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The Role of Cotranscriptional Recruitment of RNA-Binding Proteins in the Maintenance of Genomic Stability

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1.1
Introduction

All steps in transcription and pre-mRNA processing are extensively coordinated. The carboxy-terminal domain (CTD) of the large subunit of RNA polymerase (RNAP) II plays an important role in cotranscriptionally recruiting factors necessary for capping, splicing, polyadenylation, and other mRNA processing events [1–4]. The CTD acts as a platform for these factors to bind, and this process is coordinated by phosphorylation changes that occur during transcription [5]. Although transcription and RNA processing steps are not obligatorily coupled, as seen by the fact that these processes have been studied for many years as individual steps, some posttranscriptional modifications have been shown to be functionally coupled *in vitro* such as transcription capping [6] and transcription 3′ end processing [7]. There has also been recent evidence for "reverse coupling," where a proximal 5′ splice site enhances the recruitment of basal transcription initiation factors to the promoter [8]. While it is still unclear if transcription and splicing are functionally coupled in the cell [9, 10], there is evidence that cotranscriptional recruitment of serine-arginine (SR) proteins onto pre-mRNA is vital in maintaining genomic stability [11, 12]. Indeed, recent work shows that ASF/SF2, an SR protein first discovered for its role in constitutive and alternative splicing [13, 14], is a component of a high-molecular-weight (HMW) complex formed on pre-mRNA during cotranscriptional splicing assays [15], reflecting the early recruitment of ASF/SF2 and other SR proteins to nascent RNA during transcription.

All the steps in transcription and RNA processing appear to function together to produce an export-competent and translatable mRNP. In yeast, where splicing is less frequent, transcription is coupled to loading of export factors and mRNP formation through the THO/TREX complex [16]. Mutation of factors in the THO/TREX complex also results in genomic instability [16]. In metazoans, transcription is linked to mRNP formation through splicing [17], formation of the exon junction complex (EJC) [18, 19], and THO/TREX recruitment [16].

Below we will discuss the coordination of transcription and pre-mRNA processes that inherently protects the genome from invasion of nascent RNA into DNA of

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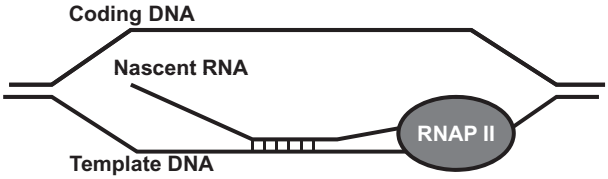

Figure 1.1 Schematic of a cotranscriptionally formed R-loop structure. Nascent RNA hybridizes with template DNA, leaving coding DNA single stranded.

the transcribing locus. The invading RNA can then hybridize to the template DNA, producing an aberrant R-loop structure, leaving the coding strand of the DNA single stranded and subject to DNA damage and strand breakage (Figure 1.1). We will describe other examples of cotranscriptionally formed R-loops and speculate on mechanisms that cause such structures to lead to genomic instability.

1.2 THO/TREX

1.2.1 THO/TREX in *Saccharomyces cerevisiae*

The THO complex proteins were first discovered in genetic screens for their role in transcription elongation of GC-rich genes in *S. cerevisiae* [20]. The complex consists of Hpr1, Tho2, Mft2, and Thp2, which are recruited to elongating RNAP II complexes. In addition to impairment of transcriptional elongation, THO mutants cause reduced efficiency of gene expression and an increase in hyper-recombination between direct repeats [21]. Mutations in *hpr1*, *tho2*, and *mft1* can also produce export defects and retention of transcripts at sites of transcription [16]. This reflects the association of THO with export factors Yra1 and Sub1 to form the TREX complex (transcription/export). TREX is recruited early to actively transcribing genes and travels entire length of genes with RNAP II [16]. Interestingly, mutants of the export machinery, Sub2, Yra1, Mex67, and Mtr2 also have THO-like phenotypes of defective transcription and hyper-recombination [16]. Further investigation of 40 selected mutants representing various steps in biogenesis and export of mRNP showed a weak but significant effect on recombination and transcript accumulation [22]. In particular, mutants of the nuclear exosome and 3′ end processing machinery showed inefficient transcription elongation and genetic interactions with THO. The TREX complex exemplifies the importance of the link between transcription and export-competent mRNP formation in yeast with the maintenance of the genomic integrity.

Further investigation of the association of the yeast TREX complex with actively transcribed DNA showed that the THO components play a critical role in the loading of the export machinery onto newly synthesized RNA [23]. Hpr1 was shown to associate with DNA templates through its association with the CTD. While Sub2 was only bound to nascent RNA, Yra1 was associated with both DNA

and RNA on intronless genes. Yra1 is recruited to THO and helps to load Sub2 onto the nascent RNA. While Hpr1 was able to associate with both intronless and intron-containing templates similarly, there was a large decrease in the ability of Yra1p and Sub2 to be deposited onto the intron-containing RNA. Data suggested that spliceosome assembly interfered with the transfer of TREX components onto the RNA in these *in vitro* transcription assays.

1.2.2 THO/TREX in Higher Eukaryotes

Recruitment of TREX may not be transcription coupled in mammals but coupled to splicing instead [24]. When Tho2 immunodepleted HeLa nuclear extracts were used for *in vitro* transcription, there was no elongation defect detected, as was seen in yeast. There was also no effect on spliceosome assembly, splicing, or RNA stability even though all components of the THO complex have previously been detected in purified spliceosomes [25]. Immunoprecipitation assays showed that human Tho2 only associated with *in vitro* spliced mRNA but not unspliced pre-mRNA. Further *in vitro* experiments showed that TREX bound to the 5′ cap-binding complex (CBC) in a splicing-dependent manner [26]. Immunoprecipitation assays showed that TREX preferentially associated with *in vitro* spliced and capped mRNA compared with uncapped or unspliced. The interaction of TREX with the 5′ cap is mediated by protein–protein interactions between REF/Aly and CBP80. Microinjection of these preassembled mRNP into *Xenopus* oocytes showed then to be export competent.

The above *in vitro* data seem to conflict with the *in vivo* data produced by Hrp1 depletion in HeLa cells. While it is evident that export-component REF/Aly directly interacts with CBP80 in a splicing-dependent manner, Hpr1 associates with DNA not RNA in yeast [23], so it is possible that recruitment of hHpr1 and hTho2 to the CBC in the immunoprecipitation assay may be due to their affinity for REF/Aly. Also, in *Drosophila melanogaster*, only the depletion of both *THO2* and *HPR1* by siRNA shows significant nuclear accumulation of poly(A)+ RNA [27], which could signify their divergent roles in transcription elongation and formation of export-component mRNP. THO is essential for heat-shock mRNA export in *D. melanogaster*, which may perhaps reflect its role in stress conditions. If the recruitment of REF/Aly is only dependent on cap formation and splicing, this might also explain the THO-independent recruitment of UAP56 in *D. melanogaster*. In any event, these data together indicate that the recruitment of the export machinery in higher eukaryotes is not linked to THO/TREX in a manner similar to *S. cerevisiae*.

1.2.3 THO/TREX and R-loop Formation

How do defects in THO/TREX cause hyper-recombination? Huertas and Aguilera proposed that mutations affecting THO/TREX components cause cotranscriptional production of aberrant R-loop structures [28]. They provided evidence of R-loop formation utilizing a hammerhead ribozyme to release hybridized nascent RNA.

This ribozyme was able to suppress transcription elongation impairment and hyper-recombination phenotype in THO mutants. Further evidence was provided by overexpression of RNase H to degrade RNA moiety of RNA:DNA hybrids, which also suppressed the THO phenotypes [28]. A more recent study of the point mutant *hpr1-101*, which has a transcriptional defect but does not cause R-loop formation, shows no hyper-recombination phenotype. This indicates that while the transcriptional defect by THO mutants may be further aggravated by R-loops, they are not mediated by them. The RNA:DNA hybrids do appear to lead to the hyper-recombination phenotype associated with THO mutants [29].

Therefore, in yeast, early recruitment of THO/TREX plays a key role in protecting against or preventing the formation of R-loop structures. In mammals though, the late recruitment of THO/TREX suggests that it plays a less important role, or perhaps no role, in protecting against genomic instability. This points to a possible role for earlier cotranscriptional processes in protecting the genome from DNA damage.

1.3 Linking Transcription to Export of mRNP

Early in transcription, the cell is already preparing to package nascent pre-mRNA into mRNP. As mentioned earlier, the RNAP II CTD coordinates the recruitment of RNA processing factors to transcribing genes. Spt5, a subunit of the DRB sensitivity-inducing factor (DSIF) transcriptional elongation factor, plays an early role in integrating the various steps of pre-mRNA processing to guarantee an export-competent mRNP. Immediately after transcription is induced, Spt5 helps to recruit a capping enzyme (CE) [30, 31], which is then activated together with the phosphorylated CTD to cap the 5′ end of the growing pre-mRNA (Figure 1.2a). *In vitro*, the recruitment of CE has been shown to cause the formation of R-loops [32]. However, these may be prevented by the recruitment of ASF/SF2 to the RNA, which prevents the formation of these aberrant RNA:DNA structures [11, 32]. Spt5 has been implicated not only in transcription elongation [33], CE recruitment, and splicing [34, 35], but also in transcription-coupled repair [36] and the recruitment of the exosome [37], which plays a key role in mRNP quality control [38, 39].

1.3.1 The Thp1-Sac3-Sus1-Cdc31 (THSC) Complex

In *S. cerevisiae*, in addition to THO/TREX, another complex has been shown to play a role in the export of properly formed mRNPs. The Thp1-Sac3-Sus1-Cdc31 (THSC) complex was shown to be recruited to transcribing genes to localize active genes to the nuclear periphery [40–42]. Sus1 is also a component of the SAGA histone acetylase complex, which is involved in facilitating transcription initiation [43]. Sus1 acts to link transcription to localization of actively transcribing genes to the nuclear pore. Deletion of *sus1* but not other genes coding SAGA components

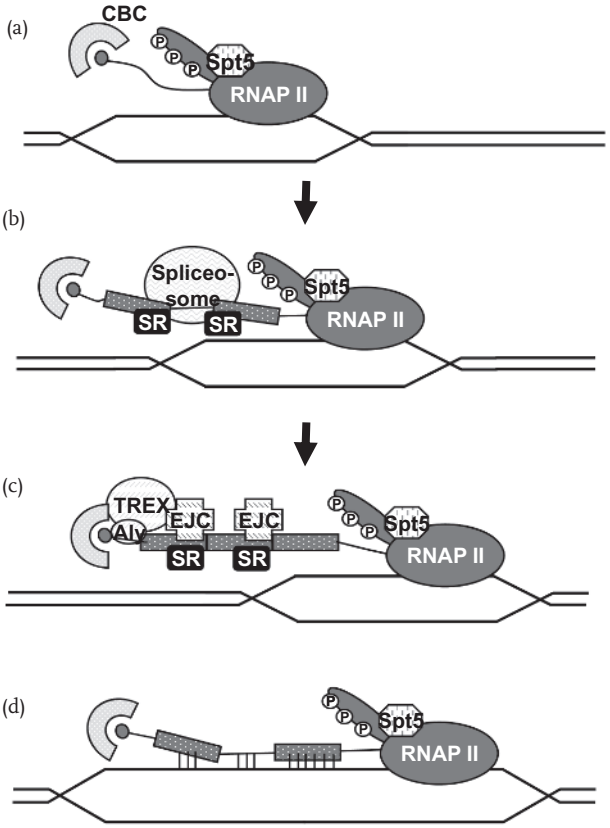

Figure 1.2 Early steps in cotranscriptional mRNA processing protect against genomic instability. (a) The phosphorylated CTD of RNAP II, along with Spt5, recruits a capping enzyme to cap the 5′ end of a growing pre-mRNA. These steps work to (b) cotranscriptionally load the spliceosome and SR proteins onto pre-mRNA to remove the introns. (c) The EJC is then deposited onto the mRNA in a splicing-dependent manner. REF/Aly and other components of the TREX complex are stabilized onto the 5′ end of the mRNA. (d) Disruption of any of these early steps of mRNA processing can leave nascent RNA open to bind to template DNA forming an R-loop structure.

confers a transcription-dependent hyper-recombination phenotype, similar to phenotypes seen in THO/TREX [40]. It is interesting to note that mutations in other nucleoporins lead to an accumulation of DNA breaks, visualized by Rad52 foci formation, but not in conjunction with transcription [44, 45].

1.3.2 SR Proteins

In metazoan cells, where most genes are spliced, SR proteins play an important role in linking transcription to spliceosome assembly in order to form an

export-competent mRNP. SR proteins can be recruited and cotranscriptionally loaded onto the pre-mRNA, indicated, in part, by interactions with the CTD of RNAP II [46] (Figure 1.2b). SR proteins can be recruited onto pre-mRNA *in vitro* by RNAP II but not by T7 RNAP during transcription assays [15]. Also, Li and Manley showed that ASF/SF2, SC35, and SRp20 can prevent the formation of cotranscriptional R-loop structures *in vitro*, most likely participating in mRNP formation on nascent transcripts and again in a CTD-specific manner [11]. It is not clear whether the function of SR proteins in the mRNP formation is directly related to their roles in splicing.

1.3.3 The Exon Junction Complex

The exon junction complex (EJC) is a complex of proteins that are deposited onto mRNA 20–24 nucleotides upstream of the exon–exon junction in a splicing-dependent fashion [47–51] (Figure 1.2c). The most recent biochemical and structural studies show that the EJC core complex consists of eIF4A3, the Y14:Magoh heterodimer, and MLN51 [47, 52–56]. All the core components have been shown to associate with the spliceosome [25]. eIF4A3 binds onto spliced RNA in a sequence-independent manner with an 8- to 10-nt footprint upstream of the splice junction [57–60] and acts as a platform for the other EJC core proteins. Various other proteins involved in nuclear export and non-sense-mediated decay have been shown to interact with this minimal core of the EJC. Early experiments showing the recruitment of the export factors UAP56, REF/Aly, and TAP/p15 to the EJC led to the belief that the EJC played a key role in export [18, 19], but later biochemical studies showed that RNPS1, REF/Aly, and UAP56 can load onto spliced RNA in the absence of the key component of the core EJC, eIF4A3 [17].

While the EJC factor is not a key factor in the recruitment of the export machinery, splicing has been shown to be mandatory for the loading of export factors [17, 26]. The core components of eIF4A3, Magoh, and Y14 also require splicing to take place in order to be deposited onto mRNA [17]. In addition, there is an enrichment of spliced mRNA in complexes containing REF/Aly and Upf3b/3a, indicating that splicing stabilizes the interactions of these proteins with the mRNA [17]. Although REF/Aly has been found to associate with the cap binding complex (CBC) on the 5′ end of the mRNA, association of the export machinery with mRNPs is splicing dependent [17] (Figure 1.2c). Therefore, splicing, not THO/TREX recruitment, plays an important role in loading of mRNP remodeling proteins to produce an export-competent mRNP.

1.3.4 The Exosome

The exosome plays a vital role in the recognition of mRNA that has not been properly capped, spliced, or 3′ end processed [38, 39, 61]. Specifically, mutations

in genes encoding THO components and components of the pre-mRNA 3′ processing complex, such as Rna14 and Rna15, can cause accumulation of improperly processed mRNA retained at sites of transcription [62]. This retention can be relieved by deletion of the gene encoding the 3′–5′ exonuclease Rrp6. Inserting a self-cleaving ribozyme can also relieve retention. Interestingly, these aberrant mRNPs can then be exported to the cytoplasm following cleavage, despite lacking properly processed 5′ ends. In higher eukaryotes, splice site mutations can also cause Rrp6-mediated nuclear retention of unspliced RNA [63, 64]. In cells expressing RNAP II with CTD truncations that affect splicing, a self-cleaving ribozyme can release an improperly processed mRNA from the site of transcription. The released transcript can then be properly spliced, while the small portion of mRNA 3′ of the cleavage site remains associated with the DNA [64]. This shows that pre-mRNA association with RNAP II is not required for splicing, but proper splicing is required for the release of mRNPs from sites of transcription in order to be exported. Interesting questions arise from these observations. Could R-loops form due to the lack of splicing? And might R-loops be a mechanism to recognize improperly processed transcripts?

1.4 Cotranscriptional R-loop Formation

It is noteworthy that defects in early steps in mRNA production appear to have a higher impact on genomic stability than steps later in the process. This is most likely due to the ability to cause cotranscriptional R-loops before the pre-mRNA is stably packaged into mRNP. As described earlier, transcription and splicing play important roles in the recruitment of the THSC, THO/TREX, and EJC, which are all loaded onto the pre-mRNA, necessary for proper export, and protect against deleterious RNA:DNA hybrid formations (Figure 1.2d). Deletion mutants of later pre-mRNA processing steps, such as 3′ end processing or quality control components of the 5′–3′ exosome, cause lower levels of hyper-recombination in deletion mutants [22]. Most likely this reflects the reduced opportunity to form R-loops since transcription has progressed to the far 3′ end of the gene.

To better understand the role of R-loops in the impairment of transcription elongation, R-loop formation was studied in an *in vitro* system [65]. RNA was hybridized to plasmid DNA to form a 300-bp R-loop structure, which was subsequently purified and studied using yeast whole cell extract. The artificial R-loops reduced transcription elongation efficiencies compared with plasmid DNA without these aberrant structures. Removal of the RNA moiety by RNase H prior to the transcription reaction improved efficiency directly proving that R-loops can cause deficiencies in transcription elongation *in vitro* [65]. Early pulse labeling experiments employing DT40 cells depleted of ASF/SF2, however, did not detect a transcriptional defect *in vivo*, suggesting that R-loops may not cause a significant elongation effect in vertebrates [66].

1.4.1
R-loops in *Escherichia coli*

Cotranscriptional R-loop formation was first suggested to occur in *E. coli* and has been extensively studied [67, 68]. RNase H was recovered in a genetic complementation screen of growth defects of topoisomerase mutants Δ*topA* in *E. coli* [69]. The ability of RNase H to digest the RNA moiety of RNA:DNA hybrids led to the belief that R-loops are involved in the observed growth defects. The negative supercoiling caused by deletion of *topA* leaves DNA more open for hybridization to RNA, most likely by nascent transcripts. Later, RNase H overproduction was shown to correct defects in transcription elongation [70] and reinstate full-length RNA synthesis in Δ*topA E. coli* [71]. Topoisomerase I is upregulated in the heat-shock response [72] likely to protect the accumulation of hypernegative supercoiling and R-loop formation at induced stress genes loci [73]. Interestingly, this is reminiscent of the need for THO in heat-shock poly(A)+ export in *D. melanogaster* [27]. The uncoupling of transcription and translation in *E. coli* by drugs that inhibit translation can also cause an increase in R-loop formation, suggesting that ribosomes in prokaryotes, like RNP structures in eukaryotes, play a role in inhibiting the re-annealing of nascent RNA to template DNA [74].

1.4.2
Naturally Occurring R-loops

Stable R-loops can also form during transcription through G-rich templates such as those used by Aguilera and colleagues in the THO/TREX mutant studies in yeast [28] and in the immunoglobulin (Ig) class switch region of mammalian B cells [75]. This is probably because rG:dC hybrids are exceptionally stable [76]. In addition, natural R-loops have also been shown to form both *in vitro* and in bacteria by transcription of the Friedreich ataxia triplet repeat, GAA-TTC, by T7 RNAP polymerase [77]. These repeats were able to form these natural R-loops in bacterial cells with normal levels of RNase H, showing that these hybrids formed more rapidly than they could be removed. We must keep in mind that T7 RNAP polymerizes at a rate of 200–400 $nt\,s^{-1}$ [78], while human RNAP II only transcribes at 15–20 $nt\,s^{-1}$ [79], which could explain the inability of RNase HI to digest the R-loops. Nonetheless, these results indicate that very stable RNA:DNA hybrids can form *in vivo*.

1.4.3
TREX Protects against R-loop Formation

When the recruitment of TREX was investigated in yeast, it was shown that Hpr1 is recruited to both intronless and intron-containing genes by RNAP II, but Yra1 and Sub2, while recruited efficiently to intronless genes, were poorly recruited to intron-containing genes [23]. Suppression by siRNA of human Hrp1 in HeLa cells, like its yeast homolog, caused gene expression and transcription elongation defects

[80]. These defects could be suppressed by expression of RNase H. Other studies showed that hTho2 immunodepleted HeLa nuclear extract did not show any transcription elongation defects [24]. These studies later concluded that the components of the human TREX complex could only be recruited via a splicing-dependent mechanism [26]. It was thus concluded that the EJC might play a role in the stabilization of the export machinery after splicing either by protein–protein interaction or remodeling of mRNP structure. This is consistent with the previously discussed enhancement of export machinery recruitment by EJC. While these studies show some discrepancies in the role of THO in transcription and R-loop formation in mammals, the data support the underlying need of cotranscriptional processes, either direct THO/TREX recruitment or recruitment of other factors, for example, SR proteins, to protect against deleterious R-loop formation and subsequent genomic instability.

1.4.4 SR Proteins Protect against R-loop Formation

As mentioned above, genetic inactivation of ASF/SF2, in the chicken B-cell line DT40, provided evidence of genomic instability, including the production of high molecular weight (HMW) DNA fragmentation, hyper-recombination, and G2 cell cycle arrest [11]. *In vitro* transcription experiments showed that cotranscriptional R-loops could be suppressed by a dose-dependent addition of ASF/SF2 in the presence of phosphorylated CTD. Later experiments showed that overexpression of RNPS1 can rescue phenotypes of HMW DNA fragmentation, hypermutation, and G2 cell cycle arrest of ASF/SF2-depleted DT40 cells, and can cause HMW DNA fragmentation when depleted from HeLa cells [12]. In contrast, SRp20 and SC35 were not able to or only partially able to rescue the HMW DNA fragmentation phenotype.

In ASF/SF2-depleted cells, it was shown that expression of RNPS1 alleviates genomic instability [12]. It is argued that since RNPS1 cannot compensate for ASF/SF2 function in splicing, RNPS1 has a role in forming RNP complexes on nascent transcripts to prevent R-loop formation. It was not investigated whether RNPS1 could prevent cotranscriptional R-loop formation *in vitro*. It is interesting to note that SRp20 and SC35 were able to partially rescue from R-loop formation *in vitro* [11] but incapable of suppression of HMW DNA fragmentation *in vivo* [12]. This could be a reflection of the role of recruitment of these factors onto the nascent transcript, for it has been shown that RNPS1 can be loaded onto RNA before the splicing reaction takes place.

RNPS1 has many functions beyond its role as a general splicing activator, especially in its diverse roles in the EJC. The cellular concentration of RNPS1 in HeLa cell strains appears to play a role in eliciting a strong non-sense-mediated decay response [81]. Another function of RNPS1 is the recruitment of Acinus and SAP18 to the EJC [54]. Acinus, SAP18, and RNPS1 are part of the apoptosis- and splicing-associated protein (ASAP) complex [82]. Microinjection of excess ASAP complex into cells causes an increased rate of apoptosis. It would be interesting if RNPS1

could function to sequester Acinus and SAP18 as a mechanism to protect against apoptosis during normal splicing and mRNP formation. If this was true, improper splicing or mRNP formation signaled by improper loading of RNPS1 and ASAP to RNA could increase the concentration of free ASAP in the cell to cause acceleration of apoptosis [82]. However, this is most likely be downstream of HMW DNA fragmentation, since ASAP only accelerates cells that are already stimulated for apoptosis. With the various functions of ASF/SF2 and RNPS1, it is difficult to distinguish the exact mechanism by which RNPS1 suppresses ASF/SF2 depletion-induced R-loop formation.

1.5 R-loop-induced Double-Stranded (ds) DNA Breaks

1.5.1 Class Switch Recombination

It is still unknown how R-loop formation can lead to dsDNA breaks in ASF/SF2-depleted cells and indeed by R-loops more generally. Investigation of naturally occurring R-loops in class switch recombination (CSR) in stimulated B cells may provide a hint to a mechanism. CSR is a DNA recombination event that switches DNA segments located upstream of each heavy chain constant region. CSR results in the switch of Ig isotype from IgM to either IgG, IgE, or IgA (reviewed in [83]). It has been shown that class switching is a transcription-dependent event that has R-loop structural intermediates [75]. Activation-induced deaminase (AID), a protein essential for CSR and somatic hypermutation, is expressed in activated B cells and specifically binds to G-quartets, tertiary structures on single-stranded (ss) DNA composed of four stacked guanidines [84]. Interestingly, it has been shown that the RNA exosome targets AID to these DNA strands [85]. Cytidines on ssDNA can then be deaminated by AID [86, 87]. Uracil, the product of cytidine deamination, is then removed by uracil-DNA glycosylase, which results in a nick or ssDNA break [88].

1.5.2 Formation of Double-Strand Breaks

How are such ssDNA breaks converted to dsDNA breaks, which then lead to DNA rearrangements, hyper-recombination, and genetic instability? There are several possibilities. For example, if two ssDNA breaks on opposing sides are in close proximity, it will form a dsDNA break. In bacteria, if an ssDNA is not properly repaired, it can be converted into dsDNA breaks [89]. This has been proposed to be by collision of the DNA replication machinery with barriers to its procession, leading to fork collapse, which will result in dsDNA breaks. Barriers can include protein bound to dsDNA or aberrant DNA structures, such as nicked DNA or possibly R-loops.

The HMW DNA fragmentation observed in the ASF/SF2-depleted DT40 [11] may be caused in conjunction with AID, whose expression is limited to activated B cells. Indeed, in *S. cerevisiae*, exogenously expressed AID activity strongly stimulated hyper-recombination by R-loop forming THO mutants [40]. However, depletion of ASF/SF2 by siRNA in HeLa cells also produced DNA breaks [11], and it is believed that dsDNA breaks occur generally when R-loops form. It is possible that related proteins in the AID family could function similarly to AID itself in these and other cell types (reviewed in [90, 91]). AID family member APOBEC1, an RNA-editing enzyme expressed in colorectal tissue, can also deaminate cytidine of ssDNA *in vitro* [91]. Overexpression of APOBEC1 induces cancer in these cells [92], reflecting its ability to cause damage to DNA *in vivo*. Thus, it is possible that related members of APOBEC family can cause dsDNA breaks observed in response to R-loop formation in other cells types. This is consistent with the fact that APOBEC family proteins are highly mutagenic [93], and this may in part reflect an ability to cause ssDNA in response to R-loops.

DNA breaks observed in cells producing R-loops can occur by mechanisms not involving APOBEC proteins. In yeast, it has been shown that *hpr1* and *tho2* mutants have increased sensitivity to DNA damage induced by UV irradiation [94]. In general, ssDNA is more susceptible to DNA damage and breaks than dsDNA. For example, spontaneous deamination of cytidine on ssDNA is 140-fold more efficient than on dsDNA [95]. Increased genetic instability was linked to impaired replication in *in vitro* experiments utilizing *HPR1* and *THO2* mutants [96]. Further evidence for this was revealed by the discovery of point mutants in *hpr1* that uncoupled hyper-recombination phenotypes from transcription elongation and transcript retention [97]. In these mutants, hyper-recombination was correlated with an impairment of replication. Impaired replication could reflect the role of fork collapse due to aberrant DNA structures and subsequent dsDNA breaks as mentioned above. This theory is supported by the fact that replication fork progression could be partially restored by hammerhead ribozyme cleavage of nascent RNA in these experiments [97].

1.5.3
Rrm3 and Pif1 DNA Helicases

Rrm3 is a 5′ to 3′ DNA helicase that facilitates replication past non-nucleosomal protein–DNA complexes, and which is conserved from yeast to humans [98–100]. This helicase is necessary for normal fork progression through an estimated 1400 discrete sites. The inability to replicate past protein–DNA complex causes fork breakage, which could lead to DNA damage and genomic instability. Indeed, *RRM3* was first identified in a genetic study because its absence increased recombination in tandemly repeated ribosomal DNA genes [101]. *In vitro* studies have shown that both Pif1 and hPif1, related proteins in the same family as Rrm3, are able to unwind RNA:DNA hybrids [101, 102]. Pif1 has been shown to inhibit the lengthening of telomeres, a structure that protects chromosome ends, by its interaction and removal of telomerase [101, 102]. Telomerase contains a component

called telomerase RNA component (TERC), a G-rich RNA that can form G-quadraplexes, which are reminiscent of the G-quartets found in CSR. It is an intriguing but interesting possibility that Rrm3p or Pif1p might play a role in protecting against R-loop formation in eukaryotic cells.

1.6 Concluding Remarks

Research for many years has been focused on functional coupling of transcription and splicing. It was surprising that a role of cotransciptional splicing, or at least recruitment of splicing factors, is to protect chromosomes from genomic instability. While a considerable amount of work has been done to understand how cotranscriptional events function to preserve genomic stability, the exact mechanism by which R-loop formation leads to dsDNA breaks is still a mystery. Understanding this process is especially interesting in light of the fact that genome instability is a key cause of cancer [103]. Dissection of the steps by which R-loop structures can form DNA breaks and consequently genomic rearrangements may indeed provide insight into cancer and degeneration.

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