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The connection between transcription and genomic instability

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Transcription is a central aspect of DNA metabolism that takes place on the same substrate as replication, repair and recombination. Not surprisingly, therefore, there is a physical and functional connection between these processes. In recent years, transcription has proven to be a relevant player in the maintenance of genome integrity and in the induction of genetic instability and diversity. The aim of this review is to provide an integrative view on how transcription can control different aspects of genomic integrity, by exploring different mechanisms that might be responsible for transcription-associated mutation (TAM) and transcription-associated recombination (TAR).
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Introduction

One intriguing question in molecular biology is how DNA replication, repair and recombination can occur in a DNA substrate that simultaneously undergoes transcription. Thus, transcription will at times take place on a DNA segment that is simultaneously being replicated or contains lesions that need to be repaired. A connection between transcription and other DNA metabolic processes has emerged over the last few years as a ubiquitous feature in all organisms from prokaryotes to higher eukaryotes, and these connections have an important impact on genetic integrity.

If transcription is blocked by a particular DNA lesion, the blocked RNA polymerase is used to sense the damage and to load the DNA repair machinery at the site of the lesion via a mechanism termed transcription-coupled repair (TCR) (Mellon *et al*., 1987; Selby and Sancar, 1993). TCR provides a good example of how the transcription process is used positively for the control of genomic stability by facilitating DNA repair (van Gool *et al*., 1997). In addition, as the transcriptional elongation apparatus advances together with proteins bound to the nascent RNA, it causes transient changes in DNA topology and chromatin structure or it can encounter the replication machinery. As a consequence, genomic stability can be compromised in the form of an increase in mutation and recombination rates. I will review here the evidence available on genetic instability associated with transcription, with the aim of discussing the possible mechanisms.

Transcription increases spontaneous and chemically induced mutations

It has been known for 30 years that mutations in a particular gene can be induced by transcription. We will refer to this phenomenon as transcription-associated mutation (TAM). Thus, in *Escherichia coli*, mutation rates of the β-galactosidase locus are stimulated by transcription in the presence of alkylating agents (Brock, 1971). In addditon, the ICR-191 mutagen reverts *lac*$^-$ mutations more frequently when transcription is activated (Herman and Dworkin, 1971). Other examples of TAM exist in yeast (Datta and Jinks-Robertson, 1995), *E.coli* (Beletskii and Bhagwat, 1996; Wright *et al*., 1999) and T7 phage (Beletskii *et al*., 2000).

There are multiple mechanisms by which mutations can be generated, including misincorporation of nucleotides during replication, failures of mismatch repair or the action of error-prone activity of DNA polymerases involved in different DNA repair pathways. However, many mutations occur as a consequence of a previous lesion in the DNA, which is caused by internal cell metabolites or external compounds. Noteworthy in this connection is the fact that many chemical reactions are much more efficient on single-stranded DNA (ssDNA) than on double-stranded DNA (dsDNA). For example, spontaneous deamination of cytosine is 140-fold more efficient on ssDNA than on dsDNA (Frederico *et al*., 1990). Interestingly, it has been observed in the *E.coli lacI* and human *hprt* genes that the non-transcribed ssDNA chain is more susceptible to mutations than the transcribed strand (Fix and Glickman, 1987; Skandalis *et al*., 1994). In the *E.coli tac* region, transcription causes a 4- to 5-fold increase in C to T mutations (Beletskii and Bhagwat, 1996). Furthermore, TAM is higher in a mutant of the T7 RNA polymerase (RNAP) with a slower elongation rate. These results suggest that C deamination in the non-transcribed strand may be dependent on the length of time that transcription maintains the DNA open during elongation (Beletskii *et al*., 2000). As will be discussed later, the strand opening facilitated by the transient accumulation of negatively supercoiled DNA behind the advancing RNAP could lead to ssDNA regions, which would be more susceptible to chemical reactions such as C deamination.

It is worth noting that TAM is likely to have an impact on evolution. Thus, 'adaptive mutation', i.e. the increase in genome-wide mutations observed during prolonged nutritional stress in cells that are not dividing and in genes whose functions are selected (Rosenberg, 1997), is associated with induction of several affected genes (Wright *et al*., 1999). Starvation-induced mutation might, therefore, be an example of TAM.

Transcription stimulates recombination

In vegetatively growing cells, homologous recombination is a major pathway for the repair of DNA breaks generated during replication or as a direct consequence of DNA-damaging agents. The frequency with which homologous recombination occurs in mitosis can be modulated by different elements and biological processes (Aguilera *et al*., 2000). Transcription is one such process. It strongly induces recombination. We will refer to this phenomenon as transcription-associated recombination (TAR).

A first example of TAR was reported in λ phage (Ikeda and Matsumoto, 1979). Other examples of TAR in prokaryotes have been shown for transduction (Dul and Drexler, 1988) and illegitimate recombination in *E.coli* (Vilette *et al*., 1995). Evidence for TAR in eukaryotes was shown with the *HOT1* DNA sequence of *Saccharomyces cerevisiae* (Voelkel-Meiman *et al*., 1987). *HOT1* contains the initiation site (I) of the 35S rRNA precursor plus the enhancer (E) of transcription by RNA polymerase I (RNAPI). *HOT1*-dependent hyper-recombination can be abolished when either the I or E element is deleted (Stewart and Roeder, 1989), when a mutant RNAPI incapable of transcribing the 35S rRNA is used (Huang and Keil, 1995), or when a transcription terminator is inserted between *HOT1* and adjacent sequences (Voelkel-Meiman *et al*., 1987). These results indicate that transcription through the recombining sequences is required for stimulation of recombination.

In yeast, RNA polymerase II (RNAPII)-mediated TAR was first shown in direct repeats transcribed under the control of the regulatable *GAL1* promoter. Induction of transcription increased deletions by ~10-fold (Thomas and Rothstein, 1989). TAR has also been reported in other recombination assays in *S.cerevisiae* (Nevo-Caspi and Kupiec, 1994; Bratty *et al*., 1996; Saxe *et al*., 2000), *Schizosaccharomyces pombe* (Grimm *et al*., 1991) and mammalian cells (Nickoloff, 1992) or in V(D)J recombination (Blackwell *et al*., 1986; Lauster *et al*., 1993). Therefore, transcription may produce structures that are not only mutagenic but also recombinogenic in all organisms from bacteria to humans.

The yeast THO complex and the Thp1 protein: a connection between RNAPII transcriptional elongation and recombination

A connection between transcription and recombination is provided by the yeast *HPR1* gene, identified by a mutation that increased recombination between DNA repeats (Aguilera and Klein, 1990) and *THO2*, identified as a multicopy suppressor of *hpr1* (Piruat *et al*., 1998). Neither *HPR1* nor *THO2* are essential genes. However, their deletion causes an increase in recombination between direct repeats as high as 3000-fold above wild-type levels. Importantly, these increases in recombination are dependent on transcriptional elongation. This was first shown with direct repeats in which, in contrast to the previously used RNAPII-dependent recombination assays of Thomas and Rothstein (1989), transcription was driven from a unique RNAPII-dependent promoter that was located outside of the repeat constructs (Chávez and Aguilera, 1997; Prado *et al*., 1997). Consequently, recombination events could occur if initiated either in or between the repeats, but not if initiated in the promoter region. Hyper-recombination in *hpr1* and *tho2* mutants depends on the type of DNA segment located between the repeats and, therefore, on the type of DNA segment through which transcriptional elongation takes place. In addition, if the *CYC1* transcription terminator is placed downstream of one repeat to impede elongation into the DNA segment flanked by the repeats, hyper-recombination is abolished (Chávez and Aguilera, 1997; Prado *et al*., 1997). Therefore, transcriptional elongation is required for hyper-recombination. As in wild-type cells, transcriptional activity is necessary for the formation of transcription-associated recombinogenic structures in *hpr1* and *tho2* mutants.

Importantly, the *hpr1Δ* and *tho2Δ* mutants are impaired in transcriptional elongation (Prado *et al*., 1997; Piruat and Aguilera, 1998). This impairment is clearly observed with DNA sequences such as *lacZ*. Full *lacZ* transcripts are recovered in *hpr1Δ* mutants with an efficiency that is below 5% of the wild-type levels (Chávez and Aguilera, 1997). This is due to the fact that transcription of either long or G+C-rich DNA sequences is particularly defective in *hpr1* mutants, at least when driven from a strong promoter (Chávez *et al*., 2001).

Hpr1 and Tho2 belong to a protein complex, termed THO, together with Mft1 and Thp2 (Chávez *et al*., 2000). Deletions in any of the four genes encoding the THO complex, plus a fifth one, the *THP1* gene (encoding another protein not found in the core THO complex) lead to similar defects in transcription-dependent hyper-recombination and transcriptional elongation (Chávez *et al*., 2000; Gallardo and Aguilera, 2001). Therefore, these proteins represent a novel group of non-essential proteins required for proper transcription elongation and genetic stability. A physical connection between Hpr1 and the transcription machinery has been indicated by its co-purification with a novel form of the RNAPII holoenzyme together with Paf1, Cdc73 and Ccr4 (Chang *et al*., 1999). This might suggest a close relationship between the THO complex and transcription. However, experimental evidence for a direct role for THO in transcription is still lacking.

Possible causes of TAR and TAM

One likely mechanism to explain TAR is the collision between the transcription and replication machineries. The stalling and collapse of replication forks has been shown to be a common event in *E.coli*, recombination thus becoming essential in underpinning replication (Vilette *et al*., 1995; Seigneur *et al*., 1998). McGlynn and Lloyd (2000) have recently shown that raising the level of the stringent response signal molecules (p)ppGpp, which modulates RNAP activity, as well as certain mutations in the β-subunit of RNAP, results in an increase in the UV survival of cells lacking the Holliday junction (HJ) resolvase RuvABC. The interpretation of this result is that RNAP might help to solve the problem of a stalled replication fork by pushing it backward, thus leading to the formation of a HJ (Figure 1). The removal of such HJs requires a recombination event. Transcription-mediated

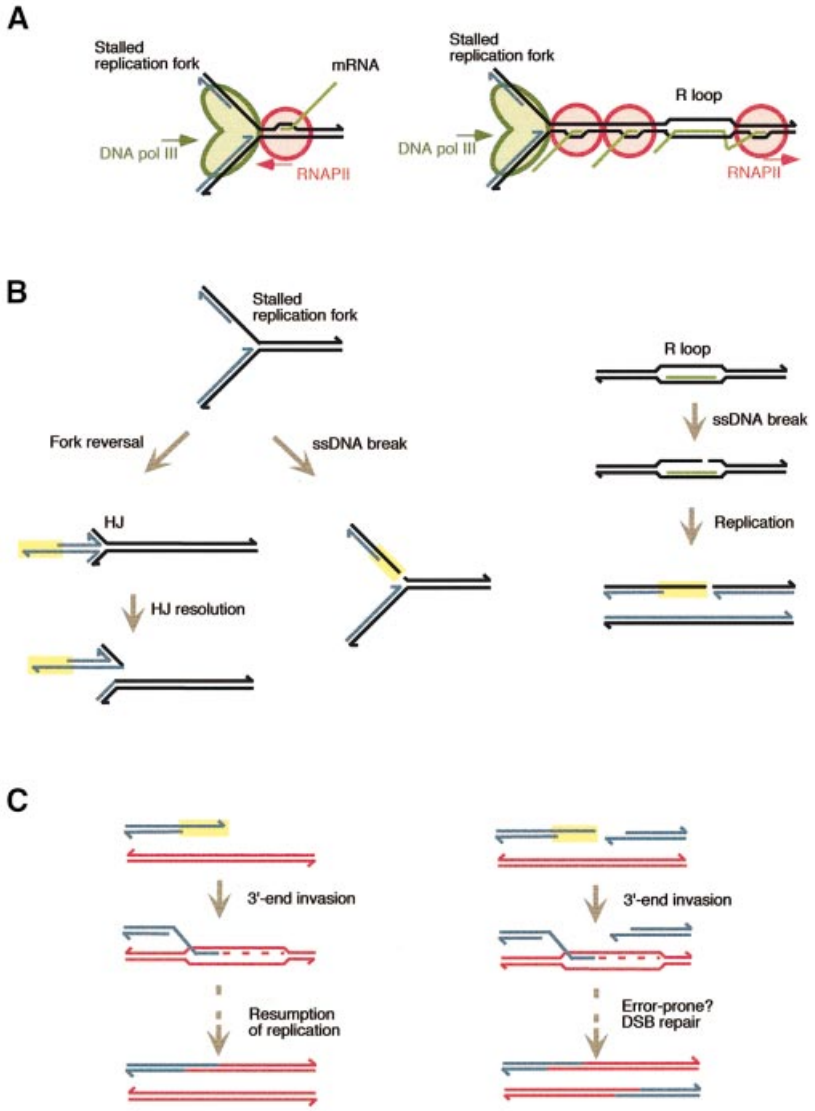

Fig. 1. Possible transcription-associated recombination mechanisms. (**A**) The replication fork could be stalled by an elongating or blocked RNAPII advancing in the opposite direction (left) or advancing in the same direction but blocked as a consequence of putative DNA–RNA hybrids (R loop) formed by the nascent RNA coming out from the RNAPII ahead (right). (**B**) The stalled replication fork can lead to recombinogenic 3′-ended ssDNA by fork reversal, leading to a HJ, or by a nick in the template DNA (left). The R loop can lead to recombinogenic ends by damage to the ssDNA template and posterior replication (right). The ssDNA region would be a consequence of the unfinished replication of the lagging strand. (**C**) Recombination could occur by strand invasion followed by replication, if only one 3′-ssDNA end is involved, or by a DSB repair mechanism, which can potentially be error prone. The latter could also explain some cases of transcription-associated mutation. A yellow box indicates the recombinogenic 3′-ssDNA ends. In (A) and (B), newly synthesized strands are shown in blue. In (C), different molecules are shown in red and blue to better visualize the recombinant products, irrespective of the newly synthesized strand.

HJ migration could conceivably explain TAR in general, including that observed in yeast wild-type cells and mutants of the THO complex and Thp1 proteins. The involvement of replication blockage in TAR would be consistent with the observations that the yeast *hpr1* mutation increases chromosome (Santos-Rosa *et al*., 1994) and plasmid loss (Chávez *et al*., 2000).

TAR can also be related to the transient accumulation of positively and negatively supercoiled DNA ahead of and behind the advancing elongating RNAPII, respectively (Brill *et al*., 1987; Wu *et al*., 1988; Tsao *et al*., 1989). Indeed, the hyper-recombination effect of yeast *top1*, *top2* and *top3* mutants suggests that changes in supercoiling modulate the recombination frequency of DNA sequences (Christman *et al*., 1988; Gangloff *et al*., 1994; Trigueros and Roca, 2001). Negatively supercoiled DNA produced by transcriptional elongation may facilitate the formation of R loops in which the nascent RNA forms a hybrid with DNA, leaving the non-template DNA single stranded. Evidence for such an event has been obtained in *E.coli* by overexpression of RNase H, which degrades the RNA moiety of the RNA–DNA hybrid. Such overexpression partially complements the growth defects and rRNA transcriptional elongation defects of *topAΔ* mutations (Hraiky *et al*., 2000). Topoisomerase I inhibits RNA–DNA hybrid formation in *E.coli* by its capacity to relax transcription-induced negative supercoiling (Massé and Drolet, 1999). As negatively supercoiled DNA facilitates

strand separation, it is conceivable that this creates a region more susceptible to attack by internal metabolites that are reactive with ssDNA, leading to both mutagenic and recombinogenic lesions. This hypothesis has the advantage that it can also explain TAM, even though more quantitative data are lacking on TAM to strengthen this possibility. In addition, the RNA–DNA hybrids may constitute roadblocks to the next transcribing RNAP (Hraiky *et al*., 2000), creating a potential block for replication (Figure 1).

In this sense, it is worth noting that the THO complex binds RNA *in vitro* (A.G.Rondón and A.Aguilera, unpublished) and that a high copy number of the putative RNA helicase *SUB2* gene suppresses *hpr1* hyper-recombination (Fan *et al*., 2001). It is therefore plausible that in mutants of the THO complex, the elevation in TAR could be mediated by the nascent RNA molecule produced during transcriptional elongation. In other words, the THO complex might be counteracting TAR by keeping the RNA transcript inaccessible for instigation of recombination.

Finally, TAR could also be related with the fact that, at least in eukaryotes, transcriptional elongation occurs through DNA organized in chromatin. As the transcription apparatus passes through a DNA region, the chromatin has to open transiently. It is likely that this contributes to a better accessibility of DNA-damaging agents and, most likely, nucleases, to the DNA. This seems to be the case in yeast mating type switching (Pâques and Haber, 1999), yeast meiotic recombination (Wu and Lichten, 1994) and V(D)J site-specific recombination of immunoglobulin (Ig) genes (McMurry and Krangel, 2000). Examples of increases in mitotic homologous recombination related to changes in chromatin structure are provided by the yeast *spt4* and *spt6* mutants, which increase DNA repeat recombination (Malagón and Aguilera, 2001), and the yeast *sir2* mutants, which show a 10- to 15-fold increase in recombination at the rDNA locus (Gottlieb and Esposito, 1989). In this locus, chromatin accessibility responds to *SIR2* dosage (Fritze *et al*., 1997).

Other mechanisms to explain TAR and TAM cannot be excluded. In any case, it is likely that TAR and TAM do not occur by a single mechanism, but by several, such as those discussed here.

Somatic hypermutation and class switching, two developmentally regulated transcription-associated mechanisms of mutagenesis and recombination

I have described above some cases in which transcription can interfere with or stimulate replication, DNA repair, mutagenesis or recombination. Evolution seems to have taken advantage of this natural connection to create specialized mechanisms for generating genetic diversity that are developmentally coupled to RNAPII-driven transcription. This is the case for somatic hypermutation and class switching recombination in Ig genes.

Class switching mediates isotype switching of Ig heavy chains during B-cell development. It is produced by an as yet unknown mechanism of recombination occurring at the 1–10 kb long S regions composed of tandem repetitive sequences that are located upstream of C_H genes. Importantly, transcription is required for class switching (Jung *et al*., 1993; Zhang *et al*., 1993). Experiments *in vitro* have shown that during transcription the S transcript hybridizes with the template DNA strand, leading to an R-loop structure (Reaban and Griffin, 1990; Reaban *et al*., 1994; Daniels and Lieber, 1995). Theoretically, such an R-loop structure could serve as a substrate for some endonuclease and, indeed, it has recently been shown *in vitro* that the XPF/ERCC1 and XPG nucleases involved in NER are able to cleave DNA in S regions, causing recombinogenic double-strand breaks (DSBs) (Tian and Alt, 2000).

Hypermutation is a mechanism by which antigen-activated B cells further diversify their Ig by introducing mutations in and around the V-region of IgH and IgL genes (Weigert *et al*., 1970). Hypermutation in the Ig genes is confined to 2 kb downstream of the Ig promoter region. The increase in mutations in this region is about six orders of magnitude above spontaneous levels (Jacobs and Bross, 2001). The mechanism underlying somatic hypermutation is also unknown. Recent reports suggest that B cells undergoing somatic hypermutation show a high frequency of DSBs in and around the targeted V(D)J region (Papavasiliou and Schatz, 2000; Jacobs and Bross, 2001). The possibility that either non-homologous end-joining (Jacobs and Bross, 2001) or the homologous recombination pathway of DSB repair (Papavasiliou and Schatz, 2000) is involved in somatic hypermutation has consequently been raised. The possibility that the homologous recombination pathway of DSB repair might be involved is particularly interesting because a link between DSB-recombinational repair and mutation has been reported in *E.coli* (Harris *et al*., 1994) and *S.cerevisiae* (Holbeck *et al*., 1997; Rattray *et al*., 2001).

Regardless of its molecular mechanism, somatic hypermutation is strongly dependent on transcription. Mutation frequencies correlate with promoter strength and transcriptional activity (Peters and Storb, 1996). DSBs found associated with hypermutation are indeed enhancer dependent and coupled to transcription (Papavasiliou and Schatz, 2000). There is no requirement for the Ig promoter itself, as hypermutation has been shown to occur also with other RNAPII promoters and with RNAPI promoters (Betz *et al*., 1994; Fukita *et al*., 1998). The observation that other genes also mutate more frequently in B cells than in other cell types (Pasqualucci *et al*., 1998; Shen *et al*., 1998) suggests that hypermutation might indeed not be limited to the region controlled by the Ig promoter. Even though transcription could facilitate hypermutation by mechanisms such as those discussed for TAM, it is difficult to imagine that such a passive mode of action is able to increase mutation frequencies by six orders of magnitude. It therefore seems plausible that there is one or more mutator factor specifically expressed in rearranged B cells that are loaded onto RNAPII or take advantage of the chromatin opening or strand separation taking place during transcription elongation to access the DNA.

In summary, somatic hypermutation and class switching may be the most sophisticated systems that have emerged in generating genetic diversity. Their coupling to transcription might not be a fortuitous coincidence, but rather a consequence of the natural connection between transcription, repair, mutagenesis and recombination.

Concluding remarks and future perspectives

A connection between transcription and other DNA metabolic processes has emerged as a ubiquitous feature in all organisms from prokaryotes to higher eukaryotes, and this connection has an important impact on genetic integrity. If a particular DNA lesion blocks the transcription apparatus, the cell takes advantage of the large blocked ternary structure at the site of damage to detect the DNA lesion and to facilitate its repair by TCR. In addition, as the transcriptional elongation apparatus advances together with proteins bound to the nascent RNA, it causes transient changes in DNA topology and chromatin structure or it can encounter the replicative machinery. As a consequence, genomic stability can be compromised, leading to the TAM and TAR phenomena reviewed here.

During elongation, RNAPII acts in concert with a number of well characterized transcriptional elongation factors, including DSIF, NELF, TFIIS, TFIIF, FACT, CSB/Rad26, Elongator, etc. (Wind and Reines, 2000; Kim *et al*., 2001; Zorio and Bentley, 2001). As transcription proceeds, the nascent RNA molecule has to undergo proper maturation, including 5′-end methylguanine capping, splicing, 3′-end cleavage and poly(A)$^+$ addition in processes that appear to occur co-transcriptionally (Hirose and Manley, 2000; Proudfoot, 2000). *In vivo*, cross-talk between transcription and splicing, poly(A)$^+$ addition and RNA export have thus been indicated by the observation that truncation of the C-terminal domain of the largest RNAPII subunit leads to the accumulation of significant amounts of unspliced pre-mRNAs in mammalian cells (Misteli and Spector, 1999). Moreover, human β-globin pre-mRNAs defective in either splicing or 3′-end formation are retained at the site of transcription (Custodio *et al*., 1999), and different blocks in yeast mRNA nuclear export lead to accumulation of hyperadenylated transcripts at the site of transcription (Jensen *et al*., 2001). All of these results are consistent with the view that transcription involves not only the elongating RNAPII complex and elongation factors, but also RNA-associated protein complexes required for transcript maturation.

Understanding the mechanisms of TAM and TAR requires the physical identification of the mutagenic and recombinogenic intermediates, such as putative R loops, blocked RNAPIIs, collapsed replication forks, HJs or DSBs. However, it also needs a detailed knowledge of the mechanism of transcript elongation and processing of the nascent RNA. These will be topics for work in many laboratories over the years to come.

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