple mutant. These results suggest that Sgs1 prevents multiple pathways of double strand break formation, including Mus81-dependent cruciform resolution.
To provide further evidence for multiple palindrome-provoked break pathways, I introduced pPX into mus81Δ sae2Δ cells and examined DNA by Southern blot. Results are shown in Figure 3-10A. As was observed in sae2Δ cells, there was a 3.25kb band in NheI-digested DNA from mus81Δ sae2Δ cells, indicating that site-specific breaks are occurring independently of Mus81. Moreover, as indicated in Figure 3-10B, mus81Δ pPX transformants contain the slow-migrating >3.25kb species in NheI-digested samples. In fact, DNA from wild-type pPX transformants are indistinguishable from mus81Δ samples. A thorough explanation of the suspected DSB pathways is presented in the discussion.
Sgs1 prevents multiple NF1 palindrome-provoked DNA break pathways. Southern blot of DNA isolated from NF1 plasmid transformants. The 3.5kb DNA break product in sgs1Δ rad51Δ cells is not suppressed by deleting MUS81. Instead, there is a less intense species migrating slightly more quickly than the 3.5kb species. The 3.5kb break band is suppressed in mus81Δ and mus81Δ rad51Δ cells, suggesting that, in MUS81+ SGS1+ cells, visualized breaks are from cruciform resolution. Sizes are in kilobases.

Figure 3-9

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>rad51Δ</th>
<th>sgs1Δ</th>
<th>mus81Δ</th>
<th>sgs1Δ mus81Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

- dimer circle 12.4
- 2X linear 12.4
- input circle 6.2

- 7.1
- 6.2
- 3.5
**Figure 3-10**

Deletion of *MUS81* does not result in the complete suppression of axis-specific break formation.  **A.** Southern blot of DNA isolated from pPX transformants. In *sae2Δ mus81Δ* cells, a 3.25kb signal is observed in NheI-digested samples, indicating the presence of 2X linear species.  **B.** Southern blots of wild-type and *mus81Δ* pPX transformants. The ~3.25kb species is more clearly observed and is present in *mus81Δ* cells. All sizes are in kilobases.
3.4 DISCUSSION

Palindromes are a class of DNA arrangement associated with both translocations and large deletions in the human genome. Through molecular analyses, it is now suggested that these instances of gross chromosomal aberrations are instigated by double strand break formation at an extruded cruciform structure. Here I found that Mus81 is required for cruciform resolution and is suspected to cross-diagonally cleave intact four-way junctions in vivo, strengthening claims that it is a Holliday junction resolvase. Cells also possess means to prevent rampant palindrome-provoked double strand breaks. Using a plasmid-based system developed for distinguishing between cruciform-provoked breaks and other types of double strand breaks, I found that the RecQ helicase Sgs1 is involved in deterring multiple pathways of double strand break formation, including cruciform resolution.

3.4.1 Evidence that Mus81 is a mitotic HJ resolvase in S. cerevisiae

Greater than 90% of cruciform resolution of the human NF1 palindrome depends on MUS81 (Figure 3-1). The MUS81 gene encodes a structure-specific endonuclease that is active when complexed with a second protein, Mms4. In mitotically growing cells, evidence indicates that Mus81-Mms4 has a major function in the repair of replication forks that have stalled or regressed at sites of DNA damage (reviewed in (Osman and Whitby, 2007)). Here, Mus81 is required for all but a minor fraction of observed cruciform resolution.

There is an ongoing discussion regarding the specificity of Mus81 in vitro and in vivo as well as its roles in DNA repair and genetic recombination (Berchowitz et al., 2007; Cromie et al., 2006; Cromie and Smith, 2007; Ehmsen and Heyer, 2008; Osman
and Whitby, 2007). With respect to *S. cerevisiae*, *mus81* mutant phenotypes were inconsistent with a major involvement in the resolution of meiotic recombination intermediates, and the bacterial HJ resolvase, RusA, could not complement those defects that were observed (De Los Santos et al., 2003). In *vitro*, preferred substrates for the *S. cerevisiae* protein are 3'-flapped and replication fork-like structures and nicked Holliday junctions, whereas only low-levels of cleavage of intact Holliday junctions are seen. Here, we have probed the substrate specificity of Mus81 in mitotically growing cells and find that it mediates cleavage of an extruded cruciform, a Holliday junction mimic. This is substantiated by the finding that RusA, an established HJ resolvase, can functionally substitute for Mus81 in the *in vivo* assay. Given current models of cruciform extrusion, in which supercoiling is necessary in order to drive and stabilize the alternative DNA structure, it seems unlikely that a four-way junction could form or persist unless the DNA were fully intact (i.e. not nicked (Sinden, 1994); a similar argument is presented in (Ehmsen and Heyer, 2008; Osman and Whitby, 2007; Taylor and McGowan, 2008)). Hence, I propose that Mus81 cleaves an intact, un-nicked four-way junction *in vivo*. It is capable of HJ resolution in mitotic cells.

As mentioned in the introduction, one earlier report investigated effects seen with a chromosomally-integrated 12bp-spaced ~300bp inverted repeat, and suggested that the observed hyper-recombination and breaks were due to cruciform resolution (Lobachev et al., 2002). Interestingly, genetic tests indicated that Mus81 was dispensable for the observations. The notion that break formation at a spaced inverted repeat may come about via a mechanism that is independent of cruciform resolution is raised by the central role in cruciform resolution demonstrated for Mus81 here. One possibility is that
breaks at spaced inverted repeats derive from a single strand hairpin- rather than cruciform-associated structure, and may initially be replication dependent “one-ended” DNA interruptions (see (Eykelenboom et al., 2008)).

Here, by the most conservative interpretation, Mus81 has either a direct or a governing role in mediating cruciform resolution in vegetative *S. cerevisiae*. Mus81-Eme1 in humans possesses the relevant biochemical HJ-resolution activity (Taylor and McGowan, 2008), and in keeping with this, our results suggest that Mus81-Mms4 in *S. cerevisiae* may be similarly endowed. The literature regarding *S. cerevisiae* Mus81 has been difficult to fully reconcile with studies in other eukaryotes and the present findings simplify the picture, and bolster the suggestion that the protein is a universal HJ resolvase in mitotic cells(Cromie et al., 2006). Not all of the cruciform resolution activity is absent in a *mus81* null cell (Figures 3-1), indicating the presence of at least one other HJ resolvase in vegetative yeast. Although Mus81 plays a much larger role in cruciform resolution than this second unidentified HJ resolvase in our assay, further work is needed to investigate the relative impact with an independent test.

The present studies put Mus81 in a new light by demonstrating that this evolutionarily-conserved repair nuclease has the inherent potential for generating damage. The liabilities engendered by Mus81 are particularly relevant to the palindrome-bearing genomes of higher eukaryotes. DNA breaks associated with palindromic sequences are a unique “thumbprint” of the cruciform resolution mechanism that matches that at reciprocal translocations observed in humans (Kurahashi et al., 2006b).

3.4.2 The role of the Sgs1 helicase in preventing cruciform resolution
The Sgs1 helicase, the sole RecQ helicase in *Saccharomyces cerevisiae*, plays several roles in the maintenance of genome stability in both mitotic and meiotic cells. During replication, Sgs1 is suggested to be required for the resolution of recombination intermediates arising at damaged replication forks (Liberi et al., 2005). Sgs1 is thought to circumvent the formation of crossover products during mitotic homologous recombination and this activity helps to prevent translocations or loss of heterozygosity (Ira et al., 2003). In meiotic cells, Sgs1 plays a role in preventing the formation of aberrant recombination intermediates that would otherwise interrupt normal chromatid segregation (Jessop and Lichten, 2008; Oh et al., 2007; Oh et al., 2008). The mitotic inter-sister chromatid hyper-recombination phenotype is observed not only in *sgs1* cells but also in human cells from Bloom's syndrome patients, carrying mutations in the gene encoding the Sgs1 homolog, Blm. Not only does Blm possess 3'-5' ATP-dependent helicase activity, *in vitro* work demonstrated that it is capable of branch migrating Holliday junctions (Karow et al., 2000). Moreover, Blm, when complexed with the type I topoisomerase, TopIIIα (homolog of yeast Top3), is capable of double Holliday junction dissolution through convergent HJ migration followed by cleavage of the resulting hemicatenane (Plank et al., 2006; Wu and Hickson, 2003). This reaction is stimulated by the addition of a third protein in the complex, Blap75 (homolog of *S. cerevisiae* Rmi1) (Bussen et al., 2007; Raynard et al., 2006; Raynard et al., 2008; Wu et al., 2006).

The substrates suspected to be acted upon by Sgs1-Top3 during meiotic and mitotic recombination as well as replication are similar in that they contain DNA four-way junctions. These are structurally equivalent to cruciform structures, which also
contain a four strand intercept. Albeit similar in structure, Holliday junctions and cruciforms differ in that the former are the products of strand exchange between two linear duplexes and the latter is formed on one continuous strand (Figure 3-11). If we extrapolate biochemical data of Blm-TopIIIα-Blap75 Holliday junction processing and apply these to cruciforms, the outcomes would differ greatly. Branch migration of a four-way junction results in the "sliding" of the four-way junction along duplexes, with no resulting loss of the junction. However, branch migration of a cruciform structure can, depending on the directionality, result in either the extension of cruciform arms (extrusion) or in their retraction (intrusion). Complete intrusion results in the complete resorption of the cruciform structure and reestablishment of lineform DNA (Figure 3-11, right panel). So how does Sgs1-Top3-Rmi1 prevent cruciform resolution? As biased directionality of Blm-provoked branched migration is suggested to be required for double Holliday junctions, it could be envisioned that Sgs1-Top3-Rmi1 may also have directionality in cruciform branch migration, favoring intrusion, thereby removing the cruciform and preventing the actions of endogenous resolvases on cruciform four-way junctions. Top3 would not be required for cleaving a hemicatenane, as needed for double Holliday junction processing, but would facilitate the energetically uphill process of resorption. Because extrusion is favored under conditions of negative superhelicity, a Top3-dependent net loss in negative supercoiling after intrusion would thereby prevent the palindrome from further rounds of extrusion. The requirement of Rmi1 may not only be to stimulate Top3's activity (Chen and Brill, 2007), it may also serve to directly bind to cruciform structures (Mullen et al., 2005).
Figure 3-11
Proposed model of cruciform "intrusion" by the Sgs1-Top3-Rmi1 complex. Left panel. A recombination intermediate is branch migrating with the help of Sgs1-Top3-Rmi1. Although the complex is suggested to perform convergent branch migration of two Holliday junctions followed by Top3-dependent hemicatenane dissolution, only one four-way junction is shown for simplicity. Right panel. An extruded cruciform in a single duplex. When in a parallel stacked configuration, it is identical in structure to the Holliday junction. Sgs1-dependent directional branch migration will lead to the eventual intrusion of the cruciform structure. Waves of negative supercoiling re-established during intrusion will be relieved by Top3.
If Sgs1-Top3-Rmi1's role is in fact, in the intrusion of cruciform structures, I anticipated an increase in the abundance of cruciforms in $sgs1\Delta$ cells. In order to detect changes in cruciform abundance \textit{in vivo}, I expressed bacterial RusA, a \textit{bona fide} HJ resolvase, in these cells and looked for a corresponding increase in RusA-dependent DSB formation at the site of the palindrome, however, upon RusA expression, an increase in DSBs was not observed (not shown). It is possible that the resolvase activity(ies) acting on these cruciforms is rapidly processing them before RusA can intervene.

\subsection*{3.4.3 Multiple types of DSBs are formed at DNA palindromes}

There are three lines of evidence that there exists at least two pathways that form axis-specific double strand breaks at palindromes: 1) deletion of \textit{MUS81} did not result in the complete suppression of breakage in $sgs1\Delta\ rad51\Delta$ cells (Figure 3-9), 2) NheI-digestion of DNA from pPX-transformed $mus81\Delta\ sae2\Delta$ cells resulted in the formation of a 3.25kb band, indicating the presence of axis-specific breaks (Figure 3-10A), and 3) albeit reduced by \textasciitilde90\%, linear products from cruciform resolution of the NF1 palindrome are detected at low levels in $mus81\Delta\ mre11-H125N$ cells (Figure 3-1). The latter also provides conclusive evidence for a second cruciform resolution pathway. It is possible that the persistent DSBs outlined in points 1 and 2 are also products of a Mus81-independent resolution mechanism, however, to irrefutably demonstrate that these are the products of resolution, a doubly hairpin-capped linear product must be observed.

Why are extruded cruciforms recognized/cleaved by more than one resolvase? Several possibilities exist. Activity of resolvases may be cell-cycle dependent. For example, Mus81 was demonstrated to be required for the formation of double strand breaks during S-phase (Froget et al., 2008; Hanada et al., 2007). Cleavage of cruciforms
by Mus81 may occur solely as a consequence of replication fork stalling at an extruded cruciform (reviewed in (Osman and Whitby, 2007)). A second possibility is that the choice of resolvase may be influenced by the actual structure of the cruciform. As shown by atomic force microscopy, cruciforms are dynamic secondary structures that can adopt unfolded or stacked (folded) conformations as well as parallel or anti-parallel orientations (Mikheikin et al., 2006; Oussatcheva et al., 2004; Shlyakhtenko et al., 2000; Shlyakhtenko et al., 1998). It is possible that Mus81, Sgs1, and other cruciform resolvases/cruciform-binding proteins have distinct non-overlapping binding affinities for these structures (as discussed, for example, in (Taylor and McGowan, 2008)). Genetic studies demonstrate that Sgs1 and Mus81 perform overlapping and distinct roles in recombination and repair, and in vitro substrate preference of Mus81 still remains controversial. My work contributes to our understanding of the substrate specificities of Mus81 and Sgs1.

The analysis of breaks in SAE2+ MRE11+ pPX transformants indicates that there is a mechanism of DSB formation in which the breaks are located in the vicinity of the XbaI axis (Figure 3-4C). In the absence of SAE2 or MRE11, hairpin-capped breaks are observed, indicating cruciform resolution as a second means of DSB formation. Using restriction enzyme analysis, the breaks in SAE2+ MRE11+ pPX transformants was mapped to ~600bps from the XbaI axis. Cells deleted for the SGS1 helicase show and increased amount of these breaks. From a comparison of the amounts of breaks observed in each pathway, I suspect that the mechanism generating breaks ~600bp away from the XbaI axis is the major pathway while resolution of cruciforms is a minor contributor to DSB formation. An ongoing focus is in determining the mechanism of formation of the
break in the vicinity of the XbaI axis. It is apparent that Sae2 and MRX are required for its formation while Mus81 is not (Figure 3-10B).

This pathway is not detected in NF1 plasmid transformants because a band migrating more slowly than the 3.5kb break species is not observed in NheI-digested DNA from wild-type or \textit{sgs1Δ} cells (Figure 3-6). Instead, in \textit{SGS1+} cells, the majority of breaks at the NF1 palindrome are observed as \textit{MUS81}-dependent resolution (Figure 3-1). For reasons that are currently unknown, in \textit{sgs1Δ} cells, I observe a large proportion of axis-specific \textit{MUS81}-independent DSBs, suggesting that Sgs1 prevents Mus81-independent DSB formation. It has not been established if these are hairpin-capped. I suspect that the main mechanisms of DSB break formation of the NF1 palindrome are cruciform resolution by Mus81 and a second unknown resolution activity. Therefore, Sgs1 prevents cruciform resolution as well as the elusive break formation pathway observed in pPX.

3.4.4 Palindrome-provoked genome instability

The impact of palindromes on human genome integrity is only beginning to be understood. The discovery of several disease-associated palindromes and near-palindromes has motivated efforts to determine how palindromes contribute to rearrangements in the genome and what, if any, preventative measures exist to circumvent palindrome-provoked damage. One proposed method of palindrome "repair" is defined as "revision". A chromosomal 15.6kb perfect palindrome acquires small, asymmetric, stabilizing deletions in a transgenic mouse line [(Cunningham et al., 2003) and reviewed in (Lewis and Cote, 2006)]. Importantly, gross genomic rearrangements, such as translocations, gene amplifications, chromosome arm loss, or very large
deletions, have never been observed (Akgün et al., 1997; Lewis et al., 1999; Zhou et al., 2001). Therefore, palindrome revision events observed in the Line 78 palindrome may be intentional and protective. By deliberately converting a perfect palindrome into an interrupted inverted repeat, cruciform extrusion is prevented.

Cruciform intrusion by RecQ helicases can be considered as a mode of cruciform stabilization. The Sgs1 helicase, having orthologs in higher eukaryotes, is now a strong candidate for driving the intrusion process. The Bloom's syndrome helicase, one of five RecQ helicases in humans, has roles in recombination, replication, and repair (reviewed in (Brosh and Bohr, 2007)). As there exists much conservation in the repair proteins already implicated in cruciform processing [Sae2(Ctp1), the Mre11-Rad50-Xrs2(Nbs1) complex, Mus81, and now Sgs1-Top3-Rmi1(Blm-TopIIIα-Blap75)], findings in the yeast system will surely have significance in human palindrome-provoked genome aberrations.
3.5 Experimental Procedures

3.5.1 Strains and Media

All yeast strains are derivatives of the BY4741 strain. All single mutants are haploid MATa strains from the Yeast Knockout (YKO) Collection (Saccharomyces Genome Deletion Consortium). Genes tested in the candidate screen are listed in Table S1. Strains were grown in YEPD or in synthetic complete media lacking uracil. top3Δ and rmi1Δ strains, MCY329 and MCY309 respectively, were a gift from Dr. Grant Brown (University of Toronto). Double and triple mutants were created using the one step gene replacement with a PCR-amplified cassette. sgs1Δ rad51::HIS3 (SML100) and mus81Δ rad51::HIS3 (SML101) strains were constructed through replacement of wild-type RAD51 of sgs1Δ and mus81Δ strains, respectively, with a PCR-amplified rad51::HIS3 amplicon from strain HKY1039 [a gift from S. Brill (Rutgers University)]. SML102 was created by replacement of wild-type SGS1 of SML101 with a PCR-amplified sgs1Δ::NATMX. The sgs1Δ::NATMX allele was first constructed by replacement of the KANMX marker of the YNO collection strain, sgs1Δ::KANMX, with a PCR amplicon of NATMX from plasmid pAG25. SML103 (mus81Δ sae2::HPHMX) was created by one step replacement of SAE2 of mus81Δ strain with a PCR amplicon of sae2::HPHMX from strain GRY2308. All strains were confirmed by PCR, demonstrating the absence of the wild-type gene and the presence of the substituted allele.

LSY716A, the mre11-H125N mutant and the corresponding wild-type strain, W303-1A are described in (Moreau et al., 1999). Cycloheximide (CHX)-resistant derivatives of W303-1A and LSY716A strains were isolated by plating 0.5mL overnight
cultures on dishes containing YEPD supplemented with 1µg/mL CHX and incubating at 30°C for 3 days. CHX-resistant mutants were confirmed by restreaking on CHX. Deletion of MUS81 in the LSY716A cyh2R and W303-1A cyh2R strains was performed using one step gene replacement with a PCR-amplified mus81Δ::KANMX cassette from the S. cerevisiae knock out collection strain. The kar1 strain, W2108-14C (MATα kar1Δ15 CAN1 CYH2 lys2Δ his3-11,15 ura3-1 trp1-1 leu2-3,112 ade2-1) was the gift of Dr. Rodney Rothstein (Columbia University). The mre11Δ strain is from the yeast deletion collection derived from BY4741 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0).

3.5.2 Assay plasmids

All test plasmids are derived from pYES2.1/V5-His-TOPO (Invitrogen) and are described in Chapter 2.

The RusA expression vector, pKR6980 (Bastin-Shanower et al., 2003) was kindly provided by S. Brill (Rutgers University). An empty version was prepared by double digest of pKR6980 with NdeI and BamH1 and blunt end re-circularization, thereby removing the RusA coding sequence.

3.5.3 Palindromic dimer plasmid (pPX) DNA preparations

Preparation and isolation of the palindromic dimer plasmid is described fully in Chapter 2.

3.5.4 Yeast plasmid DNA preparation

DNA was isolated from 10 ml cultures of S. cerevisiae grown in synthetic complete media lacking uracil using the method of Hoffman and Winston (Hoffman and Winston, 1987) except for those shown in Figures 3-4 and 3-5, which were isolated by spheroplasting as described in (Holm et al., 1986). Briefly, a 10mL culture was pelleted
by centrifugation and washed once with water. The pellet was resuspended in 150µl SCE buffer, 10µl Zymolase was added (3mg/mL Zymolase and 10% β-mercaptoethanol in SCE), and the cells were incubated at 37°C for one hour. The pellet was centrifuged for 10 seconds, drained, and resuspended in 150µl GuHCl solution (4.5M GuHCl, 0.1M EDTA, 0.15M NaCl, 0.05% sarkosyl, pH 8.0). The resuspended sample was incubated at 65°C for 10 minutes then 150µl ethanol was added. After centrifugation for 5 minutes, the pellet was drained and resuspended in 300µl 10X TE with 2µl 10mg/mL RNase A. The pellet was incubated at 37°C for 60 minutes. 5µl 10mg/mL proteinase K was then added and incubation was continued at 65°C for one hour. This was followed by two phenol:chloroform extractions and the aqueous phase was precipitated with ethanol. DNA was resuspended in 100µl TE.

3.5.5 Yeast transformations

Yeast transformations are described in detail in Chapter 2.

3.5.6 Plasmoductions

Figures 3-1 and 3-2 contain DNA samples from plasmoductants. Plasmoductions were performed as described in Georgieva and Rothstein (Georgieva and Rothstein, 2002) and are described in detail in Chapter 2. Plasmoductions of RusA plasmid recipient strains were performed as outlined except that matings were replica plated on media also lacking leucine. RusA plasmids were introduced by transformation into recipient strains, and single transformants were isolated prior to testing for complementation.

3.5.7 Nb.BsmI treatment

Nb.BsmI treatments were performed as described in Chapter 2.
3.5.8 ExoV treatment

Exonuclease V treatments were performed as described in Chapter 2.

3.5.9 Southern blot analysis

Southern blots were performed as described in Chapter 2. To avoid detecting the RusA expression vector, membranes shown in Figure 3-2 were probed with a 760bp fragment from the plasmid *URA3* open reading frame. This was generated by PCR with oligos 5'-GGAACGTGCTGCTACTCATCC-3' and 5'-CAAATAGCTTCCAGCCTGC-3'. The *URA3* probe hybridized to the same NheI-generated plasmid fragment as the pSH1-derived probe but in addition hybridized to chromosomal DNA fragments in the analyzed samples.
Chapter 4

Structural and genetic analysis of palindrome instability in mammalian cells

I performed all experiments shown. Cell lines were kindly provided by John Petrini (Sloan Kettering Institute).

The work outlined in section 4.3.1 is published in Molecular and Cellular Biology, Vol 23(23), Cunningham, L.A., Cote, A.G., Cam-Ozdemir, C. and S.M. Lewis. Rapid, stabilizing palindrome rearrangements in somatic cells by the Center-Break Mechanism, 8740-8750, Copyright (2003), American Society for Microbiology.
4.1 Abstract

Mammalian cells have the ability to activity rearrange palindromes using a process called revision. When an extrachromosomal palindromic dimer circle is introduced into cells, the dimer undergoes an axis-specific rearrangement process that results in the formation of monomer circles that can be recovered in bacteria. An ongoing question was the survivability of an intact palindrome in mammalian cells. In order to investigate the survival of perfect palindromes, I chose to determine the structure of the Line 78 transgene. Using digestion-circularization PCR followed by cloning and sequencing, I demonstrate that the Line 78 transgene contains a perfect symmetry center, proving that long perfect palindromes can persist in higher eukaryotes. Next, I wanted to determine if mutations in the Mre11-Rad50-Nbs1 complex affect the formation of revision products. A palindromic dimer was introduced into mouse cells expressing either $Mre11^{ATLD1}$ or $Nbs1^{AB}$ hypomorphic alleles. Each murine cell line produced revision monomers at frequencies that were not significantly different than those from wild-type cells. Sequence analysis of revision junctions recovered from the mutant cell lines showed that there was no qualitative effect on junction formation. Therefore, these mutations do not have a measurable effect on palindrome revision.
4.2 Introduction

There are two models used to investigate palindrome instability in mammalian cells. First, a mouse strain called "Line 78" has aided in understanding the underlying basis for palindrome instability. This mouse bears a 15.6kb transgene comprised of a tail-to-tail integration of an injected fragment (Figure 4-1). The Line 78 transgene undergoes a reproducible type of rearrangement and is characterized by the acquisition of central, asymmetric deletions (Akgün et al., 1997; Cunningham et al., 2003). After central modification occurs, no further rearrangement of the Line 78 transgene is detected (Cunningham et al., 2003). I participated in developing this model which provides an unparalleled opportunity to observe and track palindrome-induced rearrangement events by Southern blot analysis.

The second experimental approach is to transiently transfect a fully-palindromic circle into mouse cells and monitor instability by scoring the production of monomer circles (Figure 4-2) (Lewis, 1999). The assay is based upon the fact that the input dimer circles do not transform E. coli, but that the monomer revision products will. When introduced into mammalian cells, the perfectly palindromic plasmid substrate rearranges in a reproducible manner. The analysis of recovered products suggests that double strand breaks occur at each of the symmetry axes and that they are joined by non-homologous end joining. This approach is useful for identifying gene functions that affect palindrome instability.

Rearrangements observed in the Line 78 transgene and in the palindromic dimer are explained by the Center Break mechanism of palindrome revision (Lewis and Cote, 2006). Long palindromes extrude to create a cruciform structure and it is this structure,
rather than the palindrome itself, that is what undermines DNA function. In the Center-Break model, a cruciform is recognized and cleaved by a hairpin endonuclease. The double strand break is then repaired by end joining. Because end joining is an imprecise mode of repair, the palindrome is converted into an inverted repeat with a small central spacer.

An important but largely unexplored aspect of palindrome biology is their persistence in the genome. Perfect palindromes are lethal elements in all tested replicons in bacteria (Leach, 1994). Even some near-palindromes are unable to be stably maintained in *E. coli* (Lewis et al., 2005). I and others have demonstrated that long perfect palindromes can be maintained as episomes in the model eukaryote *S. cerevisiae* (Rattray, 2004). The question remained whether a non-microbial eukaryotic genome responsible for far more complex developmental programs was likewise tolerant of perfect palindromes, or whether, in this context, they are lethal. To date, no concrete evidence proved the existence of a long (longer than 200bps) perfect palindrome in mammalian cells nor was it certain that the Line 78 transgene was perfectly symmetric. It was possible that Line 78 contained very small modifications that bypass detection by Southern blot. This also places uncertainty in how the Line 78 rearrangement events are interpreted.

A second uninvestigated aspect of mammalian palindrome metabolism is the involvement of the Mre11-Rad50-Nbs1 complex in palindrome revision. Several groups have demonstrated that Mre11 plays a significant role in palindrome and inverted repeat metabolism in *E. coli, S. pombe*, and *S. cerevisiae* (Farah et al., 2005; Farah et al., 2002;
Lobachev et al., 2002; Rattray et al., 2001; Rattray et al., 2005). The potential role(s) of the MRN complex in mammalian palindrome instability has never been addressed.

Here, using digestion-circularization PCR followed by sequencing, I provide conclusive evidence that the Line 78 transgene contains a perfectly palindromic symmetry axis. This work demonstrates that, unlike in bacteria, long perfect palindromes can be maintained in a mammalian genome. By establishing the state of the intact Line 78 palindrome, we can make conclusions regarding spacer-induced stability. It strengthens the notion of spacer-induced stabilization of palindromes as proposed for Center-Break revision. I also show that hypomorphic mutations in Mre11 and Nbs1 that mimic those found in Ataxia Telangiectasia-Like Disorder (ATLD) and Nijmegen Breakage Syndrome (NBS), respectively, do not have a measurable effect on extrachromosomal dimer palindrome revision.
4.3 Results

4.3.1 The Line 78 transgene is a perfect palindrome

Up until the point at which I worked on this project, the configuration of the Line 78 transgene (Figure 4-1) was monitored solely by Southern blot, where the presence or absence of the central PstI site was determined. The transgene in the founder mouse had a PstI site in the center of the putative palindrome, and among its progeny, some retained the site and some rearranged as well. The method is not sensitive enough to detect very small central deletions or insertions that do not remove the central PstI site, therefore, the status of the Line 78 transgene in the founder and in apparently unrearranged offspring was uncertain. Prior studies have demonstrated that deletions as small as 20bps can occur that do not remove the central PstI site. Such small changes in band migration are exceedingly difficult to detect. It was a priority to confirm whether or not the founder transgene was indeed a perfect palindrome. In addition, it was important to design a method for identifying rearrangements that resulted in very small central insertions or deletions that were otherwise difficult to identify by Southern blot alone.

As diagrammed in Figure 4-1, the Line 78 transgene arose as a tail-to-tail integration of two PstI fragments. From Southern blot analysis, it appeared that the flanking PstI sites on each side of the transgene were intact. Therefore, I applied an approach called digestion-circularization PCR (DC-PCR) to determine the sequence of the symmetry center of the transgene. DNA from Line 78 cells was digested with PstI, ligated at a low DNA concentration to encourage intramolecular ligation events, and then used in a PCR reaction with transgene-specific oligos (Les1 and DC3) that would amplify
Digestion-circularization of the Line 78 transgene. The palindrome arose when two 7.8kb PstI fragments integrated in a tail-to-tail orientation. Each fragment contains two copies of a defective LacZ gene (blue). The outer PstI sites were lost upon integration (lost sites are in parentheses), however, there is a chromosomal PstI site ~1kb from each side of the chromosome/transgene flank (chromosome DNA is indicated by a wavy line). Digestion with PstI followed by ligation creates two circles, joining the chromosome sequence to the palindrome arm. PCR across the junction using transgene oligos (red) and chromosome-specific oligos specific for the left (blue) or right (green) flank separately amplifies the left and right palindrome arms.
across the PstI ligation junction (see Figure 4-1). Because prior studies suggested that the outer PstI sites of the transgene were intact, I expected a sole band of a predicted size if the transgene was a perfect palindrome. A sole band was amplified, however, it was ~1kb larger than expected. Cloning and sequencing of the PCR product revealed that the amplicon contained both a segment of transgene DNA and a 1kb segment of DNA from mouse chromosome 17 (accession no. NT_039658.1). Using primers specific for the left and right transgene flanks, this sequence was verified to be the integration site of the Line 78 palindrome (see Experimental Procedures). This analysis also revealed that the flanking PstI sites were in fact lost upon integration; the next PstI sites were found in chromosome 17 to the left and the right of the transgene, 1kb away from each transgene flank. Had the outer PstI sites of the transgene been preserved upon integration, the integration site of the transgene could not have been identified using the DC-PCR approach.

Now that the integration site was known, chromosome 17-specific primers were implemented in the DC-PCR strategy in order to individually amplify the left and right arms of the transgene (Figure 4-1 and Experimental Procedures). Individual PCR amplicons were generated, cloned, and sequenced. Analysis showed that the originally injected PstI fragment inserted tail-to-tail between two T's on mouse chromosome 17. The injected fragment and the integration site were completely preserved, except that the outer PstI sites were destroyed. Because the next chromosomal PstI sites flanking the transgene were equidistantly spaced from the transgene, only one band was observed from the original DC-PCR and from PstI-digested DNA analyzed by Southern blot. Sequence comparison of the left and right arms revealed that the Line 78 transgene
contains a perfect axis of symmetry, demonstrating that long perfect palindromes are not lethal in the mouse genome. The DC-PCR assay provides a means to more accurately define the nature of very small rearrangements and enables for a more exact measurement of palindrome rearrangement rates.

4.3.2 Quantitative analysis of palindrome revision in MRN mutant cell lines

With the finding that long perfect palindromes can be maintained in the mammalian genome, it strengthened the validity of the Center-Break Model because, as discussed in section 1.1, very small spacers can significantly impede upon cruciform extrusion. In order to further characterize the Center-Break mechanism, it was a priority to identify proteins that affect revision.

Because of Mre11's known roles in hairpin metabolism in bacteria and yeast, I was interested in determining if MRN played a role in revision in higher eukaryotes. Null mutations in Mre11, Rad50, or Nbs1 result in mouse embryonic lethality, however, mouse lines have been successfully created that harbor hypomorphic mutations in Mre11 and Nbs1 (Theunissen et al., 2003; Williams et al., 2002). Immortalized fibroblast cell lines from $\text{Mre11}^{\text{ATLD1}/\text{ATLD1}}$ and $\text{Nbs1}^{\text{AB/AB}}$ mice were used here to determine if these mutations have an effect on revision.

Mutant and wild-type cell lines were transfected with *in vitro*-prepared dimer derived from the pJH298 plasmid (Figure 4-2). The mammalian dimer is similar to the pPX dimer used in Chapters 2 and 3: they are both constructed by self-ligation of a linearized shuttle vector. Each is a fully palindromic dimer circle with two perfect symmetry axes. The cells were also transfected, in parallel, with the pJH298 source
Figure 4-2
**Dimer revision protocol in mammalian cells.** Two copies of a linearized vector are ligated in a head-head/tail-tail fashion to create a dimer circle. The plasmid is made from a mouse/E. coli shuttle vector. The large circular palindrome that is created *in vitro* cannot transform bacteria. The assay plasmid has a palindromic symmetry axis at each “pole” and thus possesses two sites where cruciform extrusion can occur. (i) The DNA is introduced into mouse cells by transfection, (ii) the cells are cultured for 2 days, and (iii) the DNA is harvested. (iv) In contrast to the assay plasmid, which is non-transforming for *E. coli*, monomer revision products are not palindromic and therefore, they can be isolated by bacterial transformation. DNA from the bacterial colonies is isolated for qualitative analysis of monomer formation.
plasmid as a transfection efficiency control. 48 hours after the transfection procedure, extrachromosomal DNA was harvested and one tenth of the harvest was used to transform *E. coli*. The total number of bacterial Amp<sup>R</sup> colonies (transformed with replicated DNA) recovered from each harvest are given in Table 4-1. The revision efficiencies for each cell line are depicted in Figure 4-3. Each cell line produced monomers efficiently, indicating that there was no major impact of these mutations on revision. The difference in revision frequencies for the mutants versus the wild-type controls did not reach significance (p=0.16 for the *Mre11<sup>ATLD1/ATLD1</sup>* cells and p=0.18 for the *Nbs1<sup>ΔB/ΔB</sup>* cells). Therefore, these mutant cells have no detectable change in revision frequency.

### 4.3.3 Qualitative analysis of revision events in MRN mutant cell lines

Even though it was found that the *Mre11<sup>ATLD1/ATLD1</sup>* and *Nbs1<sup>ΔB/ΔB</sup>* cells produced revision monomers at wild-type frequencies, it was possible that these mutations could have an effect on the revision joints. In order to analyze the fine structure of the revision products, monomers were recovered from each cell line and sequenced across the joint. The sequences of the joints are listed in Table 4-2. There was no gross difference in the fine structure of the joints recovered from either of the mutant cell lines when compared to wild-type. When each was assessed for deletion size, presence of BamHI and SalI sites (Figure 4-4), and microhomology usage (Figure 4-5), no difference was observed. Table 4-3 summarizes the characteristics for each set of sequenced joints. Taken together, these results demonstrate that the *Mre11<sup>ATLD1</sup>* and *Nbs1<sup>ΔB</sup>* mutations have no measurable effect on palindrome revision in murine cells.
Figure 4-3
Revision frequencies in MRN mutant cell lines. Error bars represent the standard error for 4 separate experiments (3 experiments were performed with the \( Mre11^{ATLD1/ATLD1} \) cells). The y axis represents the mean number of revision colonies normalized for the number of control colonies obtained in 4 separate experiments.
Table 4-1. Quantitative analysis of revision frequencies in MRN mutant cell lines.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. Revision colonies</th>
<th>No. control colonies</th>
<th>Revision efficiency*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mre11</em>+/+</td>
<td>33</td>
<td>848</td>
<td>0.0389</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>96</td>
<td>0.0208</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>265</td>
<td>0.0642</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>328</td>
<td>0.0579</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.0455</td>
</tr>
<tr>
<td><em>Mre11</em>ATLD1/ATLD1</td>
<td>1</td>
<td>345</td>
<td>0.0029</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>196</td>
<td>0.0306</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>30</td>
<td>0.0333</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.0223</td>
</tr>
<tr>
<td><em>Nbs1</em>+/+</td>
<td>4</td>
<td>1804</td>
<td>0.0022</td>
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<td>7</td>
<td>475</td>
<td>0.0147</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>202</td>
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<td>2546</td>
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</tr>
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<td>23</td>
<td>295</td>
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<tr>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.0870</td>
</tr>
</tbody>
</table>

*Revision efficiency is calculated as the no. revision colonies divided by the no. control colonies. In bold is the average revision efficiency.
Table 4-2. Sequences of revision joints from MRN mutant analysis. Underlined are sites of microhomology. Bolded are sequences from symmetry axis restriction sites. In red is inserted sequence. In green is inserted sequence derived from the plasmid.

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<th>(BamHl)</th>
<th>Clone ID</th>
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<td>-1261</td>
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<td></td>
<td>-921</td>
<td>1-11</td>
</tr>
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</tr>
<tr>
<td></td>
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<td>GCTC ATA CAAAAGGCTC</td>
<td>GGAATACGTCT</td>
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</tr>
<tr>
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<tr>
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<td>AGCTGGGCTGCAAGTC</td>
<td>AGGTGGGATCAGTC</td>
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<td></td>
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<td>T</td>
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Table 4-3. Summary of junction analysis of MRN mutant cell lines.

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<tr>
<th>Genotype</th>
<th>Insertions</th>
<th>Left side deletion</th>
<th>Right side deletion</th>
<th>Microhomology usage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>1-10</td>
<td>10+</td>
<td>Intact Sall</td>
</tr>
<tr>
<td>Mre11+/+</td>
<td>16</td>
<td>3 (39)</td>
<td>3 (39)</td>
<td>2 (13)</td>
</tr>
<tr>
<td>Mre11&lt;sup&gt;ATLD1/ATLD1&lt;/sup&gt;</td>
<td>5</td>
<td>1 (20)</td>
<td>0 (20)</td>
<td>0 (20)</td>
</tr>
<tr>
<td>Nbs1+/+</td>
<td>7</td>
<td>2 (29)</td>
<td>1 (14)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Nbs1&lt;sup&gt;^B/D_B&lt;/sup&gt;</td>
<td>30</td>
<td>4 (13)</td>
<td>1 (3)</td>
<td>4 (13)</td>
</tr>
</tbody>
</table>
Table 4-4. Primers used for DC-PCR of the Line 78 palindrome.

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
<th>3' end point</th>
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</thead>
<tbody>
<tr>
<td><strong>Transgene-specific</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Les1</td>
<td>CCT GTG TAG GTT CCA AAA TAT CTA GTG</td>
<td>Inward, 167bp to center axis</td>
</tr>
<tr>
<td>E1</td>
<td>AGC ATT ATC CTT CCA AAA TAT CTA GTG</td>
<td>Inward, 99bp to center axis</td>
</tr>
<tr>
<td>DC4</td>
<td>GAA GCT GAA AGG TGG ACA GGA AAC</td>
<td>Outward, 1,110bp to edge</td>
</tr>
<tr>
<td>DC5</td>
<td>GTC CAA CAA TCC AGC TTC AGG</td>
<td>Outward, 12bp to edge</td>
</tr>
<tr>
<td><strong>Chromosome-specific</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC171</td>
<td>CAT GGT AGG AAG CAT GGC AGC</td>
<td>Inward, 402bp to edge</td>
</tr>
<tr>
<td>AC172</td>
<td>GGA AAT GTG TCT AGC TCT CAC TGG C</td>
<td>Inward, 313bp to edge</td>
</tr>
<tr>
<td>AC173</td>
<td>CTTA TCA CAT TCA TGA CGC TGG CC</td>
<td>Inward, 202bp to edge</td>
</tr>
<tr>
<td>AC174</td>
<td>CCG TGG CAG TAA CCA TTA AGA GC</td>
<td>Inward, 148bp to edge</td>
</tr>
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<td>ACLF1</td>
<td>CTT TCC AGA TAC GAC GCA GG</td>
<td>Outward, 52bp to left PstI</td>
</tr>
<tr>
<td>ACRF1</td>
<td>GGT GAC ATC ACA GTC TAA GGA GG</td>
<td>Outward, 53bp to right PstI</td>
</tr>
</tbody>
</table>
Figure 4-4A
Proportion revision products with symmetry axis BamHI restriction site bases.

Figure 4-4B
Proportion revision products with symmetry axis SalI restriction site bases.

Blue; Mre11\(^{+/+}\), Red; Mre11\(^{ATLD1/ATLD1}\), Purple; Nbs1\(^{+/+}\), Green; Nbs1\(^{AB/\Delta B}\).
Figure 4-5
Proportion revision joints with microhomology.

Blue; Mre11^{+/+}, Red; Mre11^{ATLD1/ATLD1}, Purple; Nbs1^{+/+}, Green; Nbs1^{AB/AB}. 
4.4 Discussion

4.4.1 Long perfect palindromes are maintained in mammalian cells

Here, using DC-PCR (also known as inverse PCR), I demonstrate that the Line 78 transgene contains a perfect axis of symmetry. The structure of the transgene allowed for individual PCR amplifications of the left and right arms of the symmetry axis. This is the first example of a proven perfect palindrome in the mouse genome. Even though there are examples of suspected palindromic transgenes (Collick et al., 1996; Honchel et al., 2001), to date, no other perfectly palindromic transgene has been proven to exist in mammalian genomes. This piece of information was crucial for establishing a fundamentally important feature of higher eukaryotes: an enormous, perfect palindrome is NOT lethal as it is in *E. coli*. Thus, long perfect palindromes residing in mammalian genomes are not immediately eliminated. Such genomes can replicate and by persistency, can suffer palindrome-provoked rearrangements. The Center-Break mechanism of palindrome revision may therefore have evolved as a means to deal with problematic palindromes that are extrusion prone.

The discovery that experimentally-introduced long palindromes can be replicated in the mouse genome implies that such perfect palindromes may exist in the human genome. With the development of techniques for cloning and sequencing unstable human sequences, it will be possible to identify such palindromes.

4.4.2 Molecular genetic dissection of the Center-Break pathway

Here, I show that mutations in *Mre11* and *Nbs1* that mimic those found in patients with ATLD and NBS, respectively, do not affect revision of an extrachromosomal palindromic dimer. However, these results do identify specific roles of the MRN
complex that are not required for palindrome revision. \(Mre11^{ATLD1/ATLD1}\) and \(Nbs1^{AB/AB}\) cells exhibit chromosome instability, sensitivity to ionizing radiation, and possess multiple checkpoint defects (DNA damage induced checkpoint, G2/M checkpoint, and G1/S checkpoint), suggesting that in these cells, ATM function is impaired (Theunissen et al., 2003; Williams et al., 2002). Our laboratory has found that ATM-deficient cells are functional with respect to palindrome revision (unpublished data). Therefore, the findings here strengthen the proposal that ATM signaling is not required for efficient palindrome revision.

Although not tested directly, I suspect that the nuclease activity of Mre11 is not compromised in \(Mre11^{ATLD1/ATLD1}\) cells. The \(Mre11^{ATLD1}\) allele results in the production of a 75 amino acid truncated protein and still retains an intact N-terminus harboring the nuclease domain (Theunissen et al., 2003). Because I have determined that nuclease activity of Mre11 is involved in palindrome processing in yeast, it would be ideal to test revision in Mre11-nuclease dead cells.
4.5 Experimental Procedures

4.5.1 DC-PCR analysis of the Line 78 transgene

Genomic DNA was digested to completion with PstI. After heat inactivation, samples were ligated overnight at room temperature at a concentration of 7µg/ml. The next day, samples were ethanol precipitated and resuspended in 100µl TE. One µl (approximately 100ng) DNA was used to template two PCR reactions: the left side was amplified using Les1 and ACLF1 oligos and the right side using Les1 and ACRF1. PCR reactions contained the following: 1X PCR buffer, 3mM MgCl2, 0.2mM dNTPs, 0.4mM oligos, and 2.5U Taq polymerase (Invitrogen). 1µl of each PCR sample was used in a semi-nested PCR reaction using the same conditions as above except that primer E1 was used instead of Les1. Primer sequences are listed in Table 4-4. PCR products were cloned using the Invitrogen TA-cloning kit following the manufacturer's recommendations.

4.5.2 Identification of the Line 78 transgene integration site

Each side of the Line 78 integration site was amplified using nested PCR. To amplify the left side, the first PCR reaction contained oligos AC173 and DC4 and was used in a nested reaction containing oligos AC174 and DC5. The right side PCR contained primers AC171 and DC4 and was used in a nested PCR with primers AC172 and DC5. Reaction conditions are identical to those listed above. Oligo sequences are listed in Table 4-4.

4.5.3 Dimer preparation

The dimer was prepared from the large BamHI to SalI fragment of the polyoma-based shuttle vector pJH298 (Hesse et al., 1989). DNA was purified from a 0.8% agarose
gel containing 0.5µg/ml ethidium bromide. The DNA was extracted with a Geneclean DNA isolation kit following the manufacturers protocol. I verified that this method removed residual ethidium bromide (not shown). 50µg of DNA was incubated in 1X T4 DNA ligase buffer with 2000 units T4 DNA ligase (New England Biolabs) in a final volume of 500µl for 16 hours at room temperature. The dimeric circle was again gel purified.

4.5.4 Cell lines

Cell lines were kindly provided by John Petrini (Sloan Kettering). Nbs1<sup>AB/AB</sup> and Mre11<sup>ATLD1/ATLD1</sup> SV40-immortalized fibroblasts are described in detail in (Williams et al., 2002) and (Theunissen et al., 2003), respectively. Each was maintained in DMEM (high glucose; GIBCO) supplemented with 10% FBS.

4.5.5 Transfections

~80% confluent 10cm dishes were washed twice with 1X PBS and then once with TBS-D (TBS + 0.1% dextrose) solution. 1mL transfection solution (TBS-D with 250ng DNA and 1mg/ml DEAE-dextrose) was added to each plate dropwise and incubated at room temperature for 1hr. Cells were then washed once in TBS-D then twice in 1X PBS. Fresh DMEM + 10% FBS was added to each plate and cells were incubated for 48 hours.

4.5.6 Transfection harvests

Cells were washed once with PBS and were trypsinized and pelleted in an Eppendorf tube. Harvests of plasmid DNA was performed using a rapid alkaline lysis protocol as previously described (Lewis, 1999). DNA was resuspended in 20µl TE.

4.5.7 Monomer recovery and analysis
2µl harvest from dimer-transfected cells or 5µl DpnI-digested harvest from pJH298 transfections (digests contained 1X buffer, 4µl harvest, and 5U DpnI and were incubated for 1 hour at 37°C) were used to transform chemically competent DH10B. Ampicillin-resistant colonies were selected. Plasmid DNA was isolated from AmpR colonies was isolated using the GenElute Plasmid Miniprep Kit (Sigma). Monomers were first mapped by restriction digest analysis then revision junctions were sequenced by Macrogen Inc. (Seoul, Korea).
Chapter 5

Summary and Future Directions
5.1 Thesis Summary

5.1.1 Cruciform extrusion in yeast chromatin

For over 20 years, the notion of *in vivo* cruciform extrusion has been debated. Even though several groups provided evidence for *in vivo* cruciform extrusion, the studies were not universally accepted as definitive proof (discussed in section 1.1.4). In Chapter 2, I provided the strongest evidence to date that both an *in vitro*-prepared perfect palindrome and a near-palindrome from the human genome extrude cruciform structures when present in a yeast plasmid. Extrusion is monitored by observing the products of cruciform metabolism. This biological readout is the cross-diagonal cleavage of a four-way DNA junction. Such an event yields a linear molecule that is capped on each side by a hairpin. Such a molecule can be easily distinguished from other “open-ended” DNA species by its resistance to degradation by *M. luteus* Exonuclease V. The cruciform resolution products are only observed in yeast mutants deleted for *SAE2* or *MRE11*, whose gene products are implicated in the removal of hairpin-capped DNA ends. In order to refute the possibility that extrusion occurred prior to establishment of the plasmid within the cell, I introduced the plasmids into *sae2* and *mre11* strains by plasmodyduction (Figure 2-18).

Proving the occurrence of *in vivo* cruciform extrusion in chromatin strengthens the validity of cruciform-provoked models of human genome aberrations. It also identifies a new source of endogenous double strand breaks. Cruciform extrusion followed by resolution results in the formation of a product that will resist repair unless the cell has functional hairpin processing activity. As demonstrated here and by others (Lobachev et al., 2002; Narayanan et al., 2006; Rattray et al., 2005), the consequences of
first producing hairpin-capped DNA ends but then eliminating their repair can have devastating effects to genome stability.

With the design of a technique for monitoring \textit{in vivo} cruciform extrusion, it can be implemented: i) to examine the extrusion capabilities of other palindromes and near-palindromes in the human genome, and ii) to determine the length and symmetry requirements of cruciform extrusion \textit{in vivo} (see section 5.3.1 and 5.3.2).

5.1.2 A novel palindrome-provoked DNA amplifying process

If not repaired by Sae2 and the MRX complex, hairpin-capped double strand breaks can provoke the formation of amplifying genome aberrations (Narayanan et al., 2006). In Chapter 2, I showed that a linear hairpin-capped DNA molecule is replicated to become a palindromic dimer circle with two perfect symmetry axes and results in the doubling of DNA copy number (Figure 2-8). I observed that the newly formed palindromic symmetry axes can subsequently extrude cruciforms that are resolved, forming an inverted dimer linear molecule that is also hairpin-capped (Figure 2-8). In theory, ongoing cycles of extrusion, resolution, and replication would result in an exponential increase in DNA copy number. Such a mechanism of DNA amplification could be instigated in mammalian cells through extrusion and resolution of two cruciforms within a single chromosome, resulting in the “Lift-Out” of a hairpinned linear extrachromosomal molecule that could undergo rounds of replication, extrusion, and resolution (Figure 2-24). Lift-out can produce copy number increments even in cases in which recombination and non-homologous end joining are compromised (discussed in section 2.4.4).

5.1.3 Variable extrusion in variants of the human NF1 palindrome
The Neurofibromatosis-1 palindrome, associated with the t(17;22) translocation, is highly polymorphic (Lewis et al., 2005). In Chapter 2, I assessed in vivo cruciform extrusion for five variants of the NF1 palindrome each with differing lengths and degrees of symmetry. I showed that levels of the products formed by cruciform resolution vary between individual alleles. Assuming that cruciform cleavage is not saturated so that the amount of hairpin-capped linear species present in mre11 cells is directly related to the number of cruciforms available for cleavage, this means that cruciform extrusion and resolution are measurably different between variants (Figure 2-21). There are several implications. First, these findings demonstrate that a human palindrome involved in a recurrent translocation extrudes a cruciform, supporting the proposal that extrusion of a cruciform at 17q11 provokes t(17;22) (Kurahashi et al., 2006b). Furthermore, the variation in extrusion between NF1 alleles demonstrates that not all NF1 variants are prone to such secondary structure transitions. More generally, these findings demonstrate that individuals have differing risks of cruciform extrusion and extrusion-instigated instability (Kato et al., 2006). This may signify the importance of a largely overlooked type of DNA arrangement that, depending on the variants present, results in heterogeneous instability within the population.

5.1.4 Mus81 resolves cruciform structures in mitotic cells

Both in vitro and in vivo evidence for Mus81’s role in the resolution of four-way junctions has been the subject of much controversy (for a discussion, see (Ehmsen and Heyer, 2008)). Biochemical work on Mus81’s substrate specificity shows that it possesses relatively low activity on intact four-way junctions, while its preferred substrate is a nicked four-way junction as well as other types of three-way junctions (for
example, see (Ehmsen and Heyer, 2008; Gaskell et al., 2007)). This finding prompted the proposal that the primary role of Mus81 is to cleave D-loop recombination intermediates rather than Holliday junctions (Gaskell et al., 2007). In Chapter 3, ~90% of the cruciform resolution activity that I measured in mitotically-growing budding yeast requires the Mus81 endonuclease (Figure 3-1). I suspect that Mus81 is responsible for performing cross-diagonal cleavage of the cruciform four-way junction. My work provides evidence that Mus81 is competent for resolution of an intact Holliday junction mimic in mitotically growing cells.

As shown in Chapter 3, I detect evidence for a second cruciform resolution activity in mitotically growing cells (Figure 3-1). As discussed in section 3.4.3, each cruciform resolvase may preferentially cleave the cruciform at a particular stage of the cell cycle and/or may recognize a specific cruciform conformation. Proposed experiments for identifying gene(s) responsible for regulating Mus81 as well as methods for determining Mus81's preferential resolution conditions are listed in section 5.3.3. Strategies for the identification of the second resolvase are presented in section 5.3.4.

5.1.5 The Sgs1 helicase prevents palindrome-provoked DSBs

In Chapter 3, I presented the results of a screen of candidate genes for an altered ability to maintain a palindromic plasmid. Among the candidates, sgs1, top3, and rmi1 mutants were severely compromised in the maintenance of pPX (Figure 3-5). Sgs1 is the sole RecQ helicase in budding yeast. Furthermore, I found that in strains deleted for SGS1, TOP3, or RMI1, breakage of the NF1 palindrome is also increased (Figure 3-6). This work is the first in eukaryotic cells identifying genes that play a role in palindrome metabolism other than hairpin processing. It also defines a new role for Sgs1-Top3-Rmi1
in the maintenance of genome instability; it is required to prevent resolution of extruded cruciforms in continuous DNA, possibly through the "intrusion" of cruciforms, thereby removing the DNA four-way junction target of resolvases. Strategies for: i) further characterizing the observed DSBs in sgs1Δ cells, and ii) identifying genes responsible for generating DSBs that are normally prevented by Sgs1 are presented in section 5.3.4.
5.2 Palindrome revision as genome-maintenance

As reviewed in Chapter 1, mammalian cells possess a means with which to convert extrusion-prone sequences into inert ones called palindrome revision. This is accomplished by a relatively small modification of the palindrome’s symmetry center. In the process of accumulating the data presented herein, I did not observe any evidence suggesting that revision was occurring at the NF1 palindrome. Moreover, revision was not observed for the pPX palindromic dimer. I have limited evidence for the occurrence of a single revision event at one of the symmetry axes of pPX, however, further experiments are required before this can be confirmed. Regardless, revision at a frequency resembling that in mammalian cells was never observed.

Why doesn’t budding yeast revise palindromes while higher eukaryotes actively perform revision? First, the discrepancy may be related to genome composition. The yeast genome possesses few, if any, long palindromes or near-palindromes (Lisnic et al., 2005). If an extrusion-prone sequence arises, for example, as a consequence of replication error, it could be rapidly purged from the population. As a unicellular organism, a palindrome that causes ongoing DSB formation would reduce fitness and prevent its persistence in the population. In contrast, higher eukaryotes do contain such sequences (Lewis et al., in preparation; Lu et al., 2007), one of which has been experimentally verified to extrude here. Perhaps higher eukaryotes acquired machinery to revise palindromes as a response to the genome’s accumulation of palindromic arrangements. Our ability to live with palindromes may be due to a difference in extrusion between cell types with varying transcriptional profiles; a palindrome may lie
"dormant", being passed on through the germline while in certain cell types of the soma, it extrudes and creates problems.

Therefore, upon introduction of a cruciform-forming palindrome in budding yeast, it is rapidly resolved, hairpin ends are opened, and repair by homologous recombination restores the palindrome. Ongoing breakage would eventually select against its persistence in the population. Their first (and perhaps only) line of defense again cruciform resolution is through cruciform "intrusion" by the Sgs1-Top3-Rmi1 complex.

In mammalian cells, an extruded cruciform may be revised if not first intruded by Sgs1-Top3-Rmi1. The revision pathway may directly target the cruciform itself, preventing resolvases from accessing the four-way junction (Figure 5-1A) or revision may instead come into play after resolution (Figure 5-1B). In either case, the goal of revision is to eliminate the structure-prone features of the palindrome. If revision is compromised, unbridled resolution can occur, leading to the occurrence of chromosomal aberrations. The examples of palindrome-associated translocations and large deletions observed in the human genome may be examples of revision gone awry or may be caused by a complete absence of revision, allowing for resolution to target cruciform structures.

If yeast do not revise palindromes, then why utilize budding yeast as a model to study palindrome metabolism? It is possible that other palindrome-provoked processes such as cruciform resolution or intrusion may overlap significantly between yeast and higher eukaryotes. An exciting avenue of palindrome research will be to determine the potential role(s) of mammalian Mus81 in instigating resolution and of Blm in its prevention. In higher eukaryotes, Sae2 and Mre11 may not only open hairpins after
cruciform resolution, they may cleave the hairpins of the cruciform itself, initiating revision (Figure 5-1). Unfortunately, the impact of these proteins on mammalian palindrome revision will remain a mystery until additional research groups tackle such endeavors.
Figure 5-1
Possible mechanisms of palindrome revision in the prevention/interruption of resolution. Left: Revision independent of resolvase activity. i-ii) Torsional strain induces extrusion, (iii) the hairpins are tethered by the MRN complex, (iv) a single strand nick is introduced at each hairpin terminus, (v) branch migration resorbs the cruciform, revealing the double strand break, (vi) deletion of sequence and other end processing steps, and (vii) ligation reconnects the ends. Right: Cruciform resolution followed by Center-Break revision. Steps i, ii (induction of a cruciform) occur as to the left following which resolvase attacks the cruciform structure. (iiiia) Tethering by Center-Break revision proteins occurs concurrent with resolvase attack or (iiib) after the cruciform has been fractured. Because DNA ends are produced directly by cruciform resolution, this scheme should not require branch migration.
5.3 Future Directions

5.3.1 Extrusion of other human palindromes and near-palindromes

The studies presented herein focus on one human palindrome situated on chromosome 17. Utilizing the procedures outlined in this thesis, other human palindromes and near-palindromes could also be assessed for their relative in vivo cruciform extrusion potential as measured under a uniform set of conditions. Palindromes and near-palindromes of high priority would include other palindromic AT-rich repeats and the β-globin gene palindrome. Such palindromes could be PCR-amplified, stably cloned and propagated in wild-type S. cerevisiae, then plasmiducted to sae2- or mre11-deficient strains. Because the chromosome 22 and 11 palindromes participate in the largest number of identified PATRR-related translocations (Kurahashi et al., 2006b), it is predicted that they would have the highest relative cruciform extrusion potential. If instead, PATRR-11 and PATRR-22 are found to be less extrusion-prone than the NF1 palindrome, it would suggest that factors other than cruciform extrusion propensity affect translocation. Regardless whether they are found to extrude more or less than other pathogenic palindromes, if they are found to extrude at all in budding yeast, the finding would strengthen the validity of cruciform resolution-based models of double strand break formation. Also, such a result would provide further evidence that cruciform-provoked rearrangements are a significant contributor to genomic instability.

5.3.2 In vivo characterization of sequence requirements in extrusion

The yeast cruciform resolution and escape assay provides a unique opportunity to perform analyses of the symmetry requirements for in vivo cruciform extrusion. A suitable first set of experiments would be to engineer constructs where the palindrome is
interrupted with an incrementing size of spacers (for example, having spacer sizes begin at 1bp and increase by 1bp until cruciform resolution is no longer observed). An absence of cruciform resolution would be achieved when the ratio of hairpin-capped linear species to intact circle equals that of a control plasmid. Other critical parameters govern palindrome extrusion that before now, have not been testable. These would include, palindrome arm length, arm mismatch content, AT content of the arms and of the spacer, and the presence of certain types of flanking sequences (such as AT-rich regions (Sullivan and Lilley, 1986)) in the vicinity of the palindrome in question. These studies could provide a framework with which to estimate the cruciform extrusion potential of their sequence of interest or to more accurately identify extrusion-prone sequences in the human genome. The information provides may also benefit the fields of personal genomics, as individuals could be potentially screened for predicted extrusion-associated disease risk factors.

5.3.3 Defining cruciform resolution activity of Mus81

From the results presented in sections 3.3.1, 3.3.2, and 3.3.7, I have been able to experimentally differentiate between three pathways responsible for generating double strand breaks at palindromes. Mus81-dependent resolution is one such mechanism and from genetic analysis, is observed to be the major route of DSB formation in $SGS1^+\ TOP3^+\ RMI1^+$ cells (Figure 3-1). Because there are numerous pathways leading to DSB formation at palindromes, it would be ideal to determine the overall contribution of each to the total amount of instability that is observed. In order to determine the contribution of the Mus81-dependent pathway in palindrome instability, I propose to determine the cellular conditions in which Mus81 preferentially resolves the NF1
palindrome by determining if there is a preference with respect to a particular cell cycle stage and/or cruciform conformation. The former could be accomplished by examining suppression of resolution and escape in *mre11 mus81Δ* cells that are first synchronized by blocking cell cycle progression at the G1/S phase transition using alpha factor and then collecting samples at various time points after release from the block. Evidence from mammalian cells shows that Mus81 generates DSBs in order to restart stalled replication forks (Hanada et al., 2007). If it is found that Mus81 preferentially cleaves cruciforms during S-phase, this may signify that the protein is misrecognizing the cruciform as a stalled fork or that the role of Mus81 during S-phase is to remove DNA secondary structures that could impede upon fork progression.

As for other HJ resolvases, Mus81 may recognize a specific type of cruciform structure (open vs. folded, parallel vs. anti-parallel). Exploration of this idea would require solving the crystal structure of Mus81-Mms4 bound to an intact four-way junction then determining the conformation of the cruciform. Cruciform conformation structures have been characterized for numerous resolvases and have provided insight into the recognition of DNA substrates, however, it remains unknown if the protein i) recognizes and traps a structure already formed in free DNA or, ii) recognizes a structure in free DNA then induces the formation of a second structure in the DNA-protein complex (reviewed in (Declais and Lilley, 2008)).

The observation of palindrome-provoked resolution of the NF1 palindrome by Mus81 provides the opportunity to further characterize the regulation of Mus81’s cruciform resolvase activity. As discussed in (Ehmsen and Heyer, 2008), Mus81’s interaction with other proteins may be required for it to be shuttled into a particular
Therefore I propose to identify genes that, in addition to \textit{MUS81}, are required for cruciform resolution of the NF1 palindrome. A genome-wide screen could be performed in which a readout of escape could be detected through the escape-specific expression of a marker. Genes that when mutated suppress escape in a \textit{sae2} or \textit{mre11} background may include those required for activation of Mus81's nuclease activity or for its localization to cruciforms. It is also likely to detect mutants with an increase in escape, and may include genes that downregulate Mus81. A plasmid substrate that would allow such a screen is presented in Figure 5-2.

5.3.4 Continuation of studies on the Sgs1 helicase’s role(s) in preventing NF1 palindrome-provoked DSBs

Sgs1 is required to prevent high frequency DSB formation at both pPX and the human NF1 palindrome. Sgs1 suppresses breaks that are introduced at palindromes by at least two different pathways. This fits in with the proposal that Sgs1 acts upstream of break formation by counteracting the tendency of self-complementary sequences to assume alternative structures. I provided evidence for a second cruciform resolution pathway, as I have observed an incomplete suppression of resolution in \textit{mus81}Δ mutant cells (Figure 3-1). However, when shown that break formation at the NF1 palindrome was not fully suppressed in \textit{sgs1 rad51 mus81} cells (Figure 3-9), it was not determined if they arose from resolution or another mechanism because the breaks are open-ended.

Therefore, in order to determine if breaks observed in \textit{sgs1}Δ \textit{rad51}Δ \textit{mus81}Δ cells are generated by cruciform resolution, I propose to examine resolution products in an \textit{sgs1 rad51 mus81 sae2} mutant, where hairpin opening activity is compromised. In the event that this mutant is synthetic lethal, two possible alternatives are to test a \textit{top3 rad51}
Figure 5-2
Monomer substrate for screening for defects in palindrome resolution. The plasmid contains a URA3 gene (red) that is interrupted by an intron (green). The intron contains the NF1 palindrome. Resolution and escape will result in the formation of a dimer. This rearrangement would result in the elimination of URA3 gene expression. Cells having undergone resolution and escape could be screened for FOA resistance.


**mus81 sae2** strain or to introduce a conditional allele of sae2 into the triple mutant. The observation of hairpin-capped linear species in any of these mutants would prove the existence of a MUS81-independent cruciform resolution pathway.

Because elimination of MUS81 in sgs1Δ cells does not significantly diminish break formation in the NF1 palindrome, I suspect that deletion of a gene responsible for break formation in the MUS81-independent pathway(s) would result in a significant decrease in the observed amount of breaks. Therefore, I propose to perform a screen for candidate genes that, when mutated, reduce escape and/or DSBs in an sgs1Δ background. Such a screen may identify genes required for resolution and/or DSB formation in a MUS81-independent pathway(s). Candidates include TOP2 and YEN1 (Bermejo et al., 2007; Ip et al., 2008). The screen could be expanded to a genome-wide scale using the construct in Figure 5-2.

### 5.3.5 Determining the role of repair proteins in mammalian palindrome revision

Yeast Sgs1, Mus81, and Mre11 are highly conserved in eukaryotes. Additionally, the human homolog of Sae2, CtIP, has been recently identified and possesses significant functional overlap with its yeast counterpart (Sartori et al., 2007). Because of the known impact of these proteins on palindrome instability in single celled eukaryotes, I suspect that they also function in the metabolism of palindromes in higher eukaryotes. This could be tested in at least two ways: 1) determining their impact on extrachromosomal palindrome dimer revision in mammalian cells, and 2) determining their roles in revision and stability of the Line 78 palindrome. The palindromic dimer circle can be transfected into mutant cell lines as well as cells depleted of the protein of interest using small
interfering RNA technology. RNAi can also be used to knock down expression of a gene of interest in Line 78 transgenic cells. A priority would be to examine the fate of Line 78 as well as the dimer circle in cells depleted for Mre11 or CtIP.
5.4 Conclusion

The study of palindrome instability is in its infancy; the degree to which palindromes affect the integrity of the human genome is not understood. It is only recently that researchers have identified DNA palindromes as a class of arrangement associated with gross chromosomal aberrations in the human genome. Human studies barely moved beyond descriptive characterization of palindrome-associated translocations and deletions.

Studies aimed at characterizing palindrome instability in model organisms have been instrumental in providing clues into the relationship between palindromes and genome instability. Models that attempt at explaining palindrome-provoked genome aberrations all include the formation of a secondary structure in the instigation of the rearrangement process. One of the many obstacles that hinder the advancement of palindrome research is the fact that a palindrome can potentially adopt two secondary structures, hairpins and cruciforms, each of which could instigate several types of rearrangements. Such rearrangement pathways may possess overlapping and distinct properties; a challenge is to develop scientific means to tease apart and isolate one such pathway and characterize it solely in detail.

In this thesis, I make the first significant advance into establishing the underlying mechanism of cruciform-mediated instability in eukaryotes. Research of DNA secondary structure transitions is being pursued with increased interest (Smith, 2008). With the ongoing discovery of palindrome-associated diseases, the work presented herein will hopefully benefit the medical community.
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