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tRNA genes in eukaryotic genome organization and reorganization

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The primary function of *tRNA* genes is to provide the templates for the transcription of essential tRNA molecules. However, there is now evidence that these dispersed repetitive elements have the potential to mediate the spatial and functional organization of the genome and to drive genome change and evolution. Indeed, *tRNA* genes and related Pol III promoter elements can occupy distinct subnuclear positions and also provide barriers which functionally separate domains of chromatin. Furthermore, *tRNA* genes can also represent barriers to DNA replication fork progression and accordingly, *tRNA* genes can contribute to the formation of genomic fragile sites and have been implicated in genome evolution. Here we give insight into our current understanding of these "extra transcriptional" functions of *tRNA* genes and discuss how these functions may impact upon genome regulation and evolution.

Key words: *tRNA* gene, chromatin boundary, genome rearrangement, recombination, TFIIIC, RNA polymerase III, *B-box*

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tRNA Genes, *B-box* Elements and Genome Organization

Transcription of *tRNA* genes is mediated by RNA polymerase III (Pol III) and is dependent upon intragenic promoter elements, *A-box* and *B-box*, which serve as the binding site for a multi-subunit transcription factor, TFIIIC. The *B-box* consensus (5'-GGTTCGANTCC-3') is highly conserved with the central C-residue being invariant and essential for its function. Once bound TFIIIC directs the assembly of another multisubunit factor, TFIIIB onto a region upstream of the transcription start-site. TFIIIB-DNA complexes are extremely stable and capable of directing multiple rounds of Pol III recruitment and initiation.[1] Quantitative chromatin immunoprecipitation experiments have demonstrated high levels of TFIIIB and Pol III occupancy at *tRNA* genes,[2] suggesting that many of these elements are constantly being transcribed. Consistent with this, tRNA accounts for approximately 15% of total RNA in actively dividing yeast cells.[3] Given these considerations, it is perhaps not surprising that *tRNA* genes exert position-dependent effects on a range of chromosomal processes. Indeed, it has been known for a long time that *tRNA* genes can have a repressive effect on neighboring RNA polymerase II (Pol II) promoters[4] and as discussed below they can represent an obstacle to the progression of replication forks.[5,6] It has also become apparent that some eukaryotic cells exploit the properties of *tRNA* genes as boundary elements that demarcate the limits of chromatin domains.[7]

Boundary elements functionally separate chromatin domains so that one domain does not exert an unwanted influence upon its neighbors.[7] Some boundary elements limit the spread of heterochromatin into euchromatic regions, while others serve to insulate promoters from the action of inappropriate enhancers or silencers. Both of these types of boundary element activity have now been ascribed to *tRNA* genes. The first demonstration that a *tRNA* gene could function as a heterochromatin barrier came from analysis of the *Saccharomyces cerevisiae HMR* mating type locus which demonstrated that a $tRNA^{Thr}$ gene inhibits the spread of silencing from this region.[8] A *tRNA* gene (*TRT2*) has also been shown to prevent the spread of repressive chromatin from the **a**-specific *STE6* gene that is silenced in *MAT*α cells.[9] More

recently, a *tRNA*^{Gln} gene has been shown to be an integral component of a barrier element that prevents the spread of chromatin structures that are associated with the *S. cerevisiae* rDNA (*RDN1*) locus.[10] The role of *tRNA* genes as heterochromatin barriers is not restricted to budding yeast as they have been shown to function in this manner in the large complex centromeres of *Schizosaccharomyces pombe.*[11,12] These centromeres are composed of distinct chromatin domains, with a central core region that is flanked by two large domains of heterochromatin.[13] The central domain is associated with nucleosomes that contain a homologue of the H3 histone variant CENP-A, whereas the pericentric heterochromatin is characterized by methylation of histone H3 on lysine 9 and the association of an HP1 homologue, Swi6.[13,14] The barrier between these domains in the centromere of chromosome I, *cen1,* contains transcribed *tRNA*^{Ala} genes and deletion of these causes encroachment of heterochromatin into the central core.[11] Furthermore, loss of barrier activity results in abnormal meiotic chromosome segregation indicating that it is required for proper centromere function.[11] The transcriptional competence of these *tRNA* genes is required for barrier function as mutations in the *A-box* that abolish promoter function have a similar effect upon barrier function.[11] This is also true for the *tRNA* gene barriers at the *S. cerevisiae HMR* and *RDN1* loci.[8,10] However, there are now other documented cases where assembly of the complete Pol III transcription apparatus does not seem to be required for barrier function. A perhaps surprising development has been the identification in both budding and fission yeast of ectopic *B-box* elements that are not associated with functional Pol III genes. Chromatin immunoprecipitation (ChIP) experiments indicate that these sites are associated with TFIIIC alone, or partial assemblies of the Pol III machinery.[15-17] These sites are termed *ETC* (extra TFIIIC) in *S. cerevisiae* and *COC* (chromosome-organizing clamps) in *S. pombe*. In both cases these elements have been shown to have barrier function. In *S. pombe* the inverted repeat (*IR*) elements that flank the heterochromatic mating type (*mat*) locus contain *B-box* sequences which are critical for barrier activity.[16] Furthermore, when the *ETC4* element was located downstream of the *S. cerevisiae HMR* locus it prevented the spread of heterochromatin without recruiting TFIIIB and Pol III.[18] The *ETC4* site has also been shown to have insulator function, as it was able to block activation of *GAL* genes when inserted in between the promoter and upstream activating sequences.[18] An intact *tRNA* gene (*TRT2*) has also been shown to have similar activity. Evidence also suggests that the *ETC6* in its natural context has insulator-like function.[18] This is interesting because the *ETC6* element is located upstream of the *TFC6* gene which encodes a TFIIIC subunit. Deletion of *ETC6* was found to result in a modest, but reproducible, increase in *TFC6* mRNA[18] suggesting that to some degree yeast TFIIIC regulates its own synthesis.

The precise mechanism by which *tRNA* genes and *B-box* elements exert their barrier function is not understood, however there are a number of possibilities. The assembly of the Pol III transcription machinery results in the redistribution of nucleosomes to either side of the *tRNA* gene creating a region that is devoid of nucleosomes.[19] This is significant because nucleosome free regions have been proposed to prevent the spread of heterochromatin.[20] However, mutations that restored nucleosomes to the *HMR-tRNA* gene barrier resulted in only a small loss of function suggesting that a nucleosome free region is not necessary for barrier activity.[21] Boundary element function may require the recruitment of specific chromatin modifying and remodelling activities. The remodelling complex ISW2 is known to be recruited to *tRNA* genes through interaction with TFIIIB[22,23] and furthermore the RSC remodeler is also recruited to Pol III transcribed loci.[24] In chickens barrier elements are known to recruit histone acetyl transferases (HATs)[25] and similarly the barrier functions of *tRNA* genes at both the *RDN1* and *HMR* loci involve HATs.[10,21] In addition, human TFIIIC has also been reported to have an intrinsic acetyltransferase activity[26] although no such activity has yet to be identified for yeast TFIIIC. It is also possible that boundary function is achieved through the partitioning into higher order chromatin structures such as loops. It is therefore relevant that *tRNA* genes and *B-box* elements have been implicated in the spatial organization of the genome.[16,27] In *S. cerevisiae* although *tRNA* genes are dispersed throughout all sixteen chromosomes they are clustered at the nucleolus in a manner that is dependent upon condensin, which has been shown to load at these elements.[28,29] Furthermore, the ectopic TFIIIC binding sites in *S. pombe* have been proposed to act as chromatin organizing centres (hence the name) and are found associated with the nuclear periphery.[16] So, at least in these unicellular eukaryotes, *tRNA* genes and *B-box* elements play important roles in the three dimensional organization of the genome. The precise role that subnuclear positioning plays in boundary element function remains to be determined, although, it is interesting to note that in *S. cerevisiae* mutations that disrupt nucleolar clustering also result in the loss of *tRNA* gene-mediated silencing of nearby Pol II genes.[29,30] Experiments have also established that the *tRNA* gene barrier neighboring *HMR* is also required for establishment of cohesion at that locus[31] and cohesion is implicated in *HMR* barrier function because it is impaired in *smc1* and *smc3* mutants.[32] Furthermore, the *RDN1-tRNA*^{Gln} barrier activity is also dependent upon cohesin.[10]

At present the evidence for the role of TFIIIC binding sites in boundary function is essentially restricted to yeast, however it is worth noting that there is a potential for such sites to play important roles in genome organization in mammals. Indeed large portions of the human genome (approximately 43%) are made up of repetitive sequences, consisting mostly of retrotransposons, some of which are non-autonomous.[33-35] Short interspersed elements (SINEs) are the most abundant of these and they require factors expressed by long interspersed elements (LINEs) for their transposition.[33-35] SINEs, are transcribed by Pol III and are thought to be ancestrally derived from *tRNA* and 7SL RNA genes[36-38] and as such harbor *B-box* TFIIIC binding sites. Analysis of the distribution of SINES has revealed that >40% human promoters contain one of these retroelements, which has led to the suggestion that their TFIIIC binding sites may protect promoters from the spread of repressive chromatin modifications from adjacent sequences.[39] In support of this, an *Alu* SINE element has been shown to

protect reporter genes from interference from neighboring transcription units in transgenic mice.[40] Given that *S. cerevisiae HMR* barrier function has been found to be dependent upon cohesin,[32] it is perhaps also significant that *Alu*-containing elements have been shown to be cohesin binding sites on human chromosomes.[41]

tRNA Genes and Genome Change

There are multiple *tRNA* genes through the genomes of eukaryotes; for example, fission yeast and budding yeast have 186 and 286 reported *tRNA* genes respectively, coding for tRNAs decoding standard codons, whilst humans have 513, mouse 430 and Thale Cress has 630.[42] *tRNA* genes are found to be enriched in some genomic regions, such as the centromeres of the fission yeast.[43-45] As yet, little is known about the factors influencing their location; although a positive correlation between *tRNA* gene locations and origins of DNA replication has been made.[46] Specific *tRNA* genes are represented more than once per genome.[42] These genes provide areas of homology which could act as substrates for intragenomic non-allelic recombination events, which, in turn, could potentially contribute to changes in genomic structure. Furthermore, the physical clustering of these elements into specific sub-nuclear regions, which is observed in yeast, would be expected to potentiate recombination.[16,27]

The DNA sequences of distinct *tRNA* genes within genomes exhibit high levels of identity. For example, the 9 $tRNA^{Asp}$ genes of the fission yeast are 100% identical.[42] Whilst high levels of identity are observed in all species for a particular group of *tRNA* gene paralogues, there are some paralogue groups which exhibit divergence; for example, the 14 $tRNA^{Gly}$ genes of fission yeast can be split into three groups, with almost 100% identity within each sub-group but up to 34% divergence between sub-groups.[42] In organisms with higher numbers of members of a paralogue family, there is a greater propensity for sub-grouping divergence, although high levels of identity can still be found; for example, of the 12 $tRNA^{His}$ genes in humans 9 are 100% identical.[42] These high levels of identity within families and sub-groups indicate that there must be genetic 'cross talk' to conserve sequence identity, a process that has been termed rectification. In fission yeast it has been demonstrated that sequence rectification is driven by non-allelic gene conversion.[47-49] Whilst the mechanisms for this remain unclear, it can occur between *tRNA* genes located on the same or distinct chromosomes.[47] Rectification gene conversion is more prevalent during meiosis than mitosis, but it remains unclear whether this meiotic elevation is influenced by the induction of meiosis-specific recombination factors or the complex changes to chromosomal architecture during meiosis (reviewed in refs. 50 and 51). The observation that *tRNA* gene isotypes can be divided into sub-groups might suggest that rectification gene conversion only occurs between subgroup family members and it is tempting to speculate that this could be controlled by relative genomic position. Transfer of favorable genetic changes or removal of deleterious changes within a family of paralogues in this fashion has the advantage that it permits a gene family to rapidly respond to change relative to selective pressures and evolutionary cues.

This phenomenon suggests that *tRNA* genes have a significant recombination potential, a hypothesis that has now been tested in a number of studies. Firstly, tandemly repeated *tRNA* genes were placed on the *S. cerevisiae* genome flanking a reporter gene; by measuring the frequency of loss of the reporter gene it was established that recombination between *tRNA* gene repeats is elevated when the genes are transcriptionally active.[52] It was postulated that the mechanism for this was a single-stranded annealing reaction. Secondly, a single *tRNA* gene was placed centrally between two direct repeat elements on a plasmid in *S. cerevisiae* and mitotic recombination between the repeats was measured.[53] The presence of a *tRNA* gene resulted in a small, but significant increase in inter-repeat recombination (~2-fold), in an orientation-dependent fashion. Again, the increased level of recombination was dependent upon the *tRNA* gene being transcriptionally competent suggesting that active Pol III stimulates recombination. Mutation of the *RRM3* gene resulted in a moderate increase in the *tRNA* gene-specific inter-repeat plasmid recombination. The *RRM3* gene encodes a helicase which is referred to as the 'sweepase', as it is proposed to function at the forefront of the DNA replication machinery removing potential barriers to DNA replication.[54] This suppression by Rrm3 indicates that *tRNA* gene-associated recombination has some dependence upon DNA replication. Indeed, it is proposed that *tRNA* genes stimulate recombination due to collisions between the *tRNA* gene/Pol III complex and the DNA replication machinery to generate recombinogenic lesions.[53] In support of this it has been known for some time that *tRNA* genes can generate measureable pauses in the progression of the DNA replication machinery and that such pausing requires the binding of TFIIIC to the *tRNA* gene *B-box*.[5] Not all *tRNA* genes have the same potential for pausing DNA replication,[55] but DNA replication pausing increases when *RRM3* is mutated, consistent with a model in which collisions between RNA and DNA polymerases generate substrates for recombinases. Other work using fission yeast has demonstrated that two distinct chromosomally located *tRNA* genes, both of which generate non-polar DNA replication fork barriers (RFBs), did not stimulate inter molecular homologous recombination; however, a control RFB generated by a distinct genetic element did.[6] However, loss of Swi1 function resulted in the *tRNA* genes becoming inter molecular recombination hot spots.[6] Swi1 is a component of the replisome progression complex (RPC) and is a member of the TIMELESS family of proteins, which have been implicated in circadian rhythm regulation although a direct connection between circadian rhythm control and RPC function has not been demonstrated.[56] This demonstrates there is a requirement for the RPC to suppress *tRNA* gene-mediated recombination and indicates that when the DNA replication machinery, the replisome, is perturbed *tRNA* genes can become the source of recombinogenic lesions. In addition, the Smc5/6 complex, which is implicated in DNA repair and replication,[57] associates with all *tRNA* genes within the fission yeast genome[58] and it has been demonstrated that in the absence of this complex in the budding yeast gross chromosomal rearrangements associated with *tRNA* genes increase, suggesting the Smc5/6 complex functions to prevent *tRNA* genes becoming fragile.[59] How the Smc5/6 complex interacts with

the RPC, if at all, remains an open question, although it has been proposed that the Smc5/6 complex serves to maintain stalled DNA replication forks in a configuration which permits recombination-mediated re-establishment of a functional replication fork.[60] The loading of the Smc5/6 complex to *tRNA* genes mirrors the loading of both the sister chromatid cohesin complex loader Scc2/4 and the Smc2/4 condensin complex which is required for genome condensation, but the precise functional importance of the co-loading of these complexes to *tRNA* genes remains conjecture.[61]

Collectively, these findings suggest that single Pol III transcribed elements, such as *tRNA* genes, do not represent a major source of recombinogenic lesions under normal conditions. However, when there is a negative influence on the DNA replication machinery, then these elements can become fragile in nature creating unscheduled recombination potential.

Interestingly, chromosomal translocations occurring as a consequence of recombination between non-allelic *tRNA* genes appear to be rare. Indeed, in the fission yeast, where rectification gene conversion has been demonstrated,[47-49] there is little evidence for *tRNA* genes being the sites of reciprocal chromosomal translocations and/or gross chromosomal rearrangements and only one stable reciprocal translocation event at a *tRNA* gene has been identified to date.[48]

As for genome organization, most studies on *tRNA* gene recombinogenic potential has been confined to yeasts (see above). However, in mammals SINES provide potent sources of homology throughout the genome, which could provide sites for homologous recombination. *Alu* elements are the most abundant human SINE element and whilst *Alu-Alu* exchanges have only rarely been associated with carcinogenesis, they are associated with other genetic diseases, germ line rearrangements and evolution. More frequent exchange events occur between *Alu* elements and non-*Alu* DNA sequences, or *Alu* elements located proximal to breakpoints without making up the breakpoint sequences themselves.[33,35,62-67]

So, is there evidence for *tRNA* genes as a source of genetic instability and change? The studies to date (see above) appear to indicate that the recombination potential of these genes is limited, but can be enhanced when the replisome is perturbed in some way. Therefore *tRNA* genes could become hot spots for the formation of recombination initiating lesions and recombination-mediated change when the cell is under a replicative stress. This argument is supported by some key studies. Firstly, work to elucidate the nature of naturally occurring fragile sites within the *S. cerevisiae* genome which result in chromosomal changes, such as translocations, identified sites which were complex in nature, consisting of a variety of distinct types of genetic elements including retrotransposon LTRs, mitochondrial DNA regions and *tRNA* genes.[68] For one of these sites, the so called 403 site, the deletion of the *tRNA* genes reduces the relative fragility of this site, although the site retains some reduced ability to drive unprogrammed genetic changes, indicating that *tRNA* genes alone are not responsible for the fragile nature of this site, rather the *tRNA* genes contribute to fragility.[69] These sites become more fragile when DNA replication is perturbed, indicating an intimate functional link between the recombination potential of *tRNA* genes and DNA replication progression. Secondly, it is known that the checkpoint machinery is required to ensure that the replication fork is stabilized when it encounters barriers to its progression. In *S. cerevisiae*, when the gene for the central checkpoint signalling kinase Mec1 is mutated, so called replication slow zones (RSZs) become more unstable and form recombinogenic lesions.[70] These RSZs have been demonstrated to be enriched for *tRNA* genes, indicating that they may contribute to genome instability when the checkpoint pathways are perturbed. Lastly, there is evidence that *tRNA* genes are strongly associated with the sites within yeast genomes which have driven significant evolutionary change.[71] The study of evolutionarily related yeast species has demonstrated that sites of gene gain and evolutionary breakpoints are associated with *tRNA* genes, inferring that *tRNA* genes were associated with the recombination events which resulted in stable, and presumably advantageous, evolutionary changes to the structure of evolving yeast genomes.[71]

Closing Remarks

The emerging picture indicates that *tRNA* genes are associated with a variety of activities and as a result they present both opportunities and challenges to cells. They can provide mechanisms for the functional and spatial organization of the genome and but may also under certain circumstances represent obstacles that potentiate genome rearrangements. These elements appear to be associated with a remarkable number of proteins complexes. Precisely how the activity of these complexes is co-ordinated to manage the balance between these challenges and opportunities remains to be determined.

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