Spatial coordination of tRNA genes

by

Dave A. Pai

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biological Chemistry)
in the University of Michigan
2012

Doctoral Committee:

Professor David R. Engelke, Chair
Associate Professor Ruthann Nichols
Associate Professor Thomas E. Wilson
Assistant Professor Aaron C. Goldstrohm
Assistant Professor Patrick J. O'Brien
Adjunct Assistant Professor Daniel A. Bochar

Acknowledgements

I will start by recognizing the various funding sources that have made the work in this dissertation possible: The Rackham Merit Fellowship, the Michigan Predoctoral Training Program in Genetics, the Rackham International Research Award, and the Michigan Endowment for the Development of Graduate Education Award. These have all been instrumental to my success here at Michigan.

My dissertation committee, more than being just a hurdle to graduation, has been very helpful throughout this process, so I thank the members for all their help, both for experimental advice and personal discussions. I would additionally like to thank Tom Wilson and John Moran for their guidance during my rotations in their labs. They were useful experiences, and I take much personally and professionally from my time there. I have accumulated a number of collaborators, both here at the University of Michigan and outside, and they are listed at the ends of the relevant chapters. Additional gratitude goes to Frank Uhlmann at the London Research Institute for graciously inviting me to spend several weeks performing experiments in his lab. That experience is without parallel, and I learned much from Frank and his lab members, particularly Lidia. I also want to thank Maitreya Dunham, my adviser at Princeton and currently at the University of Washington. My experience in her lab prior to coming to Michigan provided a crucial foundation for my scientific education. Maitreya was always an excellent adviser, and I have had several useful discussions with her during my time here too.

I would like to thank the various members of the Engelke lab throughout my time here for help with experiments and useful discussion, particularly Becky Haeusler, Matt Pratt-Hyatt, Mike Marvin, Scott Walker, and Sara Rivera. Of particular note are: May Tsoi for everything that she does to keep the lab running, and for providing us on occasion with delicious food; and Paul Good, without whom it would be hard to imagine the Engelke lab existing in a recognizable form. The staff of the Department of

Biological Chemistry has been incredibly supportive and gracious, and I especially want to single out Beth Goodwin, who always knows what to do when the rest of us don't.

I graciously thank Dave Engelke for all of his guidance during my time here. The measure of an adviser for a graduate student is not necessarily the volume of data generated or how many high profile papers one can publish but how much one can learn. In this regard I could not have asked for a more fulfilling graduate school experience. Dave is truly a mentor without parallel.

The past five and a half years could maybe have been possible without a great support system of friends, but I can't imagine exactly how that would be. I have been truly fortunate to have surrounded myself with great people, peers and colleagues, both in the department and outside of it. Of particular note is anyone who has regularly joined me in my ritual of participating in Monday night trivia at Conor O'Neill's. While it may seem like a silly thing, having a regular event week after week, regardless of how science or life was otherwise treating me, helps keep one's head clear.

I would like to thank Sarah for all of her love and support, and her family for their support as well. I thank my sister for all of her support as well and wish her luck as she embarks on her exciting career in medicine. And lastly, I would like to thank Mom and Dad for everything they have ever done for me. Since elementary school even, not only have they been overtly supportive of all of my academic and extracurricular endeavors, but they have also been active participants in my education. They have always pushed me to be my absolute best and have helped me out when I would run into roadblocks. I believe strongly that I am a product of how I was raised, and I cannot imagine being where I am today without their unflinching guidance and encouragement to do what makes me happy in life.

Table of Contents

List of Figures

Figure

List of Appendices

Appendix

Abstract

Genetic information is stored in a manner that facilitates retrieval and promotes regulation of cellular processes. In eukaryotic genomes the largest collection of co-regulated genes is the transfer RNA (tRNA) gene family, transcribed by RNA polymerase III (Pol III). The budding yeast, *Saccharomyces cerevisiae*, has 274 tRNA genes widely dispersed throughout the 16 nuclear chromosomes, yet in three dimensions these genes cluster together at the nucleolus. This work investigates the mechanism and consequences of this spatial organization of tRNA genes.

Clustering of tRNA genes had initially been observed by fluorescence microscopy, but limits on resolution prevented seeing associations for individual tRNA genes. Here, in vivo chemical crosslinking identified physical interactions between genomic loci that are closely associated in three dimensions. This confirmed nucleolar clustering of tRNA genes and further demonstrated that specific association of tRNA genes along the nucleolar ribosomal RNA (rRNA) gene repeats is dependent upon tRNA gene identity. Although tRNA gene clustering is not necessarily the primary driving force of genome organization, the results suggest they are local organizers.

The mechanism of tRNA gene clustering was examined. Previous work showed the conserved condensin complex is required for clustering and is directly bound to tRNA gene transcription complexes in vivo. This work shows that binding of the Pol III transcription factor TFIIIC to the tRNA gene is necessary and sufficient for condensin to specifically recognize the tRNA gene.

Clustering of tRNA genes contributes to “silencing” of nearby transcription by RNA polymerase II (Pol II), but the molecular mechanisms are unknown. Work in both bacterial and mammalian systems has shown that other tRNA-related RNAs bind Pol II and inhibit transcription. However, this work shows not specific RNAs but a broad spectrum of RNAs directly binds to purified yeast Pol II, preventing it from subsequently binding DNA template. Globally, this result necessitates immediate ribonucleoprotein

assembly and transport of nascent transcripts to sequester inhibitory RNAs away from the polymerase.

Overall, the findings from this dissertation further our understanding of how families of genes are spatially organized and reveal important consequences of nuclear organization on cellular processes.

Chapter I

Introduction

Abstract

The DNA of living cells is highly compacted. Inherent in this spatial constraint is the need for cells to organize individual genetic loci so as to facilitate orderly retrieval of information. Complex genetic regulatory mechanisms are crucial to all organisms, and it is becoming increasingly evident that spatial organization of genes is one very important mode of regulation for many groups of genes. In eukaryotic nuclei it appears not only that DNA is organized in three-dimensional space but also that this organization is dynamic and interactive with the transcriptional state of the genes. Spatial organization occurs throughout evolution and with genes transcribed by all classes of RNA polymerases in all eukaryotic nuclei, from yeast to human. There is an increasing body of work examining the ways in which this organization and consequent regulation are accomplished. The following is a discussion of the diverse strategies that cells use to preferentially localize various classes of genes.

Introductory remarks

It has long been realized that DNA is often organized in a manner that contributes to the regulated and efficient expression of gene products. Even so, the fact that most collections of co-regulated genes, or “regulons,” are not co-linear has led to the tacit assumption that co-regulation of linearly scattered genes is achieved by diffusible transcription factors and other regulators. This assumption of diffusible, location-

independent regulation is consistent with the fact that the linear arrangement of most genes in chromosomes is not tightly conserved, even when the sequences of the genes themselves are. A growing body of work indicates, however, that preferential three-dimensional positioning of many genes in eukaryotic nuclei is part of their transcriptional programming and, at least in some cases, facilitates use of their RNA transcripts.

Operons and other linear organizational strategies

In bacteria it is common to have all or part of a regulon made as a single transcription unit, a polycistronic operon. The operon was the earliest genetic regulatory system to have its physical DNA arrangement elucidated in the study of the *lac* operon, which controls lactose utilization in *Escherichia coli* (Jacob et al., 1960). The prokaryotic operon exemplifies how cells use linear organization to achieve regulation in one dimension and is perhaps the simplest example of spatial regulation of gene expression.

Although it was thought for some time that only bacteria and archaea contain operons, it is now known that some eukaryotes also have genomic regions that fit the classical definition of an operon. The completion of the genome sequence of the trypanosome *Leishmania major* reveals global arrangement of genes in polycistronic clusters of various sizes (Ivens et al., 2005). There are several examples of operons in other metazoans such as flatworms and certain primitive chordates (Ganot et al., 2004), but the best studied example of operons in eukaryotes remains the nematode *Caenorhabditis elegans*, the first eukaryotic organism in which extensive operons were discovered (Spieth et al., 1993). It is estimated that approximately fifteen percent of *C. elegans* genes are present in operons (Blumenthal et al., 2002; Blumenthal and Gleason, 2003). Unlike with prokaryotic operons, though, the products of the individual genes encoded by most of the operons in *C. elegans* are mostly not functionally related. Thus, it has been suggested that *C. elegans* operons are evolutionarily distinct from those present in bacteria and may have arisen, not for purposes of co-regulation as with prokaryotic operons, but from a need either to select for a smaller genome or to confer a more optimal spatial arrangement for the genes themselves. That being said, the genome of *C. elegans*

does contain a few polycistronic transcripts whose component genes do encode related protein products (Clark et al., 1994; Huang et al., 1994; Page, 1997; Treinin et al., 1998). In these instances there is an argument to be made in favor of preferential localization of gene products for co-regulation. In some cases it has been suggested that *C. elegans* operons serve purposes of co-regulation in response to a global signal (Blumenthal and Gleason, 2003), and in fact there is emerging evidence consistent with this idea (Baugh et al., 2009; Garrido-Lecca and Blumenthal, 2010). Thus, in the case of *C. elegans*, the same type of gene structure might have arisen for different needs, whether for pure spatial compaction or for regulation of gene expression.

In addition to operons, there are other varieties of linear clusters of genes in eukaryotes (Figure 1.1). Some have probably arisen originally by gene duplication, but in many cases the linear arrangements appear to benefit from regional regulatory signals that control substantial distances on the linear chromosomes. Homeotic genes, and in particular the HOX gene clusters, provide one example. First discovered in *Drosophila*, HOX genes encode several transcription factors responsible for establishing the pattern of development along the anterior-posterior axis. They are linearly positioned within the clusters in the order in which they are developmentally expressed (Kaufman et al., 1980; Lewis, 1978). HOX genes were subsequently discovered in metazoans (Akam, 1989; Duboule and Dolle, 1989; Graham et al., 1989), and their clustering has been preserved throughout evolution, although the reasons for this conservation are not fully understood (Garcia-Fernandez, 2005; reviewed in Kappen and Ruddle, 1993; Kmita and Duboule, 2003; Lappin et al., 2006; Mann, 1997). There are also several non-HOX homeotic genes that are arranged in linear clusters, and these too have evolutionary conservation in higher metazoans (reviewed in Holland, 2001). Another well-studied linear grouping is the mammalian globin genes (Cao and Moi, 2002; Liang et al., 2008; Noordermeer and de Laat, 2008; Palstra et al., 2008; Proudfoot et al., 1980; Shen et al., 2001). As with the HOX clusters, they are arranged linearly in the order in which they are expressed and are particularly interesting in that there is not only linear but also spatial coordination (discussed below).

An emerging body of work shows that many small noncoding RNAs are expressed as polycistronic units and then cut up into their functional components. In

particular, these small functional RNAs include the small nucleolar (sno) RNAs and microRNAs (Lee et al., 2002; Tycowski and Steitz, 2001). In yeast, while most snoRNAs are encoded by dispersed monocistronic genes, there are also five polycistronic clusters of two to seven snoRNA genes; the precursor transcripts are then processed by RNase III family members (Chanfreau et al., 1998; Lowe and Eddy, 1999; Qu et al., 1999). While some snoRNAs in yeast and most in mammals are intron-encoded, in plants most snoRNAs are polycistronic. In *Arabadopsis thaliana*, the majority of snoRNA genes are present in clusters transcribed from a single promoter and then processed (Leader et al., 1997). There are also some intron-encoded clusters of snoRNAs in plants, particularly in the rice genome (Liang et al., 2002). In addition, there have been dicistronic transfer RNA (tRNA)-snoRNA genes found in both *Arabadopsis* and in rice, whose precursor transcripts are processed by RNase Z (Kruszka et al., 2003). This could lead to an even higher degree of regulation between the component tRNA and snoRNA products. Likewise, microRNAs are present in clusters even more extensively in metazoans. At least 40% of microRNAs in humans have been shown to be present in clusters with pairwise distances of less than 3000 nucleotides (Altuvia et al., 2005), and while the functional relevance of these clusters is not entirely clear, at least one recent study suggests that some components of microRNA clusters do in fact have functional associations with each other (Kim et al., 2009).

Linear and spatial organization of ribosomal genes

One type of DNA sequence that is found as linear groupings in nearly all life forms is the transcription unit encoding the ribosomal RNA (rRNA) subunits (reviewed in Haeusler and Engelke, 2006). The ribosomal DNA (rDNA) is transcribed as a single polycistronic unit and then processed into its component RNAs while being assembled into pre-ribosomal particles with protein subunits. In prokaryotes the mature RNA components are the 16S, 23S, and 5S rRNAs. In eukaryotes RNA polymerase I (Pol I) transcribes the rDNA polycistronic unit, which is then processed into the 18S, 28S, and 5.8S mature ribosomal RNAs. The rDNA transcription unit is typically found as many tandem linear

repeats in genomes in all phyla of life—approximately 150-200 copies in *S. cerevisiae* and about 400 copies in human. In the case of *S. cerevisiae*, a single tandem array of rRNA genes is located on the right arm of chromosome XII. In other metazoans the ribosomal genes are also present as clusters, although there is typically not one single cluster but rather a few chromosomal locations. For example, the human tandem repeat clusters are located on the five acrocentric chromosomes (Henderson et al., 1972).

Whether present in the linear genome map as a single cluster or as multiple clusters, the ribosomal gene arrays act as the organization points of dense nuclear subcompartments termed nucleoli, the location of rRNA transcription, processing, and assembly into pre-ribosomal nucleoprotein particles (reviewed in Boisvert et al., 2007; Gerbi et al., 2003; Pederson, 1998; Prieto and McStay, 2005). Because of this role of the ribosomal clusters, they are often termed Nucleolar Organizer Regions (NORs). Yeast contain a single nucleolus visible by fluorescence in situ hybridization (FISH) microscopy or electron microscopy as a large crescent-shaped structure at one end of the nucleus, with other species containing varying numbers of nucleoli (Figure 1.2). The nucleolus exemplifies one way in which cells have developed preferential spatial positioning of genes of like function in order to maximize efficiency of cellular processes. By concentrating rRNA genes all in one place within the nucleus, the machinery needed for their transcription, processing, and assembly into ribosomes can be localized to a distinct nuclear subdomain, resembling a “factory” that Henry Ford would have envied. The framework (RNA) moves along an assembly line while the appropriate components (proteins) are loaded on at the right time and the necessary finishing steps (processing) are carried out. While localization at the nucleolus may not be essential (Oakes et al., 1998; Oakes et al., 1993), concentration and organization of various components there could facilitate timely and efficient incorporation, rather than if the components were dispersed throughout the nucleus. This sort of “assembly line” efficiency may be particularly important for ribosome biosynthesis, since it is a massive and highly complex effort that often occupies over half the RNA synthetic expenditure of the cell. The substructures associated with the rDNA Pol I transcription units and pre-ribosome assembly have been examined extensively for several decades (Hernandez-

Verdun, 2006; Olson and Dundr, 2005; reviewed in Puvion-Dutilleul et al., 1991; Scheer and Weisenberger, 1994; Schwarzacher and Wachtler, 1991).

There is one type of rRNA gene that is not part of the large Pol I transcripts in eukaryotic nuclei: the 5S rRNA gene. The 5S genes are transcribed by RNA polymerase III (Pol III) and are organized differently from the other rRNA genes, although there are distinct similarities. In some cases, notably in *S. cerevisiae*, the 5S genes are interspersed within the Pol I-transcribed ribosomal tandem arrays (Bell et al., 1977), but in most eukaryotes they are arranged in various numbers of tandem repeat clusters that are linearly separate from the large ribosomal clusters. In some cases they are present in a single cluster, as in the chicken genome (Daniels and Delany, 2003), and in other cases 5S rRNA gene types that are expressed at distinctive times in development are found in separate clusters, as with the oocyte-type vs. somatic genes in *Xenopus* (Harper et al., 1983). Linear clustering of the 5S genes might facilitate the same types of regulatory benefits that are seen in the clustering of the large ribosomal clusters. Placement of the 5S genes away from the other ribosomal genes in higher eukaryotes may serve further regulatory roles.

In a smaller number of cases, such as in *Schizocaccharomyces pombe* (Mao et al., 1982) or in *Neurospora crassa* (Metzenberg et al., 1985), the 5S genes are more dispersed throughout the linear map. In at least one case, in the non-conventional dimorphic yeast *Yarrowia lipolytica*, while the many of the 5S genes are scattered throughout the genome, nearly half of the 5S genes appear to be present in dicistronic tRNA-5S gene clusters (Acker et al., 2008). This unique type of linear cluster could potentially allow very tightly regulated transcription between the tRNA and the 5S RNA transcripts. There is also evidence that 5S genes that are otherwise clustered can be retrotransposed and that these copies are scattered throughout the genome, some of which are expressed (Drouin, 2000). In these select cases, a further level of organization might come into play for spatial regulation of expression. This would not be much of a surprise, as a substantial body of work suggests that in metazoans with separate 5S gene clusters, there is a high degree of spatial organization throughout evolution. Since the 5S genes of *S. cerevisiae* are within the large ribosomal cluster, they are necessarily nucleolar. Additionally, FISH and electron microscopy have shown nucleolar localization of the

transcribed 5S gene clusters in other organisms as well (reviewed in Haeusler and Engelke, 2006). Thus, the three-dimensional localization of 5S genes to the nucleolus, whether arranged in linear clusters or scattered across the genome, might be a component of coordinating the overall regulation of ribosomal processing and assembly.

Yeast tRNA genes: Co-localization of many linearly dispersed loci

The ribosomal RNA genes are not the only class of genes that are thought to be localized to the nucleolus. Recently it has been shown in *S. cerevisiae* that the 274 tRNA genes, which are Pol III transcription units scattered throughout the linear map of the sixteen chromosomes, are preferentially localized to the nucleolus (Thompson et al., 2003). At the time of this finding, there was some knowledge in the field about global positioning of specific regions of the yeast genome—centromeres, telomeres, and the silent mating loci are all localized to the nuclear periphery (reviewed in Gasser, 2001; Loidl, 2003). The finding that tRNA genes dispersed throughout the genome were localized to a single nuclear substructure was a somewhat astonishing observation, though it had been foreshadowed by earlier findings that components of the pre-tRNA processing pathway are found there. Imaging by FISH had shown that pre-tRNA transcripts are localized primarily to the nucleolus in *S. cerevisiae*, and some early tRNA processing enzymes in *S. cerevisiae*—specifically the endoribonuclease, RNase P, and the tRNA isopentenyltransferase, Mod5—are also nucleolar (Bertrand et al., 1998; Tolerico et al., 1999). Direct visualization of tRNA gene clusters at the nucleolus has since been confirmed by higher resolution technologies involving chemical crosslinking of nuclei and high throughput sequencing across crosslinked regions of the genome (Rodley et al., 2009). In retrospect, the concentration of tRNA genes and early processing machinery to nucleolus makes a certain amount of logistical sense, since it is the site of massive synthesis of the other non-messenger RNAs involved in translation—5S, 5.8S, and the large ribosomal RNAs. Synthesis of these RNAs is co-regulated under various conditions with tRNAs (Briand et al., 2001), and spatial coordination of these biosynthetic pathways

could provide possibilities for co-regulation, in addition to an assembly line for tRNAs, as well as ribosome synthesis and transport.

The spatial organization of tRNA genes is not likely to be a static situation. Although the majority of the tRNA genes appears to remain associated with the nucleolus throughout the cell cycle in yeast, even during late mitotic division (Haeusler et al., 2008), individual genes could vary. In fact, individual tRNA genes do dissociate into the nucleoplasm if transcription by Pol III is interrupted (Hull et al., 1994; Thompson et al., 2003). Disruption of nucleolar architecture also disperses tRNA genes and pre-tRNA transcripts throughout the nucleoplasm (Wang et al., 2005b), and thus individual tRNA genes likely become transiently dissociated from the cluster during division of the replicated nucleoli, even while the bulk of the genes seems to remain associated with the nucleolus. It is also possible that the tRNA genes can be either transiently or for long periods dissociated from the nucleolus in response to more dominant positioning imperatives from neighboring genes. No pattern of which other genes surround tRNA genes has yet made itself obvious, although genes that exist very near tRNA genes might need to be adapted to the environment. In general, transcription promoters for Pol II tend to be severely underrepresented within 500 base pairs of tRNA genes (Bolton and Boeke, 2003), yet the Ty retrotransposon elements have developed a strong preference for inserting near tRNA genes, by at least two different mechanisms (Chalker and Sandmeyer, 1990, 1992; Devine and Boeke, 1996). This suggests not only that the retrotransposons have adapted but that there is some selective advantage to the Ty or the host cell in this genomic arrangement. There is evidence that the proximity to the tRNA gene influences expression of the Ty element (Hull et al., 1994), consistent with a transcriptional regulatory adaptation.

The potential for condensation by Pol III complexes in mammals

Since the discovery of tRNA gene clusters in budding yeast, clustering of tRNA genes has also been observed in fission yeast, although these clusters co-localize with centromeres rather than the rDNA (Iwasaki et al., 2010). In metazoans tRNA genes can

be found in single or multiple copies, but little is known about their expression or localization. There is some recent information about which families of tRNA genes are actively transcribed in humans and in mice (Coughlin et al., 2009; Dittmar et al., 2006; Lowe and Eddy, 1997), but we still know relatively little about their localization. While there is no direct evidence of clustering of Pol III-transcribed genes outside of *S. cerevisiae*, it would seem possible that global organization by clustering of this type of transcription unit is not evolutionarily restricted to a single species. This hypothesis is supported by data that the clustering of tRNA genes in yeast appears to be mediated by at least one protein complex that is highly conserved throughout evolution. Chromatin immunoprecipitation followed by hybridization to high resolution oligonucleotide microarrays revealed that the multi-subunit protein complex condensin is present throughout the cell cycle over all tRNA genes and over a small number of other sites bound by Pol III transcription factors across the entire *S. cerevisiae* genome (D'Ambrosio et al., 2008). Additionally, FISH microscopy of *S. cerevisiae* nuclei in cells containing temperature-sensitive alleles of all five subunits of condensin shows a dispersal or gross mislocalization of tRNA genes away from the nucleolus (Haeusler et al., 2008). Co-immunoprecipitation experiments further show association of condensin with a DNA-mediated complex of the general transcription factors TFIIIC and TFIIIB, although not with Pol III itself, suggesting that a potentially direct interaction of condensin with the tRNA gene transcription complex may be mediating the clustering of tRNA genes to the nucleolus. Condensin is also responsible for mediating centromeric localization of tRNA gene clusters in fission yeast (Iwasaki et al., 2010).

The involvement of condensin in the dynamic positioning of chromosomal loci, while not immediately intuitive, is not overly surprising. Condensin is a member of the Structural Maintenance of Chromosomes (SMC) family of protein complexes, whose components have a high degree of structural and functional conservation throughout evolution (reviewed in Cobbe and Heck, 2000; Hirano, 2002, 2006; Huang et al., 2005; Jessberger, 2002; Losada and Hirano, 2005; Uhlmann and Hopfner, 2006). At least three distinct eukaryotic SMC complexes evolved from a single prokaryotic SMC complex, whose structure is remarkably similar to its eukaryotic counterparts. SMC complexes are thought to associate directly with DNA to mediate various activities such as chromosome

condensation and cohesion and to be essential for processes such as replication and repair. Most studies on the condensin complex in the decade and a half since its identification have focused on its functions during mitosis and meiosis. There is, however, emerging evidence for a more widespread role for condensin during interphase in various eukaryotic models (reviewed in Hirano, 2005; Legagneux et al., 2004; Tsang et al., 2007b). Specific examples come from studies on gene regulation and transcriptional control in Drosophila (Cobbe et al., 2006; Dej et al., 2004; Lupo et al., 2001) and maintenance of genome structure in yeast (Tsang et al., 2007a). Thus, it would not be surprising if, as the eukaryotic genome grew larger and the job of organizing the genome became more complex, cells evolved alternate functions outside of mitosis and meiosis for condensin in the localization of genes during interphase. Additionally, the presence of condensin at tRNA genes located at the nucleolus is consistent with data from several groups showing that condensin is highly enriched at the rDNA cluster in both budding yeast and in fission yeast and is required to maintain proper compaction of the rDNA cluster during interphase (Freeman et al., 2000; Lavoie et al., 2004; Nakazawa et al., 2008; Tsang et al., 2007a; Wang et al., 2005a; Wang and Strunnikov, 2008).

The involvement of highly conserved protein complexes at tRNA gene clusters brings into question whether clustering of Pol III elements occurs in higher eukaryotes. While the 5S and tRNA genes encode the most abundant gene products of Pol III-transcribed genes, there are other, far more abundant DNA elements containing tRNA-class Pol III promoters. In many organisms, particularly vertebrates, the most abundant Pol III elements are the short interspersed nuclear elements (SINEs) (reviewed in Belancio et al., 2008; Deininger and Batzer, 2002; Okada, 1991). Some of the better studied SINEs include the five major families of mouse SINEs, which are retrotransposons derived from pre-tRNA and 7SL RNA and make up about 7% of the murine genome (Waterston et al., 2002). In humans, the predominant SINEs are the Alu elements, which arose from 7SL RNA transcripts and are thought to comprise at least 10% of the human genome (Lander et al., 2001). Since the discovery of SINEs forty years ago, various hypotheses have been advanced for possible evolutionary advantages conferred by these “junk” sequences in the large genomes of higher eukaryotes. In many cases they have been proposed to serve as regulatory sequences, both positive and

negative (Ferrigno et al., 2001; Saffer and Thurston, 1989; Saksela and Baltimore, 1993; Thorey et al., 1993; Tomilin et al., 1990). In support of this idea, it has been shown that mouse B2 SINE transcripts can directly bind to Pol II and negatively regulate Pol II transcription (Allen et al., 2004; Espinoza et al., 2004). Newer studies reveal that human Alu transcripts can also bind to Pol II and repress its transcriptional activity (Mariner et al., 2008; Yakovchuk et al., 2009). Additionally, B2 SINE DNA can act as a chromatin "boundary element" (Lunyak et al., 2007), a block to propagation of nucleosome-mediated chromatin regulation, similarly to the function of tRNA genes and even partially assembled Pol III transcription complexes as boundary elements in yeast (Donze et al., 1999; Donze and Kamakaka, 2001; Simms et al., 2008).

In vitro, SINEs can be transcribed efficiently by tRNA gene-like complexes, although they are not normally expressed at significant levels inside cells (Carey et al., 1986; Jang and Latchman, 1989; Kim et al., 1995). It is not known if the SINEs—or a significant percentage of them—have complexes similar to the tRNA gene complexes (TFIIIC + TFIIIB + Pol III) associated with them in vivo. If they do have similar complexes, it may very well be possible that, as with yeast, they are similarly able through clustering to serve as chromosomal compaction and organizational signals. This would be consistent with at least one study showing clustering of SINE elements in human lymphocyte nuclei (Kaplan et al., 1993). Since mammalian genomes are up to a hundred times larger than those of yeast, but have only two or three times as many *bona fide* tRNA genes, one could surmise that SINE elements might serve the compaction function that tRNA genes assume in yeast, even without making stable, functional transcripts. Furthermore, since metazoans have at most a few nucleoli, the potential compaction function of SINE elements is likely to be independent of localization to any specific nuclear subcompartment (Figure 1.3).

Transcription factories and chromosome territories

The nucleolus can be thought of as a specialized version of what has been termed a "transcription factory" (Bartlett et al., 2006; Carter et al., 2008; Faro-Trindade and Cook,

2006; reviewed in Pombo and Cook, 1996; Pombo et al., 2000; Sexton et al., 2007). Just as localization of Pol I and Pol III transcription to the nucleolus serves to regulate coordinated cellular processes, so too has it been suggested that some actively elongating RNA polymerase II (Pol II) complexes are localized to factories of Pol II transcription. These were initially identified as foci of nascent transcription and later found to contain high local levels of Pol II (Iborra et al., 1996; Jackson et al., 1993; Wansink et al., 1993). Since such a significant amount of the genome is encoded by Pol II, spatially-coordinated transcription by Pol II might also have a significant impact on the three-dimensional organization of the nucleus, similarly to how clustering and localization of tRNA and rRNA genes organize the yeast genome. Indeed, while the details of transcription factory formation are not entirely clear, it appears that these factories are formed not only by genes from linearly distant regions of the same chromosome, as initially proposed, but also by genes on different chromosomes (Osborne et al., 2004; Osborne et al., 2007). Consequently, there is the possibility of a high degree of spatial organization resulting from Pol II factory formation. Nevertheless, the factory model is likely to be an oversimplified look at how genes come together, and the functional reasons for how and why various genes come together are only starting to be understood (Brown et al., 2008).

The co-localization of genes from different chromosomes is, on the surface, at odds with the idea that in most higher eukaryotes the interphase genome is widely thought to be arranged into chromosome territories (reviewed in Cremer and Cremer, 2001; Cremer et al., 2006; Gilbert et al., 2005; Meaburn and Misteli, 2007; Parada and Misteli, 2002; Parada et al., 2004). A territory is a distinct spatial region of the nucleus in which a chromosome is contained during interphase. Visualization of chromosomes in nuclei from various species, by “painting” each chromosome with distinct fluorescent probes, shows individual chromosomes occupying distinct subnuclear sections that can be easily distinguished from one another. Nevertheless, as distinct as territories may appear, within the established territory model there are examples of individual genomic loci that have been found well outside their expected territories, even for genes that are generally localized to their home territories (Chambeyron and Bickmore, 2004; Mahy et al., 2002a; Volpi et al., 2000). Given the necessarily dynamic nature of the nucleus,

though, it should not come as much of a surprise that genes can position and re-position themselves, even within the otherwise established territory model.

The majority of higher eukaryotes are now thought to have chromosome territories, although these have not been as clearly delineated in *S. cerevisiae*, despite the recent demonstration of ordered gene positions in yeast (Berger et al., 2008). The yeast genome is comparatively much smaller than those of higher eukaryotes, so it is possible that its chromosome organization evolved around a different format. It has been proposed that the yeast genome undertakes a Rabl-type organization, with centromeres and telomeres at opposite poles of the nucleus (Bystricky et al., 2004; Bystricky et al., 2005; Duan et al., 2010; Therizols et al., 2010). The existence of chromosome territories or other global organization scheme in a particular organism does not preclude the possibility of organization by Pol III elements (tRNA genes or SINEs), although it probably means that any resulting condensation is a local, rather than broadly interchromosomal, phenomenon.

A model for interactions between chromosome territories was initially proposed and termed the interchromosome domain (ICD) model (Cremer et al., 1993). The basis for this model was that transcriptionally active regions of the genome must be readily accessible to the nuclear machinery that are localized to the interchromosomal regions between territories. Individual genes can thus be strategically positioned at the interface of two or more territories to allow for maximum regulation and energetic favorability. Individual chromatin fibers would occasionally loop out into the ICD, where rare chromosomal contacts between loci would occur; some have called these contacts “chromosome kissing” (Cavalli, 2007; de Laat, 2007; Kleckner and Weiner, 1993). While this paradigm seems compelling, newer data indicate that there is a much higher degree of interaction between the territories than the ICD model suggests (reviewed in Chubb et al., 2002; Hlatky et al., 2002; Holley et al., 2002; Sachs et al., 2000; Spilianakis et al., 2005).

In order to revise the ICD model and attempt a better picture of how chromosome territories interact, Branco and Pombo developed a novel cryo-FISH method that preserves chromatin structure while providing very high microscopic resolution (Branco and Pombo, 2006). They proposed a new model for interaction between territories that

they call the interchromosomal network model (ICN). This model lays out a much more plastic arrangement of territories, where they are still localized to distinct regions of the nucleus but are much freer to intermingle with each other at their boundaries. The ICN model implies that significant interchromosomal contacts would drive the shape of territories in the nuclei of metazoans, and indeed, active transcription does affect the nature of interchromosomal contacts (Branco and Pombo, 2006). In fact, there are a variety of factors that shape territories. In particular, the overall architecture of territories has been shown to change, often drastically, in response to developmental cues in cells (Bartova and Kozubek, 2006; Kuroda et al., 2004; Stadler et al., 2004; Wegel and Shaw, 2005). Altered epigenetic marks such as methylation can also instigate changes in nuclear organization by reorganizing chromosome territories (Matarazzo et al., 2007). We should therefore look at territories as pliable, rather than rigid or impenetrable structures, especially in light of data that transcriptional machinery is shown to have the ability to access the interior of chromosome territories (Mahy et al., 2002b). In fact, most transcriptional factors are quite capable of accessing the interiors of territories. Thus, the idea that active genes are mainly positioned at the surface of chromosome territories is likely to be an oversimplification.

Positions of individual genes, in addition to overall territory architecture, can also change quite drastically in response to differentiation, developmental signals, or other changes in their transcriptional state. Much work has been done on spatial positioning of the human globin genes, in addition to their linear organization (Brown et al., 2006; Brown et al., 2001; Ragoczy et al., 2006; Tolhuis et al., 2002; Zhou et al., 2006). Active globin genes become clustered and localize to nuclear speckles (Brown et al., 2008), similar to how active tRNA genes cluster and localize at nucleoli in yeast. Studies on the oncogenes, *bcr*, *abl*, and *c-myc*, show that they change positions relative to each other in response to cell cycle or developmental cues (Bartova et al., 2000; Neves et al., 1999), suggesting that spatial positioning of developmentally important genes aids in the differentiation processes of the cell. Studies on mammalian adipogenesis genes have revealed that not only do adipogenesis-related genes change position during that process, but also that entire chromosome territories change positions, and the adipogenesis genes are often found on loops away from the main territories (Szczerbal et al., 2009). Activity-

dependent repositioning has been shown with many human genes (Lanctot et al., 2007; Meaburn and Misteli, 2008), and ligand binding to nuclear receptors can activate specific interactions between genes, which appear to be important for ligand-induced transcriptional regulation (Hu et al., 2008). Re-positioning of one gene can also bring along with it adjacent, functionally unrelated genes (Zink et al., 2004), similar to how individual yeast tRNA genes might become positioned away from the nucleolus in response to more dominant localization signals from adjacent loci. Functionally distinct alleles of the same gene can, at least in one example, occupy different positions within the nucleus (Takizawa et al., 2008).

Recently it has been shown possible to construct a map of the three-dimensional organization of the human interphase genome in relation to the transcriptome, thus tying together global genomic structure and function (Goetze et al., 2007). Indeed, there is an emerging body of work suggesting that functional interactions across chromosomes can drive gene localization (Rajapakse et al., 2009). Work with the mouse globin genes demonstrates a vast interaction network across nearly all the chromosomes with transcribed genes (Schoenfelder et al., 2010). It is becoming appreciated that transcription factor-mediated organization of linearly dispersed loci happens throughout eukaryotes. In fission yeast, highly expressed genes are often co-localized to a higher degree than average, and genes in the same gene ontology (GO) family have been shown to co-localize with each other (Tanizawa et al., 2010). Intriguingly, these spatial clusters of genes often have identical short DNA motifs in their promoter regions, further providing evidence of transcription factor-mediated co-localization. This observation is consistent with the idea of “DNA Zip Codes” that direct certain sequences of DNA to specific subnuclear regions (Ahmed et al., 2010).

Positioning of genes as a component of regulation

Several studies, particularly in yeast and flies, have provided further evidence and mechanistic insight as to how individual genes can become dramatically re-positioned based on gene activity. Multiple studies in *S. cerevisiae* have shown large-scale re-

localization of gene positions when cells are induced under certain conditions. When cells are treated with alpha-factor, the *FIG2* gene becomes highly localized to the nuclear periphery, specifically toward the side of mating projection (Casolari et al., 2005). The *SUC2* gene, which encodes a sucrose invertase, is mobile within the nucleus when repressed in glucose media, but when cells are grown in the absence of glucose to activate *SUC2*, the gene becomes tightly localized to the nuclear periphery (Sarma et al., 2007). Localization of genes to the periphery is often accompanied by physical and genetic connection to the nuclear pore complex (Cabal et al., 2006; Casolari et al., 2005; Casolari et al., 2004). One would suspect certain signaling cues to direct this localization, and it has in fact been demonstrated that phosphorylation of nuclear pore components is one of potentially many ways in which the localization of active genes is mediated (Brickner and Brickner, 2010). Additionally, there appears to be some pathway-dependence on gene dynamics. Transcriptional activation of the subtelomeric gene *HXK1* by growth on a non-glucose carbon source has been shown to relocate it to the nuclear pore complex (Taddei et al., 2006); however, when the same gene is activated via an alternative pathway, using the VP16 activator, nuclear pore association is eliminated. This result would suggest that a single gene would need to be differentially positioned within the nucleus in order to be regulated by different pathways. Recent work in *Drosophila* indicates that the *hsp70* gene cluster is anchored to the nuclear periphery, and proteins that are involved in retaining the *hsp70* cluster at the periphery are also implicated in its transcriptional regulation (Kurshakova et al., 2007). Additional work in *Drosophila* suggests a potential link between dosage compensation and localization of the X-chromosome to the nuclear pore complex (Mendjan et al., 2006).

Several studies in yeast demonstrate that artificial tethering of genes to the nuclear periphery can alter the expression of those genes or other regulatory genes. Tethering the yeast *INO1* locus to the nuclear periphery activates the *INO1* gene itself and can additionally promote either silencing or activation of other regulatory genes (Brickner and Walter, 2004). Doing the same to the *HXK1* gene also promotes its own transcriptional activation (Taddei et al., 2006). Tethering genes to the nuclear periphery can also be sufficient to activate an artificial promoter (Menon et al., 2005). These data

appear to be contrary to the original view that the nuclear periphery is a transcriptionally silenced domain in yeast.

In addition to these observations from yeast, several intriguing studies on artificial tethering of loci have been done recently in human cells. It has been shown that a gene can be artificially targeted to the periphery and is subsequently able to recruit its transcriptional machinery (Kumaran and Spector, 2008). Other work shows repression of certain loci when artificially targeted to the nuclear lamina (Reddy et al., 2008), while another report indicates that when specific chromosomes are tethered to the nuclear periphery, it is possible to change the expression patterns of certain genes to varying degrees (Finlan et al., 2008). This latter finding could have intriguing effects on human health, as aberrant gene positioning of selected marker genes may be a diagnostic tool to identify diseased alleles (e.g., Meaburn and Misteli, 2008).

In addition to the relatively recently discovered phenomenon of activation at the yeast nuclear periphery, there can be a type of transcriptional "memory" at the periphery (reviewed in Ahmed and Brickner, 2007). Upon activation, the *INO1* and *GAL1* genes are recruited to the periphery, but when repressed they continue to remain associated with the periphery for several generations before returning to their previous location in the nucleoplasm. Studies with the *GAL1* gene show that retention at the periphery allows the cells to turn these genes back on after re-activation more rapidly than nucleoplasmic *GAL1* loci. Brickner and colleagues propose that cells have a mechanism to identify recently repressed genes and suggest that this is a novel type of epigenetic memory for the cell (Brickner, 2009). Rapid re-activation of both *GAL1* and *INO1* genes and retention of either locus at the periphery are dependent on the histone variant H2A.Z (Brickner et al., 2007). In the case of the *GAL1* gene, rapid re-activation is also dependent on the SWI/SNF chromatin remodeling complex and the Gal1 protein itself (Kundu et al., 2007; Zacharioudakis et al., 2007). We can compare these requirements of specific nuclear factors in the efficient expression and localization of these loci to the requirement of condensin in positioning tRNA genes to the nucleolus, where pre-tRNA synthesis and initial processing might be readily coordinated with 5S and other rRNA biosynthetic pathways.

Concluding remarks

Computers use a tool called a cache for temporary storage of data that will likely be accessed again in the near future. Access to that data from the cache is easier or faster than from where it was originally located. In other words, once a specific memory location is accessed, that location or nearby locations can be made easier to access in the short term. We can relate this to the idea of epigenetic transcriptional memory. The nuclear periphery might act as a sort of "cache" for the *GAL1* and *INO1* genes, and retention of these genes in the "cache" of the periphery allows for faster gene activation than if those genes needed to be accessed from their original location, the nucleoplasm.

Indeed, the overall idea that cells use preferential positioning of genetic loci to regulate expression and use of gene products can be seen as one of efficient information retrieval. For thousands of years, library science has dealt with the problem of storing large numbers of documents so that they may be found and accessed readily. Computers eventually made it possible to store information in numbers that were previously beyond human limits, and the subsequent science of information retrieval allowed the creation of models to facilitate orderly and efficient access of information. The prompt recovery of genetic material from within the three-dimensional space of our nuclei is a problem not entirely different from this. As an example, the placement of tRNA genes at the nucleolus facilitates orderly retrieval of those "documents" by those who are looking for them, i.e., the tRNA processing and assembly machineries. The yeast genome contains in total 6000 genes, and humans are thought to contain 20,000-25,000 genes by most recent estimates. The rapid recovery of this large amount of information from as compact and complex of a space as the nucleus necessitates cells to develop "models" of their own for searching and using this data.

Aims of this work

The mechanics and consequences of the spatial organization of tRNA genes provides a useful system in which to study eukaryotic genome organization, as tRNA genes are the largest group of co-regulated genes in eukaryotic genomes. The budding yeast *S. cerevisiae* is an ideal model in which to study this phenomenon; tRNA genes in larger eukaryotes have largely not been annotated in detail. As detailed in this chapter, it has now been shown that the linearly dispersed tRNA genes in yeast are spatially clustered at the rDNA repeats (nucleolus). In Chapter II we examine the interaction of individual tRNA genes, which was previously not possible due to the low resolution of microscopy in yeast nuclei. Surprisingly, the identity of the tRNA gene affects individual gene-gene interactions; in particular, it affects where exactly along the rDNA repeats the tRNA gene associates.

The involvement of condensin in the formation of tRNA gene clusters has now been established both in budding yeast and in fission yeast. Thus, dissecting the mechanism of condensin interaction with a tRNA gene is helpful in understanding how tRNA genes cluster together. Previous work showed in vivo association of condensin with the Pol III transcription factors TFIIIC and TFIIIB. In Chapter III we show that TFIIIC bound to a tRNA gene is necessary and sufficient for condensin to specifically recognize the tRNA gene. These results are discussed in terms of TFIIIC-condensin associations facilitating tRNA gene organization and regulation.

One consequence of nucleolar clustering of tRNA genes is tgm silencing, although the mechanism of this silencing is not understood. Gene regulation by small RNAs is ubiquitous in both prokaryotes and eukaryotes, and in some cases, certain small RNAs can directly bind to RNA polymerases and inhibit their transcription. Thus, it was hypothesized that transcripts from nearby tRNA genes might directly inhibit Pol II locally. In Chapter IV we show that a wide variety of RNAs directly binds to Pol II near the enzyme's active site, in turn preventing binding of Pol II to a DNA template, thus inhibiting transcription. These results have consequences as a potential mechanism for tgm silencing but also create a need for nascent transcripts to be packaged and transported away from the site of transcription.

Acknowledgements

This chapter has been modified from the article, “Spatial organization of genes as a component of regulated expression,” published in *Chromosoma* [2010. Feb; 119(1): 13-25]. Permission to reproduce the article has been obtained from Springer. I would like to thank Tom Misteli, Tom Blumenthal, and Jason Brickner for critical reading of the manuscript prior to publication.

References

Acker, J., Ozanne, C., Kachouri-Lafond, R., Gaillardin, C., Neuveglise, C., and Marck, C. (2008). Dicistronic tRNA-5S rRNA genes in Yarrowia lipolytica: an alternative TFIIIA-independent way for expression of 5S rRNA genes. Nucleic acids research *36*, 5832-5844.

Ahmed, S., Brickner, D.G., Light, W.H., Cajigas, I., McDonough, M., Froyshteter, A.B., Volpe, T., and Brickner, J.H. DNA zip codes control an ancient mechanism for gene targeting to the nuclear periphery. Nature cell biology *12*, 111-118.

Ahmed, S., and Brickner, J.H. (2007). Regulation and epigenetic control of transcription at the nuclear periphery. Trends Genet *23*, 396-402.

Akam, M. (1989). Hox and HOM: homologous gene clusters in insects and vertebrates. Cell *57*, 347-349.

Allen, T.A., Von Kaenel, S., Goodrich, J.A., and Kugel, J.F. (2004). The SINE-encoded mouse B2 RNA represses mRNA transcription in response to heat shock. Nature structural & molecular biology *11*, 816-821.

Altuvia, Y., Landgraf, P., Lithwick, G., Elefant, N., Pfeffer, S., Aravin, A., Brownstein, M.J., Tuschl, T., and Margalit, H. (2005). Clustering and conservation patterns of human microRNAs. Nucleic acids research *33*, 2697-2706.

Bartlett, J., Blagojevic, J., Carter, D., Eskiw, C., Fromaget, M., Job, C., Shamsher, M., Trindade, I.F., Xu, M., and Cook, P.R. (2006). Specialized transcription factories. Biochemical Society symposium, 67-75.

Bartova, E., and Kozubek, S. (2006). Nuclear architecture in the light of gene expression and cell differentiation studies. Biology of the cell / under the auspices of the European Cell Biology Organization *98*, 323-336.

Bartova, E., Kozubek, S., Kozubek, M., Jirsova, P., Lukasova, E., Skalnikova, M., and Buchnickova, K. (2000). The influence of the cell cycle, differentiation and irradiation on the nuclear location of the abl, bcr and c-myc genes in human leukemic cells. Leukemia research *24*, 233-241.

Baugh, L.R., Demodena, J., and Sternberg, P.W. (2009). RNA Pol II accumulates at promoters of growth genes during developmental arrest. Science (New York, NY *324*, 92-94.

Belancio, V.P., Hedges, D.J., and Deininger, P. (2008). Mammalian non-LTR retrotransposons: for better or worse, in sickness and in health. Genome research *18*, 343-358.

Bell, G.I., DeGennaro, L.J., Gelfand, D.H., Bishop, R.J., Valenzuela, P., and Rutter, W.J. (1977). Ribosomal RNA genes of Saccharomyces cerevisiae. I. Physical map of the repeating unit and location of the regions coding for 5 S, 5.8 S, 18 S, and 25 S ribosomal RNAs. The Journal of biological chemistry *252*, 8118-8125.

Berger, A.B., Cabal, G.G., Fabre, E., Duong, T., Buc, H., Nehrbass, U., Olivo-Marin, J.C., Gadal, O., and Zimmer, C. (2008). High-resolution statistical mapping reveals gene territories in live yeast. Nature methods *5*, 1031-1037.

Bertrand, E., Houser-Scott, F., Kendall, A., Singer, R.H., and Engelke, D.R. (1998). Nucleolar localization of early tRNA processing. Genes & development *12*, 2463-2468.

Blumenthal, T., Evans, D., Link, C.D., Guffanti, A., Lawson, D., Thierry-Mieg, J., Thierry-Mieg, D., Chiu, W.L., Duke, K., Kiraly, M., *et al.* (2002). A global analysis of Caenorhabditis elegans operons. Nature *417*, 851-854.

Blumenthal, T., and Gleason, K.S. (2003). Caenorhabditis elegans operons: form and function. Nature reviews *4*, 112-120.

Boisvert, F.M., van Koningsbruggen, S., Navascues, J., and Lamond, A.I. (2007). The multifunctional nucleolus. Nature reviews *8*, 574-585.

Bolton, E.C., and Boeke, J.D. (2003). Transcriptional interactions between yeast tRNA genes, flanking genes and Ty elements: a genomic point of view. Genome research *13*, 254-263.

Branco, M.R., and Pombo, A. (2006). Intermingling of chromosome territories in interphase suggests role in translocations and transcription-dependent associations. PLoS biology *4*, e138.

Briand, J.F., Navarro, F., Gadal, O., and Thuriaux, P. (2001). Cross talk between tRNA and rRNA synthesis in Saccharomyces cerevisiae. Molecular and cellular biology *21*, 189-195.

Brickner, D.G., and Brickner, J.H. Cdk phosphorylation of a nucleoporin controls localization of active genes through the cell cycle. Molecular biology of the cell *21*, 3421-3432.

Brickner, D.G., Cajigas, I., Fondufe-Mittendorf, Y., Ahmed, S., Lee, P.C., Widom, J., and Brickner, J.H. (2007). H2A.Z-mediated localization of genes at the nuclear periphery confers epigenetic memory of previous transcriptional state. PLoS biology *5*, e81.

Brickner, J.H. (2009). Transcriptional memory at the nuclear periphery. Current opinion in cell biology.

Brickner, J.H., and Walter, P. (2004). Gene recruitment of the activated INO1 locus to the nuclear membrane. PLoS biology *2*, e342.

Brown, J.M., Green, J., das Neves, R.P., Wallace, H.A., Smith, A.J., Hughes, J., Gray, N., Taylor, S., Wood, W.G., Higgs, D.R., *et al.* (2008). Association between active genes occurs at nuclear speckles and is modulated by chromatin environment. The Journal of cell biology *182*, 1083-1097.

Brown, J.M., Leach, J., Reittie, J.E., Atzberger, A., Lee-Prudhoe, J., Wood, W.G., Higgs, D.R., Iborra, F.J., and Buckle, V.J. (2006). Coregulated human globin genes are frequently in spatial proximity when active. The Journal of cell biology *172*, 177-187.

Brown, K.E., Amoils, S., Horn, J.M., Buckle, V.J., Higgs, D.R., Merkenschlager, M., and Fisher, A.G. (2001). Expression of alpha- and beta-globin genes occurs within different nuclear domains in haemopoietic cells. Nature cell biology *3*, 602-606.

Bystricky, K., Heun, P., Gehlen, L., Langowski, J., and Gasser, S.M. (2004). Long-range compaction and flexibility of interphase chromatin in budding yeast analyzed by high-resolution imaging techniques. Proceedings of the National Academy of Sciences of the United States of America *101*, 16495-16500.

Bystricky, K., Laroche, T., van Houwe, G., Blaszczyk, M., and Gasser, S.M. (2005). Chromosome looping in yeast: telomere pairing and coordinated movement reflect anchoring efficiency and territorial organization. The Journal of cell biology *168*, 375-387.

Cabal, G.G., Genovesio, A., Rodriguez-Navarro, S., Zimmer, C., Gadal, O., Lesne, A., Buc, H., Feuerbach-Fournier, F., Olivo-Marin, J.C., Hurt, E.C., *et al.* (2006). SAGA interacting factors confine sub-diffusion of transcribed genes to the nuclear envelope. Nature *441*, 770-773.

Cao, A., and Moi, P. (2002). Regulation of the globin genes. Pediatric research *51*, 415-421.

Carey, M.F., Singh, K., Botchan, M., and Cozzarelli, N.R. (1986). Induction of specific transcription by RNA polymerase III in transformed cells. Molecular and cellular biology *6*, 3068-3076.

Carter, D.R., Eskiw, C., and Cook, P.R. (2008). Transcription factories. Biochemical Society transactions *36*, 585-589.

Casolari, J.M., Brown, C.R., Drubin, D.A., Rando, O.J., and Silver, P.A. (2005). Developmentally induced changes in transcriptional program alter spatial organization across chromosomes. Genes & development *19*, 1188-1198.

Casolari, J.M., Brown, C.R., Komili, S., West, J., Hieronymus, H., and Silver, P.A. (2004). Genome-wide localization of the nuclear transport machinery couples transcriptional status and nuclear organization. Cell *117*, 427-439.

Cavalli, G. (2007). Chromosome kissing. Current opinion in genetics & development *17*, 443-450.

Chalker, D.L., and Sandmeyer, S.B. (1990). Transfer RNA genes are genomic targets for de Novo transposition of the yeast retrotransposon Ty3. Genetics *126*, 837-850.

Chalker, D.L., and Sandmeyer, S.B. (1992). Ty3 integrates within the region of RNA polymerase III transcription initiation. Genes & development *6*, 117-128.

Chambeyron, S., and Bickmore, W.A. (2004). Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription. Genes & development *18*, 1119-1130.

Chanfreau, G., Legrain, P., and Jacquier, A. (1998). Yeast RNase III as a key processing enzyme in small nucleolar RNAs metabolism. Journal of molecular biology *284*, 975-988.

Chubb, J.R., Boyle, S., Perry, P., and Bickmore, W.A. (2002). Chromatin motion is constrained by association with nuclear compartments in human cells. Curr Biol *12*, 439-445.

Clark, S.G., Lu, X., and Horvitz, H.R. (1994). The Caenorhabditis elegans locus lin-15, a negative regulator of a tyrosine kinase signaling pathway, encodes two different proteins. Genetics *137*, 987-997.

Cobbe, N., and Heck, M.M. (2000). Review: SMCs in the world of chromosome biology-from prokaryotes to higher eukaryotes. Journal of structural biology *129*, 123-143.

Cobbe, N., Savvidou, E., and Heck, M.M. (2006). Diverse mitotic and interphase functions of condensins in Drosophila. Genetics *172*, 991-1008.

Coughlin, D.J., Babak, T., Nihranz, C., Hughes, T.R., and Engelke, D.R. (2009). Prediction and verification of mouse tRNA gene families. RNA biology *6*.

Cremer, T., and Cremer, C. (2001). Chromosome territories, nuclear architecture and gene regulation in mammalian cells. Nature reviews *2*, 292-301.

Cremer, T., Cremer, M., Dietzel, S., Muller, S., Solovei, I., and Fakan, S. (2006). Chromosome territories--a functional nuclear landscape. Current opinion in cell biology *18*, 307-316.

Cremer, T., Kurz, A., Zirbel, R., Dietzel, S., Rinke, B., Schrock, E., Speicher, M.R., Mathieu, U., Jauch, A., Emmerich, P., *et al.* (1993). Role of chromosome territories in the functional compartmentalization of the cell nucleus. Cold Spring Harb Symp Quant Biol *58*, 777-792.

D'Ambrosio, C., Schmidt, C.K., Katou, Y., Kelly, G., Itoh, T., Shirahige, K., and Uhlmann, F. (2008). Identification of cis-acting sites for condensin loading onto budding yeast chromosomes. Genes & development *22*, 2215-2227.

Daniels, L.M., and Delany, M.E. (2003). Molecular and cytogenetic organization of the 5S ribosomal DNA array in chicken (Gallus gallus). Chromosome Res *11*, 305-317.

de Laat, W. (2007). Long-range DNA contacts: romance in the nucleus? Current opinion in cell biology *19*, 317-320.

Deininger, P.L., and Batzer, M.A. (2002). Mammalian retroelements. Genome research *12*, 1455-1465.

Dej, K.J., Ahn, C., and Orr-Weaver, T.L. (2004). Mutations in the Drosophila condensin subunit dCAP-G: defining the role of condensin for chromosome condensation in mitosis and gene expression in interphase. Genetics *168*, 895-906.

Devine, S.E., and Boeke, J.D. (1996). Integration of the yeast retrotransposon Ty1 is targeted to regions upstream of genes transcribed by RNA polymerase III. Genes & development *10*, 620-633.

Dittmar, K.A., Goodenbour, J.M., and Pan, T. (2006). Tissue-specific differences in human transfer RNA expression. PLoS genetics *2*, e221.

Donze, D., Adams, C.R., Rine, J., and Kamakaka, R.T. (1999). The boundaries of the silenced HMR domain in Saccharomyces cerevisiae. Genes & development *13*, 698-708.

Donze, D., and Kamakaka, R.T. (2001). RNA polymerase III and RNA polymerase II promoter complexes are heterochromatin barriers in Saccharomyces cerevisiae. The EMBO journal *20*, 520-531.

Drouin, G. (2000). Expressed retrotransposed 5S rRNA genes in the mouse and rat genomes. Genome / National Research Council Canada = Genome / Conseil national de recherches Canada *43*, 213-215.

Duan, Z., Andronescu, M., Schutz, K., McIlwain, S., Kim, Y.J., Lee, C., Shendure, J., Fields, S., Blau, C.A., and Noble, W.S. A three-dimensional model of the yeast genome. Nature *465*, 363-367.

Duboule, D., and Dolle, P. (1989). The structural and functional organization of the murine HOX gene family resembles that of Drosophila homeotic genes. The EMBO journal *8*, 1497-1505.

Espinoza, C.A., Allen, T.A., Hieb, A.R., Kugel, J.F., and Goodrich, J.A. (2004). B2 RNA binds directly to RNA polymerase II to repress transcript synthesis. Nature structural & molecular biology *11*, 822-829.

Faro-Trindade, I., and Cook, P.R. (2006). Transcription factories: structures conserved during differentiation and evolution. Biochemical Society transactions *34*, 1133-1137.

Ferrigno, O., Virolle, T., Djabari, Z., Ortonne, J.P., White, R.J., and Aberdam, D. (2001). Transposable B2 SINE elements can provide mobile RNA polymerase II promoters. Nature genetics *28*, 77-81.

Finlan, L.E., Sproul, D., Thomson, I., Boyle, S., Kerr, E., Perry, P., Ylstra, B., Chubb, J.R., and Bickmore, W.A. (2008). Recruitment to the nuclear periphery can alter expression of genes in human cells. PLoS genetics *4*, e1000039.

Freeman, L., Aragon-Alcaide, L., and Strunnikov, A. (2000). The condensin complex governs chromosome condensation and mitotic transmission of rDNA. The Journal of cell biology *149*, 811-824.

Ganot, P., Kallesoe, T., Reinhardt, R., Chourrout, D., and Thompson, E.M. (2004). Spliced-leader RNA trans splicing in a chordate, Oikopleura dioica, with a compact genome. Molecular and cellular biology *24*, 7795-7805.

Garcia-Fernandez, J. (2005). The genesis and evolution of homeobox gene clusters. Nature reviews *6*, 881-892.

Garrido-Lecca, A., and Blumenthal, T. RNA polymerase II C-terminal domain phosphorylation patterns in Caenorhabditis elegans operons, polycistronic gene clusters with only one promoter. Molecular and cellular biology *30*, 3887-3893.

Gasser, S.M. (2001). Positions of potential: nuclear organization and gene expression. Cell *104*, 639-642.

Gerbi, S.A., Borovjagin, A.V., and Lange, T.S. (2003). The nucleolus: a site of ribonucleoprotein maturation. Current opinion in cell biology *15*, 318-325.

Gilbert, N., Gilchrist, S., and Bickmore, W.A. (2005). Chromatin organization in the mammalian nucleus. International review of cytology *242*, 283-336.

Goetze, S., Mateos-Langerak, J., Gierman, H.J., de Leeuw, W., Giromus, O., Indemans, M.H., Koster, J., Ondrej, V., Versteeg, R., and van Driel, R. (2007). The three-dimensional structure of human interphase chromosomes is related to the transcriptome map. Molecular and cellular biology *27*, 4475-4487.

Graham, A., Papalopulu, N., and Krumlauf, R. (1989). The murine and Drosophila homeobox gene complexes have common features of organization and expression. Cell *57*, 367-378.

Haeusler, R.A., and Engelke, D.R. (2006). Spatial organization of transcription by RNA polymerase III. Nucleic acids research *34*, 4826-4836.

Haeusler, R.A., Pratt-Hyatt, M., Good, P.D., Gipson, T.A., and Engelke, D.R. (2008). Clustering of yeast tRNA genes is mediated by specific association of condensin with tRNA gene transcription complexes. Genes & development *22*, 2204-2214.

Harper, M.E., Price, J., and Korn, L.J. (1983). Chromosomal mapping of Xenopus 5S genes: somatic-type versus oocyte-type. Nucleic acids research *11*, 2313-2323.

Henderson, A.S., Warburton, D., and Atwood, K.C. (1972). Location of ribosomal DNA in the human chromosome complement. Proceedings of the National Academy of Sciences of the United States of America *69*, 3394-3398.

Hernandez-Verdun, D. (2006). The nucleolus: a model for the organization of nuclear functions. Histochemistry and cell biology *126*, 135-148.

Hirano, T. (2002). The ABCs of SMC proteins: two-armed ATPases for chromosome condensation, cohesion, and repair. Genes & development *16*, 399-414.

Hirano, T. (2005). Condensins: organizing and segregating the genome. Curr Biol *15*, R265-275.

Hirano, T. (2006). At the heart of the chromosome: SMC proteins in action. Nat Rev Mol Cell Biol *7*, 311-322.

Hlatky, L., Sachs, R.K., Vazquez, M., and Cornforth, M.N. (2002). Radiation-induced chromosome aberrations: insights gained from biophysical modeling. Bioessays *24*, 714-723.

Holland, P.W. (2001). Beyond the Hox: how widespread is homeobox gene clustering? Journal of anatomy *199*, 13-23.

Holley, W.R., Mian, I.S., Park, S.J., Rydberg, B., and Chatterjee, A. (2002). A model for interphase chromosomes and evaluation of radiation-induced aberrations. Radiation research *158*, 568-580.

Hu, Q., Kwon, Y.S., Nunez, E., Cardamone, M.D., Hutt, K.R., Ohgi, K.A., Garcia-Bassets, I., Rose, D.W., Glass, C.K., Rosenfeld, M.G., *et al.* (2008). Enhancing nuclear

receptor-induced transcription requires nuclear motor and LSD1-dependent gene networking in interchromatin granules. Proceedings of the National Academy of Sciences of the United States of America *105*, 19199-19204.

Huang, C.E., Milutinovich, M., and Koshland, D. (2005). Rings, bracelet or snaps: fashionable alternatives for Smc complexes. Philosophical transactions of the Royal Society of London *360*, 537-542.

Huang, L.S., Tzou, P., and Sternberg, P.W. (1994). The lin-15 locus encodes two negative regulators of Caenorhabditis elegans vulval development. Mol Biol Cell *5*, 395-411.

Hull, M.W., Erickson, J., Johnston, M., and Engelke, D.R. (1994). tRNA genes as transcriptional repressor elements. Molecular and cellular biology *14*, 1266-1277.

Iborra, F.J., Pombo, A., Jackson, D.A., and Cook, P.R. (1996). Active RNA polymerases are localized within discrete transcription "factories' in human nuclei. Journal of cell science *109 (Pt 6)*, 1427-1436.

Ivens, A.C., Peacock, C.S., Worthey, E.A., Murphy, L., Aggarwal, G., Berriman, M., Sisk, E., Rajandream, M.A., Adlem, E., Aert, R., *et al.* (2005). The genome of the kinetoplastid parasite, Leishmania major. Science (New York, NY *309*, 436-442.

Iwasaki, O., Tanaka, A., Tanizawa, H., Grewal, S.I., and Noma, K. Centromeric localization of dispersed Pol III genes in fission yeast. Molecular biology of the cell *21*, 254-265.

Jackson, D.A., Hassan, A.B., Errington, R.J., and Cook, P.R. (1993). Visualization of focal sites of transcription within human nuclei. The EMBO journal *12*, 1059-1065.

Jacob, F., Perrin, D., Sanchez, C., and Monod, J. (1960). [Operon: a group of genes with the expression coordinated by an operator.]. C R Hebd Seances Acad Sci *250*, 1727-1729.

Jang, K.L., and Latchman, D.S. (1989). HSV infection induces increased transcription of Alu repeated sequences by RNA polymerase III. FEBS letters *258*, 255-258.

Jessberger, R. (2002). The many functions of SMC proteins in chromosome dynamics. Nat Rev Mol Cell Biol *3*, 767-778.

Kaplan, F.S., Murray, J., Sylvester, J.E., Gonzalez, I.L., O'Connor, J.P., Doering, J.L., Muenke, M., Emanuel, B.S., and Zasloff, M.A. (1993). The topographic organization of repetitive DNA in the human nucleolus. Genomics *15*, 123-132.

Kappen, C., and Ruddle, F.H. (1993). Evolution of a regulatory gene family: HOM/HOX genes. Current opinion in genetics & development *3*, 931-938.

Kaufman, T.C., Lewis, R., and Wakimoto, B. (1980). Cytogenetic Analysis of Chromosome 3 in DROSOPHILA MELANOGASTER: The Homoeotic Gene Complex in Polytene Chromosome Interval 84a-B. Genetics *94*, 115-133.

Kim, J., Kass, D.H., and Deininger, P.L. (1995). Transcription and processing of the rodent ID repeat family in germline and somatic cells. Nucleic acids research *23*, 2245-2251.

Kim, Y.K., Yu, J., Han, T.S., Park, S.Y., Namkoong, B., Kim, D.H., Hur, K., Yoo, M.W., Lee, H.J., Yang, H.K., *et al.* (2009). Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer. Nucleic acids research *37*, 1672-1681.

Kleckner, N., and Weiner, B.M. (1993). Potential advantages of unstable interactions for pairing of chromosomes in meiotic, somatic, and premeiotic cells. Cold Spring Harbor symposia on quantitative biology *58*, 553-565.

Kmita, M., and Duboule, D. (2003). Organizing axes in time and space; 25 years of colinear tinkering. Science (New York, NY *301*, 331-333.

Kruszka, K., Barneche, F., Guyot, R., Ailhas, J., Meneau, I., Schiffer, S., Marchfelder, A., and Echeverria, M. (2003). Plant dicistronic tRNA-snoRNA genes: a new mode of expression of the small nucleolar RNAs processed by RNase Z. The EMBO journal *22*, 621-632.

Kumaran, R.I., and Spector, D.L. (2008). A genetic locus targeted to the nuclear periphery in living cells maintains its transcriptional competence. The Journal of cell biology *180*, 51-65.

Kundu, S., Horn, P.J., and Peterson, C.L. (2007). SWI/SNF is required for transcriptional memory at the yeast GAL gene cluster. Genes & development *21*, 997-1004.

Kuroda, M., Tanabe, H., Yoshida, K., Oikawa, K., Saito, A., Kiyuna, T., Mizusawa, H., and Mukai, K. (2004). Alteration of chromosome positioning during adipocyte differentiation. Journal of cell science *117*, 5897-5903.

Kurshakova, M.M., Krasnov, A.N., Kopytova, D.V., Shidlovskii, Y.V., Nikolenko, J.V., Nabirochkina, E.N., Spehner, D., Schultz, P., Tora, L., and Georgieva, S.G. (2007). SAGA and a novel Drosophila export complex anchor efficient transcription and mRNA export to NPC. The EMBO journal *26*, 4956-4965.

Lanctot, C., Cheutin, T., Cremer, M., Cavalli, G., and Cremer, T. (2007). Dynamic genome architecture in the nuclear space: regulation of gene expression in three dimensions. Nature reviews *8*, 104-115.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., *et al.* (2001). Initial sequencing and analysis of the human genome. Nature *409*, 860-921.

Lappin, T.R., Grier, D.G., Thompson, A., and Halliday, H.L. (2006). HOX genes: seductive science, mysterious mechanisms. The Ulster medical journal *75*, 23-31.

Lavoie, B.D., Hogan, E., and Koshland, D. (2004). In vivo requirements for rDNA chromosome condensation reveal two cell-cycle-regulated pathways for mitotic chromosome folding. Genes & development *18*, 76-87.

Leader, D.J., Clark, G.P., Watters, J., Beven, A.F., Shaw, P.J., and Brown, J.W. (1997). Clusters of multiple different small nucleolar RNA genes in plants are expressed as and processed from polycistronic pre-snoRNAs. The EMBO journal *16*, 5742-5751.

Lee, Y., Jeon, K., Lee, J.T., Kim, S., and Kim, V.N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. The EMBO journal *21*, 4663-4670.

Legagneux, V., Cubizolles, F., and Watrin, E. (2004). Multiple roles of Condensins: a complex story. Biology of the cell / under the auspices of the European Cell Biology Organization *96*, 201-213.

Lewis, E.B. (1978). A gene complex controlling segmentation in Drosophila. Nature *276*, 565-570.

Liang, D., Zhou, H., Zhang, P., Chen, Y.Q., Chen, X., Chen, C.L., and Qu, L.H. (2002). A novel gene organization: intronic snoRNA gene clusters from Oryza sativa. Nucleic acids research *30*, 3262-3272.

Liang, S., Moghimi, B., Yang, T.P., Strouboulis, J., and Bungert, J. (2008). Locus control region mediated regulation of adult beta-globin gene expression. Journal of cellular biochemistry *105*, 9-16.

Loidl, J. (2003). Chromosomes of the budding yeast Saccharomyces cerevisiae. International review of cytology *222*, 141-196.

Losada, A., and Hirano, T. (2005). Dynamic molecular linkers of the genome: the first decade of SMC proteins. Genes & development *19*, 1269-1287.

Lowe, T.M., and Eddy, S.R. (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic acids research *25*, 955-964.

Lowe, T.M., and Eddy, S.R. (1999). A computational screen for methylation guide snoRNAs in yeast. Science (New York, NY *283*, 1168-1171.

Lunyak, V.V., Prefontaine, G.G., Nunez, E., Cramer, T., Ju, B.G., Ohgi, K.A., Hutt, K., Roy, R., Garcia-Diaz, A., Zhu, X., *et al.* (2007). Developmentally regulated activation of a SINE B2 repeat as a domain boundary in organogenesis. Science (New York, NY *317*, 248-251.

Lupo, R., Breiling, A., Bianchi, M.E., and Orlando, V. (2001). Drosophila chromosome condensation proteins Topoisomerase II and Barren colocalize with Polycomb and maintain Fab-7 PRE silencing. Molecular cell *7*, 127-136.

Mahy, N.L., Perry, P.E., and Bickmore, W.A. (2002a). Gene density and transcription influence the localization of chromatin outside of chromosome territories detectable by FISH. The Journal of cell biology *159*, 753-763.

Mahy, N.L., Perry, P.E., Gilchrist, S., Baldock, R.A., and Bickmore, W.A. (2002b). Spatial organization of active and inactive genes and noncoding DNA within chromosome territories. The Journal of cell biology *157*, 579-589.

Mann, R.S. (1997). Why are Hox genes clustered? Bioessays *19*, 661-664.

Mao, J., Appel, B., Schaack, J., Sharp, S., Yamada, H., and Soll, D. (1982). The 5S RNA genes of Schizosaccharomyces pombe. Nucleic acids research *10*, 487-500.

Mariner, P.D., Walters, R.D., Espinoza, C.A., Drullinger, L.F., Wagner, S.D., Kugel, J.F., and Goodrich, J.A. (2008). Human Alu RNA is a modular transacting repressor of mRNA transcription during heat shock. Molecular cell *29*, 499-509.

Matarazzo, M.R., Boyle, S., D'Esposito, M., and Bickmore, W.A. (2007). Chromosome territory reorganization in a human disease with altered DNA methylation. Proceedings of the National Academy of Sciences of the United States of America *104*, 16546-16551.

Meaburn, K.J., and Misteli, T. (2007). Cell biology: chromosome territories. Nature *445*, 379-781.

Meaburn, K.J., and Misteli, T. (2008). Locus-specific and activity-independent gene repositioning during early tumorigenesis. The Journal of cell biology *180*, 39-50.

Mendjan, S., Taipale, M., Kind, J., Holz, H., Gebhardt, P., Schelder, M., Vermeulen, M., Buscaino, A., Duncan, K., Mueller, J., *et al.* (2006). Nuclear pore components are involved in the transcriptional regulation of dosage compensation in Drosophila. Molecular cell *21*, 811-823.

Menon, B.B., Sarma, N.J., Pasula, S., Deminoff, S.J., Willis, K.A., Barbara, K.E., Andrews, B., and Santangelo, G.M. (2005). Reverse recruitment: the Nup84 nuclear pore subcomplex mediates Rap1/Gcr1/Gcr2 transcriptional activation. Proceedings of the National Academy of Sciences of the United States of America *102*, 5749-5754.

Metzenberg, R.L., Stevens, J.N., Selker, E.U., and Morzycka-Wroblewska, E. (1985). Identification and chromosomal distribution of 5S rRNA genes in Neurospora crassa. Proceedings of the National Academy of Sciences of the United States of America *82*, 2067-2071.

Nakazawa, N., Nakamura, T., Kokubu, A., Ebe, M., Nagao, K., and Yanagida, M. (2008). Dissection of the essential steps for condensin accumulation at kinetochores and rDNAs during fission yeast mitosis. The Journal of cell biology *180*, 1115-1131.

Neves, H., Ramos, C., da Silva, M.G., Parreira, A., and Parreira, L. (1999). The nuclear topography of ABL, BCR, PML, and RARalpha genes: evidence for gene proximity in specific phases of the cell cycle and stages of hematopoietic differentiation. Blood *93*, 1197-1207.

Noordermeer, D., and de Laat, W. (2008). Joining the loops: beta-globin gene regulation. IUBMB life *60*, 824-833.

Oakes, M., Aris, J.P., Brockenbrough, J.S., Wai, H., Vu, L., and Nomura, M. (1998). Mutational analysis of the structure and localization of the nucleolus in the yeast Saccharomyces cerevisiae. The Journal of cell biology *143*, 23-34.

Oakes, M., Nogi, Y., Clark, M.W., and Nomura, M. (1993). Structural alterations of the nucleolus in mutants of Saccharomyces cerevisiae defective in RNA polymerase I. Molecular and cellular biology *13*, 2441-2455.

Okada, N. (1991). SINEs. Current opinion in genetics & development *1*, 498-504.

Olson, M.O., and Dundr, M. (2005). The moving parts of the nucleolus. Histochemistry and cell biology *123*, 203-216.

Osborne, C.S., Chakalova, L., Brown, K.E., Carter, D., Horton, A., Debrand, E., Goyenechea, B., Mitchell, J.A., Lopes, S., Reik, W., *et al.* (2004). Active genes dynamically colocalize to shared sites of ongoing transcription. Nat Genet *36*, 1065-1071.

Osborne, C.S., Chakalova, L., Mitchell, J.A., Horton, A., Wood, A.L., Bolland, D.J., Corcoran, A.E., and Fraser, P. (2007). Myc dynamically and preferentially relocates to a transcription factory occupied by Igh. PLoS biology *5*, e192.

Page, A.P. (1997). Cyclophilin and protein disulfide isomerase genes are co-transcribed in a functionally related manner in Caenorhabditis elegans. DNA Cell Biol *16*, 1335-1343.

Palstra, R.J., de Laat, W., and Grosveld, F. (2008). Beta-globin regulation and long-range interactions. Advances in genetics *61*, 107-142.

Parada, L., and Misteli, T. (2002). Chromosome positioning in the interphase nucleus. Trends in cell biology *12*, 425-432.

Parada, L.A., McQueen, P.G., and Misteli, T. (2004). Tissue-specific spatial organization of genomes. Genome biology *5*, R44.

Pederson, T. (1998). The plurifunctional nucleolus. Nucleic acids research *26*, 3871-3876.

Pombo, A., and Cook, P.R. (1996). The localization of sites containing nascent RNA and splicing factors. Experimental cell research *229*, 201-203.

Pombo, A., Jones, E., Iborra, F.J., Kimura, H., Sugaya, K., Cook, P.R., and Jackson, D.A. (2000). Specialized transcription factories within mammalian nuclei. Critical reviews in eukaryotic gene expression *10*, 21-29.

Prieto, J.L., and McStay, B. (2005). Nucleolar biogenesis: the first small steps. Biochemical Society transactions *33*, 1441-1443.

Proudfoot, N.J., Shander, M.H., Manley, J.L., Gefter, M.L., and Maniatis, T. (1980). Structure and in vitro transcription of human globin genes. Science (New York, NY *209*, 1329-1336.

Puvion-Dutilleul, F., Bachellerie, J.P., and Puvion, E. (1991). Nucleolar organization of HeLa cells as studied by in situ hybridization. Chromosoma *100*, 395-409.

Qu, L.H., Henras, A., Lu, Y.J., Zhou, H., Zhou, W.X., Zhu, Y.Q., Zhao, J., Henry, Y., Caizergues-Ferrer, M., and Bachellerie, J.P. (1999). Seven novel methylation guide small nucleolar RNAs are processed from a common polycistronic transcript by Rat1p and RNase III in yeast. Molecular and cellular biology *19*, 1144-1158.

Ragoczy, T., Bender, M.A., Telling, A., Byron, R., and Groudine, M. (2006). The locus control region is required for association of the murine beta-globin locus with engaged transcription factories during erythroid maturation. Genes & development *20*, 1447-1457.

Rajapakse, I., Perlman, M.D., Scalzo, D., Kooperberg, C., Groudine, M., and Kosak, S.T. (2009). The emergence of lineage-specific chromosomal topologies from coordinate gene regulation. Proceedings of the National Academy of Sciences of the United States of America *106*, 6679-6684.

Reddy, K.L., Zullo, J.M., Bertolino, E., and Singh, H. (2008). Transcriptional repression mediated by repositioning of genes to the nuclear lamina. Nature *452*, 243-247.

Rodley, C.D., Bertels, F., Jones, B., and O'Sullivan, J.M. (2009). Global identification of yeast chromosome interactions using Genome conformation capture. Fungal Genet Biol *46*, 879-886.

Sachs, R.K., Levy, D., Chen, A.M., Simpson, P.J., Cornforth, M.N., Ingerman, E.A., Hahnfeldt, P., and Hlatky, L.R. (2000). Random breakage and reunion chromosome aberration formation model; an interaction-distance version based on chromatin geometry. International journal of radiation biology *76*, 1579-1588.

Saffer, J.D., and Thurston, S.J. (1989). A negative regulatory element with properties similar to those of enhancers is contained within an Alu sequence. Molecular and cellular biology *9*, 355-364.

Saksela, K., and Baltimore, D. (1993). Negative regulation of immunoglobulin kappa light-chain gene transcription by a short sequence homologous to the murine B1 repetitive element. Molecular and cellular biology *13*, 3698-3705.

Sarma, N.J., Haley, T.M., Barbara, K.E., Buford, T.D., Willis, K.A., and Santangelo, G.M. (2007). Glucose-responsive regulators of gene expression in Saccharomyces cerevisiae function at the nuclear periphery via a reverse recruitment mechanism. Genetics *175*, 1127-1135.

Scheer, U., and Weisenberger, D. (1994). The nucleolus. Current opinion in cell biology *6*, 354-359.

Schoenfelder, S., Sexton, T., Chakalova, L., Cope, N.F., Horton, A., Andrews, S., Kurukuti, S., Mitchell, J.A., Umlauf, D., Dimitrova, D.S., *et al.* Preferential associations between co-regulated genes reveal a transcriptional interactome in erythroid cells. Nature genetics *42*, 53-61.

Schwarzacher, H.G., and Wachtler, F. (1991). The functional significance of nucleolar structures. Annales de genetique *34*, 151-160.

Sexton, T., Umlauf, D., Kurukuti, S., and Fraser, P. (2007). The role of transcription factories in large-scale structure and dynamics of interphase chromatin. Seminars in cell & developmental biology *18*, 691-697.

Shen, W., Liu, D.P., and Liang, C.C. (2001). The regulatory network controlling beta-globin gene switching. Molecular biology reports *28*, 175-183.

Simms, T.A., Dugas, S.L., Gremillion, J.C., Ibos, M.E., Dandurand, M.N., Toliver, T.T., Edwards, D.J., and Donze, D. (2008). TFIIIC binding sites function as both heterochromatin barriers and chromatin insulators in Saccharomyces cerevisiae. Eukaryotic cell *7*, 2078-2086.

Spieth, J., Brooke, G., Kuersten, S., Lea, K., and Blumenthal, T. (1993). Operons in C. elegans: polycistronic mRNA precursors are processed by trans-splicing of SL2 to downstream coding regions. Cell *73*, 521-532.

Spilianakis, C.G., Lalioti, M.D., Town, T., Lee, G.R., and Flavell, R.A. (2005). Interchromosomal associations between alternatively expressed loci. Nature *435*, 637-645.

Stadler, S., Schnapp, V., Mayer, R., Stein, S., Cremer, C., Bonifer, C., Cremer, T., and Dietzel, S. (2004). The architecture of chicken chromosome territories changes during differentiation. BMC cell biology *5*, 44.

Szczerbal, I., Foster, H.A., and Bridger, J.M. (2009). The spatial repositioning of adipogenesis genes is correlated with their expression status in a porcine mesenchymal stem cell adipogenesis model system. Chromosoma *118*, 647-663.

Taddei, A., Van Houwe, G., Hediger, F., Kalck, V., Cubizolles, F., Schober, H., and Gasser, S.M. (2006). Nuclear pore association confers optimal expression levels for an inducible yeast gene. Nature *441*, 774-778.

Takizawa, T., Gudla, P.R., Guo, L., Lockett, S., and Misteli, T. (2008). Allele-specific nuclear positioning of the monoallelically expressed astrocyte marker GFAP. Genes & development *22*, 489-498.

Tanizawa, H., Iwasaki, O., Tanaka, A., Capizzi, J.R., Wickramasinghe, P., Lee, M., Fu, Z., and Noma, K. Mapping of long-range associations throughout the fission yeast genome reveals global genome organization linked to transcriptional regulation. Nucleic acids research *38*, 8164-8177.

Therizols, P., Duong, T., Dujon, B., Zimmer, C., and Fabre, E. Chromosome arm length and nuclear constraints determine the dynamic relationship of yeast subtelomeres. Proceedings of the National Academy of Sciences of the United States of America *107*, 2025-2030.

Thompson, M., Haeusler, R.A., Good, P.D., and Engelke, D.R. (2003). Nucleolar clustering of dispersed tRNA genes. Science (New York, NY *302*, 1399-1401.

Thorey, I.S., Cecena, G., Reynolds, W., and Oshima, R.G. (1993). Alu sequence involvement in transcriptional insulation of the keratin 18 gene in transgenic mice. Molecular and cellular biology *13*, 6742-6751.

Tolerico, L.H., Benko, A.L., Aris, J.P., Stanford, D.R., Martin, N.C., and Hopper, A.K. (1999). Saccharomyces cerevisiae Mod5p-II contains sequences antagonistic for nuclear and cytosolic locations. Genetics *151*, 57-75.

Tolhuis, B., Palstra, R.J., Splinter, E., Grosveld, F., and de Laat, W. (2002). Looping and interaction between hypersensitive sites in the active beta-globin locus. Molecular cell *10*, 1453-1465.

Tomilin, N.V., Iguchi-Ariga, S.M., and Ariga, H. (1990). Transcription and replication silencer element is present within conserved region of human Alu repeats interacting with nuclear protein. FEBS letters *263*, 69-72.

Treinin, M., Gillo, B., Liebman, L., and Chalfie, M. (1998). Two functionally dependent acetylcholine subunits are encoded in a single Caenorhabditis elegans operon. Proceedings of the National Academy of Sciences of the United States of America *95*, 15492-15495.

Tsang, C.K., Li, H., and Zheng, X.S. (2007a). Nutrient starvation promotes condensin loading to maintain rDNA stability. The EMBO journal *26*, 448-458.

Tsang, C.K., Wei, Y., and Zheng, X.F. (2007b). Compacting DNA during the interphase: condensin maintains rDNA integrity. Cell cycle (Georgetown, Tex *6*, 2213-2218.

Tycowski, K.T., and Steitz, J.A. (2001). Non-coding snoRNA host genes in Drosophila: expression strategies for modification guide snoRNAs. European journal of cell biology *80*, 119-125.

Uhlmann, F., and Hopfner, K.P. (2006). Chromosome biology: the crux of the ring. Curr Biol *16*, R102-105.

Volpi, E.V., Chevret, E., Jones, T., Vatcheva, R., Williamson, J., Beck, S., Campbell, R.D., Goldsworthy, M., Powis, S.H., Ragoussis, J., *et al.* (2000). Large-scale chromatin organization of the major histocompatibility complex and other regions of human chromosome 6 and its response to interferon in interphase nuclei. Journal of cell science *113 (Pt 9)*, 1565-1576.

Wang, B.D., Eyre, D., Basrai, M., Lichten, M., and Strunnikov, A. (2005a). Condensin binding at distinct and specific chromosomal sites in the Saccharomyces cerevisiae genome. Molecular and cellular biology *25*, 7216-7225.

Wang, B.D., and Strunnikov, A. (2008). Transcriptional homogenization of rDNA repeats in the episome-based nucleolus induces genome-wide changes in the chromosomal distribution of condensin. Plasmid *59*, 45-53.

Wang, L., Haeusler, R.A., Good, P.D., Thompson, M., Nagar, S., and Engelke, D.R. (2005b). Silencing near tRNA genes requires nucleolar localization. J Biol Chem *280*, 8637-8639.

Wansink, D.G., Schul, W., van der Kraan, I., van Steensel, B., van Driel, R., and de Jong, L. (1993). Fluorescent labeling of nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. The Journal of cell biology *122*, 283-293.

Waterston, R.H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J.F., Agarwal, P., Agarwala, R., Ainscough, R., Alexandersson, M., An, P., *et al.* (2002). Initial sequencing and comparative analysis of the mouse genome. Nature *420*, 520-562.

Wegel, E., and Shaw, P. (2005). Gene activation and deactivation related changes in the three-dimensional structure of chromatin. Chromosoma *114*, 331-337.

Yakovchuk, P., Goodrich, J.A., and Kugel, J.F. (2009). B2 RNA and Alu RNA repress transcription by disrupting contacts between RNA polymerase II and promoter DNA within assembled complexes. Proceedings of the National Academy of Sciences of the United States of America.

Zacharioudakis, I., Gligoris, T., and Tzamarias, D. (2007). A yeast catabolic enzyme controls transcriptional memory. Curr Biol *17*, 2041-2046.

Zhou, G.L., Xin, L., Song, W., Di, L.J., Liu, G., Wu, X.S., Liu, D.P., and Liang, C.C. (2006). Active chromatin hub of the mouse alpha-globin locus forms in a transcription factory of clustered housekeeping genes. Molecular and cellular biology *26*, 5096-5105.

Zink, D., Amaral, M.D., Englmann, A., Lang, S., Clarke, L.A., Rudolph, C., Alt, F., Luther, K., Braz, C., Sadoni, N., *et al.* (2004). Transcription-dependent spatial arrangements of CFTR and adjacent genes in human cell nuclei. The Journal of cell biology *166*, 815-825.

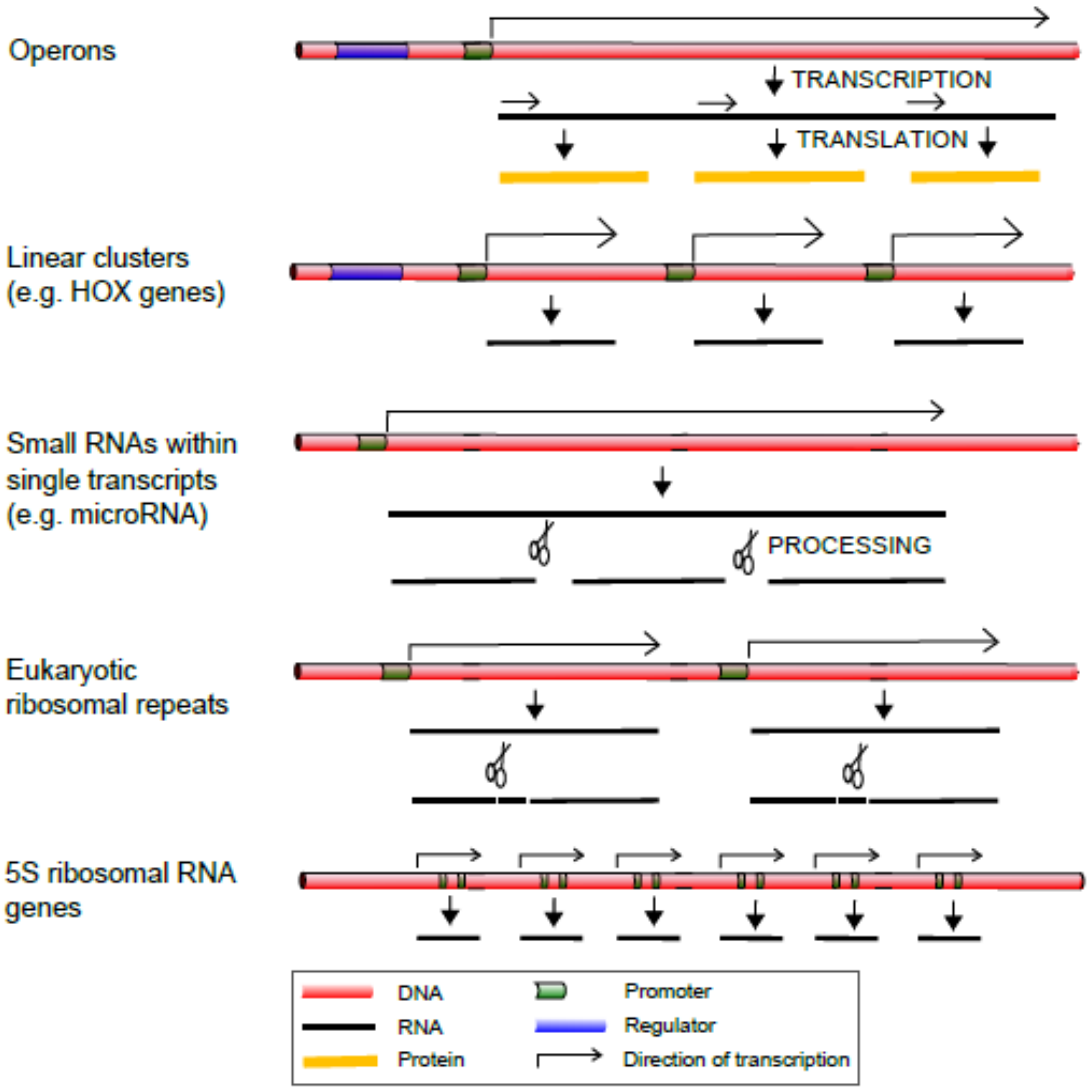


Figure 1.1. Methods of linear gene organization. Eukaryotic cells have developed a variety of ways to arrange genetic information on the linear map to regulate gene expression. From top to bottom: Operons, which are transcribed as a single polycistronic transcript under control of an upstream operator; Linear clusters, such as the HOX genes, which are under control of a common regulator; Small RNAs, such as microRNAs, which are transcribed as a polycistronic unit and then processed into smaller RNAs; the Pol I-transcribed ribosomal repeats, transcribed in eukaryotes as a 35S transcript and then processed into 18S, 5.8S, and 28S rRNAs; and the small Pol III-transcribed 5S genes, which in most eukaryotes are present in tandemly repeated linear clusters.

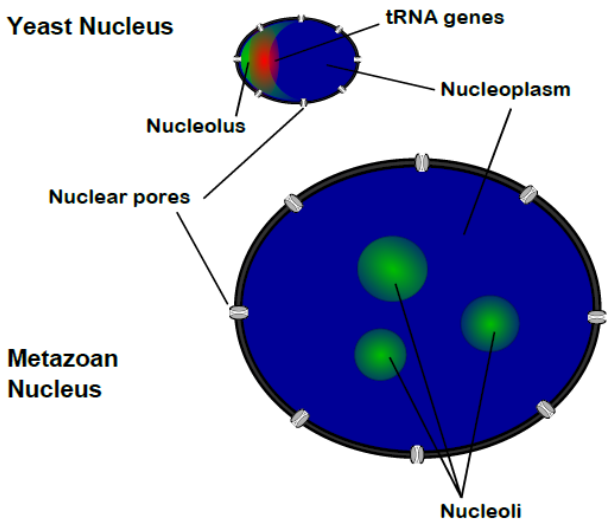


Figure 1.2. Comparison of yeast and metazoan nucleoli. The eukaryotic nucleolus is defined by the Pol I-transcribed ribosomal cluster. In yeast, the ribosomal cluster is located on the linear map in one group on chromosome XII; consequently, the yeast nucleolus can be visualized by FISH microscopy as a single crescent-shaped structure, typically localized to one side of the nucleus. In yeast, the tRNA genes can be visualized by FISH microscopy as a single cluster localized to the nucleolus. Metazoans generally have multiple clusters of ribosomal genes; thus, metazoan nuclei usually have several nucleoli spread throughout the nucleus. The metazoan nucleus is generally several times larger than the yeast nucleus.

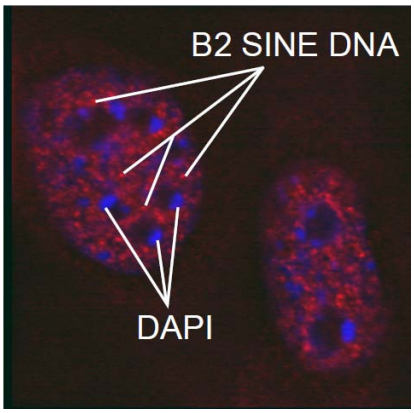


Figure 1.3. FISH microscopy showing distribution of mouse B2 SINEs. Mouse embryonic fibroblasts were fixed in 1% paraformaldehyde and adhered to slides. Fluorescent oligonucleotides complementary to B2 SINEs hybridized to genomic DNA. There appears to be speckled signal of B2 elements throughout the nucleoplasm, suggesting clusters, but this signal appears not to be preferentially associated with either the nucleoli or the nuclear periphery. Red B2 SINE DNA, blue DAPI stain of AT-rich heterochromatin. Thanks to Paul Good for data.

Chapter II

tRNA gene identity affects local partner selection but not necessarily global genome arrangement

Abstract

The three-dimensional organization of genomes is dynamic and plays a critical role in the regulation of cellular development and phenotypes. The tRNA genes in yeast provide a useful system in which to study the spatial organization of a single large family of co-regulated genes. In this chapter, proximity-based ligation methods (i.e., chromosome conformation capture [3C] and circularized chromosome conformation capture [4C]) are used to explore the spatial organization of tRNA genes and their locus-specific interactions with the ribosomal DNA. The results indicate that these interactions depend on both tRNA coding sequence identity as well as the surrounding chromosomal loci. These observations support a model whereby the three-dimensional, spatial organization of tRNA loci within the nucleus utilizes tRNA gene-specific signals to affect local interactions, though broader organization of chromosomal regions are determined by factors outside the tRNA genes themselves.

Introduction

DNA in the nuclei of eukaryotic organisms is arranged in an ordered yet dynamic manner, necessary for establishing and maintaining proper compaction and regulation of our genomes. Structural genome organization is manifested on different levels, such as linear operons (Blumenthal and Gleason, 2003) and arrays of genes, as well as spatial arrangement of chromosome territories (Cremer et al., 2006; Meaburn and Misteli, 2007).

Recent studies have implicated interactions that form between genomic loci in the regulation of genes (Brown et al., 2008; Lanctôt et al., 2007) and of cellular processes such as development and differentiation (Brown et al., 2006; Ragoczy et al., 2006). These phenomena have been studied in many eukaryotic systems (see Chapter 1 for a full review), yet the spatial organization of a single family of genes not present as a linear cluster has not extensively been investigated.

Examination of the spatial organization of gene families can provide insight into how position relates to evolutionary or functional imperatives. The largest family of co-regulated genes in the eukaryotic genome is the RNA polymerase III (Pol III)-transcribed tRNA gene family. The budding yeast *Saccharomyces cerevisiae* has 274 tRNA genes that are dispersed throughout the linear maps of the 16 chromosomes. Fluorescence *in situ* hybridization (FISH) microscopy has shown that these tRNA genes are clustered throughout the cell cycle, with the assistance of condensin complexes bound at each gene, and that clusters localize to the boundary of the nucleolus in a microtubule-dependent manner (Haeusler et al., 2008; Thompson et al., 2003; Wang et al., 2005). Condensin has also been localized to the nucleolar ribosomal DNA (rDNA) repeats, and mutants of condensin affect proper compaction of the rDNA repeats (D'Ambrosio et al., 2008; Freeman et al., 2000; Machin et al., 2004; Tsang et al., 2007; Wang and Strunnikov, 2008). Clustering of tRNA genes has also been observed in fission yeast (Iwasaki and Noma, 2010; Iwasaki et al., 2010), although their subnuclear localization is different from that seen in budding yeast.

Fluorescence microscopy provided an initial picture of the general arrangement of tRNA genes within the budding yeast nucleus, but the limited resolution of the technique combined with the small size of yeast nuclei prohibited further understanding of the details of these spatial interactions. Proximity-based ligation methodologies, which crosslink spatially adjacent loci, now permit investigation of direct physical interactions between genes in greater detail. Two of these techniques, genome conformation capture (GCC) and a variant of HiC, have previously been used to produce a yeast genome contact map (Duan et al., 2010; Rodley et al., 2009). These methods confirmed the microscopy results by showing preferential interactions between tRNA genes (Rodley et al., 2009), consistent with the physical clustering observed by fluorescence microscopy.

Since the localization of a large number of dispersed genes to a single subnuclear region necessarily requires a vast rearrangement of the genome, it is of interest to investigate whether individual tRNA gene associations are a controlling influence on the overall organization of the genome, or merely serve as non-specific "fasteners." In the latter case, the tRNA genes as "fasteners" would provide a level of local condensation, while global organization would be determined by factors other than tRNA genes. In this chapter we use three methods that rely on proximity—GCC, chromosome conformation capture (3C), and circularized chromosome conformation capture (4C)—to examine the contributions that tRNA genes make to the positioning of specific loci within the *S. cerevisiae* nucleus.

Results

Genome conformation capture was performed on unsynchronized, exponentially growing *S. cerevisiae* cells (Figure 2.1A) and (Rodley et al., 2009). Briefly, cells were chemically crosslinked in vivo, digested with the restriction enzyme *Msp*I, diluted, and ligated to promote intramolecular ligation. Crosslinks were reversed to generate a DNA library representing the set of in vivo DNA interactions in the genome. The library was analyzed as described. In agreement with FISH imaging studies (Bertrand et al., 1998; Haeusler et al., 2008; Thompson et al., 2003; Wang et al., 2005), GCC revealed that many tRNA genes formed multiple interactions with the ribosomal DNA locus (RDN) on Chromosome XII (Rodley et al., 2009), which contains multiple tandem copies of the ribosomal RNA (rRNA) genes and forms the nucleolus. While numerous of these interactions were well above background, there was an extremely strong and specific interaction between one particular DNA fragment containing a $tRNA^{Lys}$ gene on Chromosome XVI, *tK(CUU)P* (Chr XVI: 581,025-583,522; Figure 2.1B, top panel, and Figure 2.2), and the non-transcribed spacer sequence (NTS1) in the RDN locus, adjacent to the Pol III-transcribed 5S rRNA gene (Chr XII: 460,025-460,609). None of the *Msp*I restriction fragments adjacent to the *tK(CUU)P* $tRNA^{Lys}$ gene fragment interacted with NTS1 (Figure 2.2). In fact only two of the nearby fragments (Chr XVI: 585884-589137

and 549477-580469, respectively) interacted with the rDNA at levels even slightly above the background (Figure 2.2; compare "-2" and "+2" fragments with the middle fragment). It is theoretically possible that some of the *tK(CUU)P*-NTS1 interactions involve the extra-chromosomal rDNA circles (ERCs) that are present within the yeast nucleus (Ganley et al., 2009; Sinclair and Guarente, 1997). However, preliminary data indicate that high ERC copy number does not correlate with increased interaction frequencies (data not shown; communicated by Justin O'Sullivan). We conclude that the interaction is driven by signals within the fragment and, given that tRNA genes are known to cluster with the nucleolus (Haeusler et al., 2008; Thompson et al., 2003; Wang et al., 2005), we hypothesized that the tRNA gene was responsible for this interaction.

Clustering of the tRNA genes is mediated by one or more condensin complexes bound to the tRNA genes (D'Ambrosio et al., 2008; Haeusler et al., 2008). Since condensin is also bound to the rDNA repeats and concentrated adjacent to the 5S rRNA gene (D'Ambrosio et al., 2008), we hypothesized that the *tK(CUU)P*-NTS1 association might be determined by a condensin-condensin interaction between the tRNA gene complex and the NTS1 region. However, we remained open to the possibility that there might be other contributors that direct this particular tRNA gene to this specific region of the RDN locus. Therefore, to examine to what extent the tRNA gene was responsible for this tight association, we performed 3C on both a wild type (WT) strain and the same strain from which only the tRNA gene coding region, including its intragenic transcription promoter, had been precisely deleted (yDP97, *tK(CUU)P::kanMX6* (Longtine et al., 1998); Figure 2.1B, bottom panel). Quantitative analysis of locus proximities by 3C (O'Sullivan et al., 2009; Rodley et al., 2009) showed that precise deletion of the *tK(CUU)P* coding sequence did not significantly alter the frequency of interaction between the general locus (fragments F1 or F2' on Chromosome XVI) and a *Hin*dIII fragment spanning the 5S rRNA gene and including the NTS1 region (Figures 2.1C and 2.1D). Furthermore, the deletion did not significantly affect growth rate, determined by co-culturing the two strains for 100 generations (Figure 2.1E). This lack of growth defect suggests an absence of serious disruption to nuclear organization, in contrast to the strong growth defects that were previously observed in mutants that disrupted general tRNA gene clustering or nucleolar organization (Haeusler et al., 2008;

Kendall et al., 2000; Wang et al., 2005). Together, these results indicate that the *tK(CUU)P* $tRNA^{Lys}$ gene itself does not provide the major driving force that determines the proximity of this chromosomal segment to the rDNA repeats. Rather, the general spatial arrangement of these chromosomal loci is driven by factors outside the tRNA gene. Despite this, this result does not preclude the possibility that the tRNA gene might determine the positions of local contacts.

The coding regions of yeast tRNA genes contain the major transcriptional promoter elements and are bound by identical sets of transcription components (i.e., TFIIIC, TFIIIB, and Pol III) (Kassavetis et al., 1990; Moqtaderi and Struhl, 2004; Roberts et al., 2003), consistent with the finding that all tested tRNA gene loci can be expressed. Therefore, it was predicted that the nature of the tRNA coding sequences would not alter the interaction behavior of a locus. To test this prediction, we precisely replaced the mature tRNA coding regions of two different $tRNA^{Leu}$ loci—*tL(UAA)B2* on Chromosome II, and *tL(CAA)G3* on Chromosome VII. The coding regions of these $tRNA^{Leu}$ genes interacted with the RDN locus in the parental strain, although with different patterns across the rDNA repeats (Figure 2.3B). The *tL(UAA)B2* and *tL(CAA)G3* coding regions were precisely replaced with the coding region from a $tRNA^{Tyr}$ gene-variant, the *SUP4*-1 ochre suppressor (Kurjan et al., 1980), to allow selection for the insertion (Figure 2.3A). In each case the 5' and 3' flanking regions, including upstream transcription initiation and downstream termination sites, and the primary transcript processed leader and trailer sequences were retained from the original $tRNA^{Leu}$ locus. As expected, the *SUP4*-1 replacements of the $tRNA^{Leu}$ coding regions continued to allow association of both loci with the RDN locus. Yet, unexpectedly, the preferred positions of the associations along the RDN locus were altered (Figure 2.3B). The dataset represents the results of a single 4C experiment. Inter-experiment variation can arise through differences in growth, crosslinking, digestion, and ligation; these can affect comparison between different datasets, so replicate experiments were not performed. However, none of these issues affect *relative* comparisons of interaction frequencies within a single experiment.

As a control for general disruption of tRNA gene contacts, quantitative 3C analyses of the *S. cerevisiae* strains yPH499, yDP77, and yDP84 identified no significant

differences in the interactions between the wild-type *SUP4* $tRNA^{Tyr}$ gene at its native locus (*tY(GUA)J2* on Chr X: 542960-543119) and the 25S rDNA (Chr XII: 451928-452600) locus (Figure 2.4). These results are consistent with the *tK(CUU)P* $tRNA^{Lys}$ gene replacement data above, suggesting that external sequences specify general positioning within the nuclear space but that internal factors affect precise local positioning.

The finding that the identity of the tRNA gene itself affects partner selection was surprising to us, and in fact, upon further investigation of the dataset, we found many other interactions demonstrating that the identity of the internal tRNA gene sequences does indeed affect partner selection. Looking at the interactions between the replaced tRNA genes and other non-repetitive genes, some preferred interaction partners were gained following replacement of the original $tRNA^{Leu}$ gene sequence with the *SUP4*-1 sequence (Table 2.1). The most pronounced of these were the interactions between *SUP4*-1 at the new loci and the genes *SRB2* and *MED6* (Table 2.1, Lines 15 and 16, respectively). These two genes do not interact at all with the $tRNA^{Leu}$ genes, but when the $tRNA^{Leu}$ genes are replaced with the *SUP4*-1 sequence, the tRNA gene loci interact with strikingly high frequency with *MED6* and *SRB2*. Of additional note is that the original $tRNA^{Leu}$ genes do not interact with the genomic copy of *SUP4*, but the *SUP*-1 replacement gains interactions with genomic *SUP4* (Table 2.1, Line 17). This ability of the internal tRNA gene sequences to contribute to binding partner selection is at once intriguing and puzzling. However, the overall effect can be explained by the hypothesis that the flanking sequences act to direct a locus to a particular region of the nucleus. Once within this region, partner selection is influenced by the tRNA gene sequence itself.

Discussion

The effect of the tRNA gene internal sequences on the positioning of the tRNA gene was completely unexpected, given that all tRNA gene transcription complexes bind the same required components to the internal promoters, as far as is known (Kassavetis et al., 1990; Moqtaderi and Struhl, 2004; Roberts et al., 2003). However, in spite of the

unexpected nature of this finding, this phenomenon could have several causes. One possible explanation is that there might be an epigenetic component to the interaction patterns. In the recombination event that leads to the insertion of a new tRNA gene at the locus, the cells would rewrite the epigenetic state, generating a new pattern of positioning.

Another explanation involves TFIIIC, the transcription factor that initially recognizes the gene, has a bipartite binding site within the tRNA coding region (Paule and White, 2000). The spacing between these sites in the original $tRNA^{Leu}$ genes and in the replacement $tRNA^{Tyr}$ gene (i.e., *SUP4*-1) is slightly different, and the resulting change in the topology or strength of the TFIIIC-DNA interaction might subtly alter its interaction with other binding partners. For example, TFIIIC has been shown to directly interact with condensin (Haeusler et al., 2008), which is required for clustering of tRNA genes, so differences in TFIIIC-DNA binding properties could affect how individual tRNA genes interact with each other. Moreover, since the degree of occupation of all the genomic tRNA genes by TFIIIC, TFIIIB, and Pol III is variable across the genome (Moqtaderi and Struhl, 2004; Moqtaderi et al., 2010; Oler et al., 2010; Roberts et al., 2003), the nature of the complexes could be changed by having different geometries or stability of occupation by one or more components.

A more speculative yet possible explanation might also be that tRNA gene complexes recruit tRNA-specific protein components to them due to differences in their transcript sequences. For example, although both $tRNA^{Leu}$ and $tRNA^{Tyr}$ are cleaved at their 5' and 3' mature ends early in biosynthesis, the exact order and location of nucleotide modification events is not clear. Many processing reactions are tRNA-specific (Phizicky and Hopper, 2010), and some of these tRNA-specific reactions could happen either co-transcriptionally or very soon after transcription. It therefore remains possible that some enzymes associate with tRNA gene complexes through the nascent RNAs in a sequence-dependent manner.

It has never been entirely clear why tRNA genes generally cluster together at the nucleolus. The idea of a transcription factory is appealing. The tRNA genes along with the 5S rRNA genes that are embedded in the ribosomal repeats are all transcribed by Pol III, and co-localizing them would provide efficiency in transcription, as well as in early

processing steps, such as cleavage of their 5' leaders and 3' trailers. Additionally, it has been suggested that Pol III transcription is co-regulated with Pol I transcription (Briand et al., 2001), providing a further level of spatial regulation. Yet the results of this study indicate that it is not necessarily the tRNA genes themselves that provide the driving force of global genome organization. Thus, it is likely convenient and even beneficial for the tRNA genes to co-localize with the nucleolus, but their positioning in the proximity of the nucleolus is likely dictated by other chromosomal localization signals. Indeed, it is unlikely that all 274 tRNA genes in the *S. cerevisiae* genome are required to be clustered together at the nucleolus at all times. Some tRNA genes may never find their way to the nucleus, and others may be there at times and elsewhere at other times, driven by the spatial commands of neighboring genes. These tRNA genes would not lose functionality, but those that are at the nucleolus reap the benefits of more efficient transcription and processing.

Overall, this study supports a model whereby the locus-specific tRNA transcription complexes serve as "fasteners" to determine local interactions and promote clustering, but that this occurs in combination with other determinants that dominate global nuclear positioning.

Materials and Methods

Strain construction. The coding sequence of *tK(CUU)P* in *S. cerevisiae* BY4741 (*MAT*a *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*), including mature coding sequence and intron, was precisely replaced with the *kanMX6* expression cassette by recombination using PCR fragments generated from plasmid pFA6a-*kanMX6* (Longtine et al., 1998) to create yDP97. Transformants were selected on medium containing G418, and exact gene replacement was verified by PCR and sequencing.

The coding sequence and intron of *tL(UAA)B2* (Chr II, coordinates 347583 to 347699) in the wild-type strain (yPH499; *MAT*a *ura3-52 lys2-801_amber ade2-101_ochre trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1*) was precisely replaced by recombination with the *SUP4*-1 ochre suppressor $tRNA^{Tyr}$ coding sequence and intron (Kurjan et al., 1980)

and selection for suppression of the ochre *ade2* mutation to create yDP77. Correct replacement was subsequently confirmed by PCR analysis and sequencing. Similarly, *tL(CAA)G3* (Chr VII, coordinates 857511 to 857374) was precisely replaced in yPH499 using the same method to create yDP84.

Growth competition. Competitions were done essentially as described (Torres et al., 2010). Briefly, BY4741 and yDP97 were mixed in equal amounts to OD_{600} = 0.2 in 50 mL of synthetic complete medium. Cells were maintained in exponential phase by dilution back to OD_{600} = 0.2 every 18-24 hours. Cells were plated at intervals to YPD and YPD containing G418, and the ratio of G418-resistant ($G418^R$) colonies to total colonies was plotted.

Genome conformation capture (GCC) (Rodley et al., 2009). Briefly, BY4741 cells were grown in 2% glucose to mid-logarithmic phase and crosslinked with 1% formaldehyde. For each experiment, chromatin was prepared from 15 sets of 10^8 (i.e., a total of 1.36 x 10^9) cells. Chromatin was digested with *Msp*I (Fermentas) and ligated (T4 ligase; Invitrogen). Crosslinks were reversed in the presence of proteinase K (final concentration 7-11µg, Roche). Samples were treated with RNase A (final concentration 10 µg/mL) prior to purification by phenol:chloroform extraction and column purification (Zymo Clean and Concentrator, Zymo Research). Paired-end sequencing (36 bp) was performed on 5µg DNA using the Illumina Genome Analyzer platform (Allan Wilson Centre, Massey University, New Zealand & Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland). Sequences were deposited with the Gene Expression Omnibus (GEO) accession number GSE30103.

External controls for random ligation events. To experimentally control for spurious inter-molecular ligation events, during the GCC process samples were spiked with two ligation controls during library preparation. The first ligation control consisted of PCR products that were added (1:1 ratio with the nuclear genome copy number) before the GCC ligation step. These controls were designed to estimate the frequency of random inter-molecular ligation events during GCC library preparation. A maximum of 47 separate ligation events were observed, none of which occurred at levels above the

statistically defined experimental noise. The second ligation control consisted of the addition of pUC19 plasmid to the sample following the GCC ligation in order to control for random ligation events during preparation of the samples for sequencing. We observed a maximum of six interactions between pUC19 and the rest of the genome; again, none of these interactions were above the statistically defined experimental noise. In conclusion, the fact that the high copy number rDNA and mitochondrial DNA elements do not show significant levels of random inter-molecular interactions with our internal control sequences is empirical evidence that the interactions we observe result from intra-molecular ligation events. Therefore, random ligation events during sample preparation do not account for the interactions we observe.

Determination of noise threshold. Statistical analyses are performed to determine whether the GCC dataset is something other than random. 100,000 simulations of random pairings were done to determine the maximum count of a particular interaction that would be observed under this noise model, given the same number of sequences, interactions, and fragments as in the experimental data. The results of the simulations lead us to conclude that the interaction patterns cannot be attributed to noise alone with a p-value less than 10^{-5}. Secondly, we performed analyses to determine what frequency individual interactions have to achieve before they are deemed to be present at a level above experimental noise. It is justified to assume the pairings are independent and therefore the number of times one specific pairing occurs is a binomially distributed random variable. S1 (13,622) and S2 (6277.4) are the number of mitochondrial and nuclear segments, respectively, which participate in at least one interaction. We calculate the probability $P(X \geq k)$, where N is number of observed pairings (478,978), and p is 1 divided by S1 multiplied by S2, for one specific pairing to occur k or more times. L, the number of possible pairings, is S1 multiplied by S2. We then expect to see $L[P(X \geq k)] = 2.4167$ pairings occurring k or more times by chance, i.e., the expected number of false positives. Therefore, 3 or more interactions (k=3) was selected as an acceptable noise cut-off value.

Analysis of repetitive elements. Repetitive elements are those genomic features which occur more than once within the genome (e.g., tRNA genes and rRNA genes). We were particularly interested in the interactions between the rDNA repeats and the rest of

the genome, specifically Fragment #13476, containing *tK(CUU)P*, itself a repetitive element. Statistical calculations were performed on datasets in which the sequences could be uniquely positioned on the reference genome. Where the analysis was concerned with connections between repetitive elements, connections between sequences deemed "unique" and connections involving sequences deemed "repetitive" were analyzed separately. For the analyses in this chapter, only the unique datasets were used. The copy number of the rDNA repeats in our samples was calculated by aligning the sequence files against a short section of rDNA (Chr XII: 460517-460612) and comparing it against the single copy *GAL1* locus (Chr II: 279790-279909). We calculated the ratio of rDNA to the unique nuclear element (*GAL1*) to be 141.72.

Chromosome conformation capture (3C) (Dekker et al., 2002) BY4741 and yDP97 strains were grown to mid-logarithmic phase in synthetic complete media containing 2% glucose (w/v). Chromatin was prepared according to (Rodley et al., 2009) using *Hind*III or *Msp*I restriction enzyme. *Hind*III cleaves the *kanMX6* expression cassette and thus results in three restriction fragments in yDP97, as opposed to two fragments in BY4741. Interactions between F1 and F2 of the WT strain (F1 and F2' of the yDP97 strain) on Chr XVI, and the rDNA *Hind*III fragment (Chr XII: 457,910-460,634 bp) were measured for three biological replicates.

Quantitative 3C analyses (O'Sullivan et al., 2009; Rodley et al., 2009) were performed by comparison to dedicated standards using FAM labeled BHQ Probes (BioSearch Technologies) and Taqman® Gene Expression Master Mix (Applied Biosystems) on an ABI Prism 7000 Sequence Detection System (SDS7000). Samples (2 µl) were analyzed in triplicate in 20 µl reactions (final volume). Real-time analyses were performed using a 3-stage program (50°C, 2:00 min; 95°C, 10:00 min; 45x [95°C, 0:15 sec; 60°C, 1:00 min]). To standardize between samples, *GAL1* copy number was determined by qPCR using Sybr-green and a five stage program (50°C, 2:00 min; 95°C, 2:00 min; 40x [95°C, 0:15 sec; 59.5°C, 0:30 sec; 72°C, 0:30 sec]; 55°C, 1:00; followed by a dissociation analysis).

Circular chromosome conformation capture (4C) (O'Sullivan et al., 2009). YPH499, yDP77, and yDP84 strains were grown to mid-logarithmic phase in synthetic complete media containing 2% glucose (w/v). Chromatin was harvested and prepared as for 3C samples using the *Msp*I restriction enzyme.

Nested inverse PCR primers were designed to amplify out of the 'bait' *Msp*I fragments that contained the intact *tL(UAA)B2* or *tL(CAA)G3* gene. *SUP4*-1 contains an additional *Msp*I site, which was compensated for by performing nested amplifications of the two fragments independently upon the same 4C library. PCR conditions were as follows 1) first round (95°C, 2:00 min; 35x [95°C, 0:30 sec; 59°C, 0:30 sec; 72°C, 2:00 min]; 72°C, 5:00) and 2) nested second round (95°C, 2:00 min; 35x [95°C, 0:30 sec; 62°C, 0:30 sec; 72°C, 2:00 min]; 72°C, 5:00). The primer annealing temperatures for fragment 1 and 2 of the yDP77 strain were 68.1°C and 60°C, respectively. Nested primers contained unique 6 bp tags (TCTCTG [yPH499 wild type arrangement of the yDP84 strain], TGATGC [yDP84 fragment 1], and AGCACG [yDP84 fragment 2], AGAGAC [yPH499 wild type arrangement of the yDP77 strain], ACAGAG [yDP77 fragment 1], TAGATC [yDP77 fragment 2]) to enable pooling of the 4C PCR products for sequencing (100 bp paired end) on an Illumina Genome Analyser (Allan Wilson Centre, Massey University). Sequences were mapped onto the *S. cerevisiae* S288c genome sequence using Topography v1.19 (Rodley et al., 2009). Sequence files are available from GEO (series record GSE30103).

A total of 73,010,074 100 bp sequences were generated for the pooled 4C libraries, sorted, trimmed (17 bp either side of the *Msp*I restriction site) and mapped onto the *S. cerevisiae* S288C genome using Topography (v1.19; (Rodley et al., 2009)). No mismatches were allowed. Similar numbers of inter- and non-adjacent intra-chromosomal interactions were observed for the *tL(UAA)B2* and *tL(CAA)G3* loci in the wild-type background (233,456 and 250,195, respectively). The tRNA replacement on Chr VII (yDP84) resulted in the most interactions associated with it overall (1,513,787), while the yDP77 mutant resulted in considerably less (173,546) map able interactions.

Sorting involved some pre-processing of sequence tags. Each of the individual samples was isolated from the sequence files according to its 6 bp tag and primer sequence and trimmed to 34 bp (with the *Msp*I recognition sequence in the centre). The

sequences for fragment 1 and 2 for each mutant (yDP77 and yDP84) were pooled. Since *SUP4*-1 shares considerable identity with the 9 copies of the *tY(GUA)* $tRNA^{Tyr}$ gene in the yeast genome, particularly around the *Msp*I restriction site, the primer sequences which hybridized adjacent to the novel restriction site within this locus could not be uniquely positioned to the bait fragment on the reference genome. Therefore, the unique primer sequences from the opposite ends of the bait fragments were substituted for the 17 bp repetitive sequences that abut the novel restriction site within *SUP4*-1, prior to analysis. Thus, sequences that crossed the *SUP4*-1 restriction site within the bait fragments were accurately mapped to either *tL(UAA)B2* or *tL(CAA)G3*, depending on the interaction under investigation. For analysis, adjacent interaction frequencies were used to correct for between sample comparisons.

Acknowledgements

This chapter is modified from the manuscript, "tRNA Gene Identity Affects Nuclear Positioning," published in *PLoS ONE* [2011; 6(12): e29267. Epub 2011 Dec 19.]. The experiments and content of the chapter were done in collaboration with Dr. Justin M. O'Sullivan and Chris D. M. Rodley of Massey University in Auckland, New Zealand. I would also like to thank Sara R. Rivera for help with the preliminary data interpretation.

References

Bertrand, E., Houser-Scott, F., Kendall, A., Singer, R.H., and Engelke, D.R. (1998). Nucleolar localization of early tRNA processing. Genes & development *12*, 2463-2468.

Blumenthal, T., and Gleason, K.S. (2003). Caenorhabditis elegans operons: form and function. Nature reviews *4*, 112-120.

Briand, J.F., Navarro, F., Gadal, O., and Thuriaux, P. (2001). Cross talk between tRNA and rRNA synthesis in Saccharomyces cerevisiae. Mol Cell Biol *21*, 189-195.

Brown, J.M., Green, J., das Neves, R.P., Wallace, H.A., Smith, A.J., Hughes, J., Gray, N., Taylor, S., Wood, W.G., Higgs, D.R., *et al.* (2008). Association between active genes

occurs at nuclear speckles and is modulated by chromatin environment. J Cell Biol *182*, 1083-1097.

Brown, J.M., Leach, J., Reittie, J.E., Atzberger, A., Lee-Prudhoe, J., Wood, W.G., Higgs, D.R., Iborra, F.J., and Buckle, V.J. (2006). Coregulated human globin genes are frequently in spatial proximity when active. The Journal of cell biology *172*, 177-187.

Cremer, T., Cremer, M., Dietzel, S., Muller, S., Solovei, I., and Fakan, S. (2006). Chromosome territories--a functional nuclear landscape. Curr Opin Cell Biol *18*, 307-316.

D'Ambrosio, C., Schmidt, C.K., Katou, Y., Kelly, G., Itoh, T., Shirahige, K., and Uhlmann, F. (2008). Identification of cis-acting sites for condensin loading onto budding yeast chromosomes. Genes & development *22*, 2215-2227.

Dekker, J., Rippe, K., Dekker, M., and Kleckner, N. (2002). Capturing chromosome conformation. Science *295*, 1306-1311.

Duan, Z., Andronescu, M., Schutz, K., McIlwain, S., Kim, Y.J., Lee, C., Shendure, J., Fields, S., Blau, C.A., and Noble, W.S. (2010). A three-dimensional model of the yeast genome. Nature *465*, 363-367.

Freeman, L., Aragon-Alcaide, L., and Strunnikov, A. (2000). The condensin complex governs chromosome condensation and mitotic transmission of rDNA. The Journal of Cell Biology *149*, 811-824.

Ganley, A.R., Ide, S., Saka, K., and Kobayashi, T. (2009). The effect of replication initiation on gene amplification in the rDNA and its relationship to aging. Mol Cell *35*, 683-693.

Haeusler, R.A., Pratt-Hyatt, M., Good, P.D., Gipson, T.A., and Engelke, D.R. (2008). Clustering of yeast tRNA genes is mediated by specific association of condensin with tRNA gene transcription complexes. Genes & development *22*, 2204-2214.

Iwasaki, O., and Noma, K.I. Global genome organization mediated by RNA polymerase III-transcribed genes in fission yeast. Gene.

Iwasaki, O., Tanaka, A., Tanizawa, H., Grewal, S.I., and Noma, K. Centromeric localization of dispersed Pol III genes in fission yeast. Molecular biology of the cell *21*, 254-265.

Kassavetis, G.A., Braun, B.R., Nguyen, L.H., and Geiduschek, E.P. (1990). S. cerevisiae TFIIIB is the transcription initiation factor proper of RNA polymerase III, while TFIIIA and TFIIIC are assembly factors. Cell *60*, 235-245.

Kendall, A., Hull, M.W., Bertrand, E., Good, P.D., Singer, R.H., and Engelke, D.R. (2000). A CBF5 mutation that disrupts nucleolar localization of early tRNA biosynthesis

in yeast also suppresses tRNA gene-mediated transcriptional silencing. Proceedings of the National Academy of Sciences of the United States of America *97*, 13108-13113.

Kurjan, J., Hall, B.D., Gillam, S., and Smith, M. (1980). Mutations at the yeast SUP4 tRNATyr locus: DNA sequence changes in mutants lacking suppressor activity. Cell *20*, 701-709.

Lanctôt, C., Cheutin, T., Cremer, M., Cavalli, G., and Cremer, T. (2007). Dynamic genome architecture in the nuclear space: Regulation of gene expression in three dimensions. Nature Reviews Genetics *8*, 104-115.

Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953-961.

Machin, F., Paschos, K., Jarmuz, A., Torres-Rosell, J., Pade, C., and Aragon, L. (2004). Condensin regulates rDNA silencing by modulating nucleolar Sir2p. Curr Biol *14*, 125-130.

Meaburn, K.J., and Misteli, T. (2007). Cell biology: chromosome territories. Nature *445*, 379-781.

Moqtaderi, Z., and Struhl, K. (2004). Genome-wide occupancy profile of the RNA polymerase III machinery in Saccharomyces cerevisiae reveals loci with incomplete transcription complexes. Molecular and Cellular Biology *24*, 4118-4127.

Moqtaderi, Z., Wang, J., Raha, D., White, R.J., Snyder, M., Weng, Z., and Struhl, K. (2010). Genomic binding profiles of functionally distinct RNA polymerase III transcription complexes in human cells. Nat Struct Mol Biol *17*, 635-640.

O'Sullivan, J.M., Sontam, D.M., Grierson, R., and Jones, B. (2009). Repeated elements coordinate the spatial organization of the yeast genome. Yeast *26*, 125-138.

Oler, A.J., Alla, R.K., Roberts, D.N., Wong, A., Hollenhorst, P.C., Chandler, K.J., Cassiday, P.A., Nelson, C.A., Hagedorn, C.H., Graves, B.J., *et al.* (2010). Human RNA polymerase III transcriptomes and relationships to Pol II promoter chromatin and enhancer-binding factors. Nat Struct Mol Biol *17*, 620-628.

Paule, M.R., and White, R.J. (2000). Survey and summary: transcription by RNA polymerases I and III. Nucleic Acids Research *28*, 1283-1298.

Phizicky, E.M., and Hopper, A.K. (2010). tRNA biology charges to the front. Genes & Development *24*, 1832-1860.

Ragoczy, T., Bender, M.A., Telling, A., Byron, R., and Groudine, M. (2006). The locus control region is required for association of the murine beta-globin locus with engaged transcription factories during erythroid maturation. Genes Dev *20*, 1447-1457.

Roberts, D.N., Stewart, A.J., Huff, J.T., and Cairns, B.R. (2003). The RNA polymerase III transcriptome revealed by genome-wide localization and activity-occupancy relationships. Proc Natl Acad Sci U S A *100*, 14695-14700.

Rodley, C.D., Bertels, F., Jones, B., and O'Sullivan, J.M. (2009). Global identification of yeast chromosome interactions using Genome conformation capture. Fungal Genet Biol *46*, 879-886.

Sinclair, D.A., and Guarente, L. (1997). Extrachromosomal rDNA circles--a cause of aging in yeast. Cell *91*, 1033-1042.

Thompson, M., Haeusler, R.A., Good, P.D., and Engelke, D.R. (2003). Nucleolar clustering of dispersed tRNA genes. Science *302*, 1399-1401.

Torres, E.M., Dephoure, N., Panneerselvam, A., Tucker, C.M., Whittaker, C.A., Gygi, S.P., Dunham, M.J., and Amon, A. Identification of aneuploidy-tolerating mutations. Cell *143*, 71-83.

Tsang, C.K., Li, H., and Zheng, X.S. (2007). Nutrient starvation promotes condensin loading to maintain rDNA stability. EMBO J *26*, 448-458.

Wang, B.D., and Strunnikov, A. (2008). Transcriptional homogenization of rDNA repeats in the episome-based nucleolus induces genome-wide changes in the chromosomal distribution of condensin. Plasmid *59*, 45-53.

Wang, L., Haeusler, R.A., Good, P.D., Thompson, M., Nagar, S., and Engelke, D.R. (2005). Silencing near tRNA genes requires nucleolar localization. J Biol Chem *280*, 8637-8639.

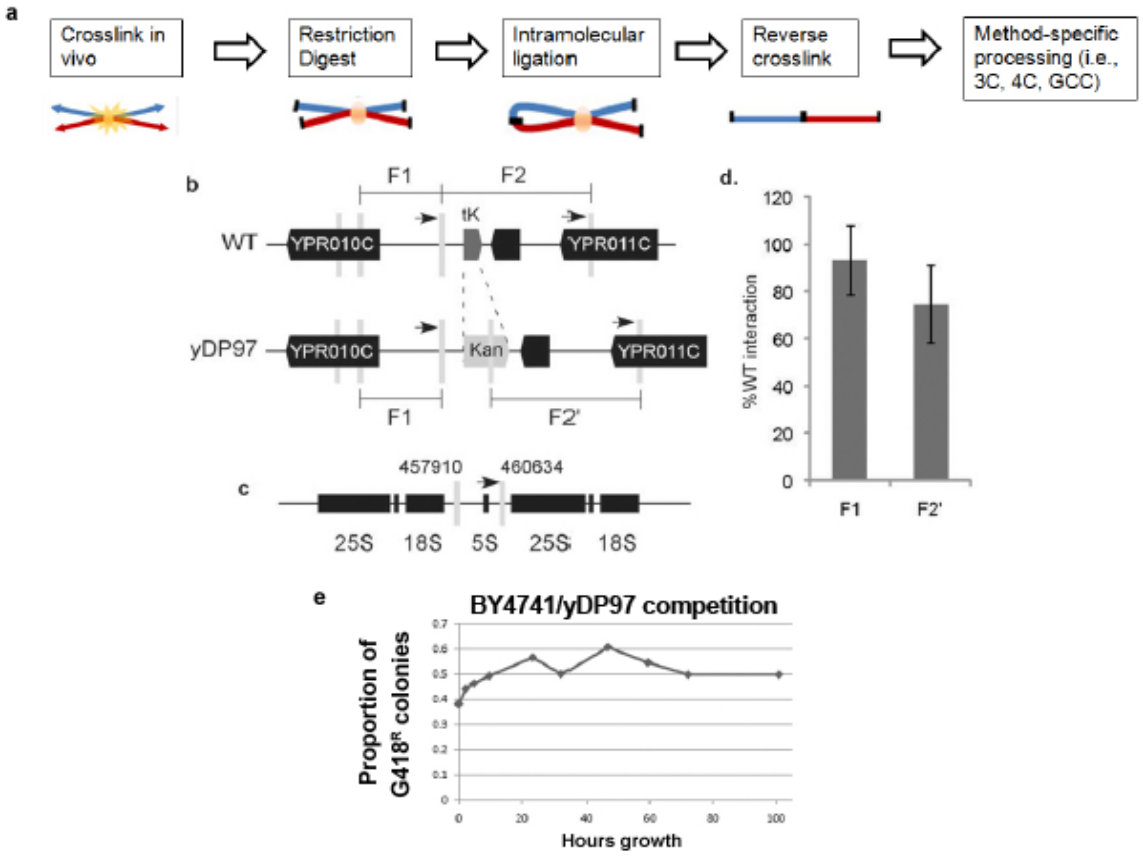


Figure 2.1. Deletion of a tRNA gene retains interactions between that locus and the rDNA repeats. a) Schematic of proximity-based ligation methodologies. b) The wild-type (WT) *S. cerevisiae* strain BY4741 had the *tK(CUU)P* tRNA gene replaced with the *kanMX6* expression cassette. c) The 3C partner sequence was located across the 5S rDNA. Gray bars denote *Hind*III restriction sites; arrows denote 3C primer positions. d) Quantitative 3C PCR demonstrates no significant reduction in interaction frequency for the F1 or F2/F2' fragments due to removal of the tRNA gene. Results are expressed as percentage of WT F1 or F2 interactions, and *GAL1* was used to standardize between samples (Rodley et al., 2009). e) Co-culturing BY4741 and yDP97 for 100 generations in a batch competition assay and measuring relative rate of G418 resistance demonstrates that the deletion strain has no major growth defects.

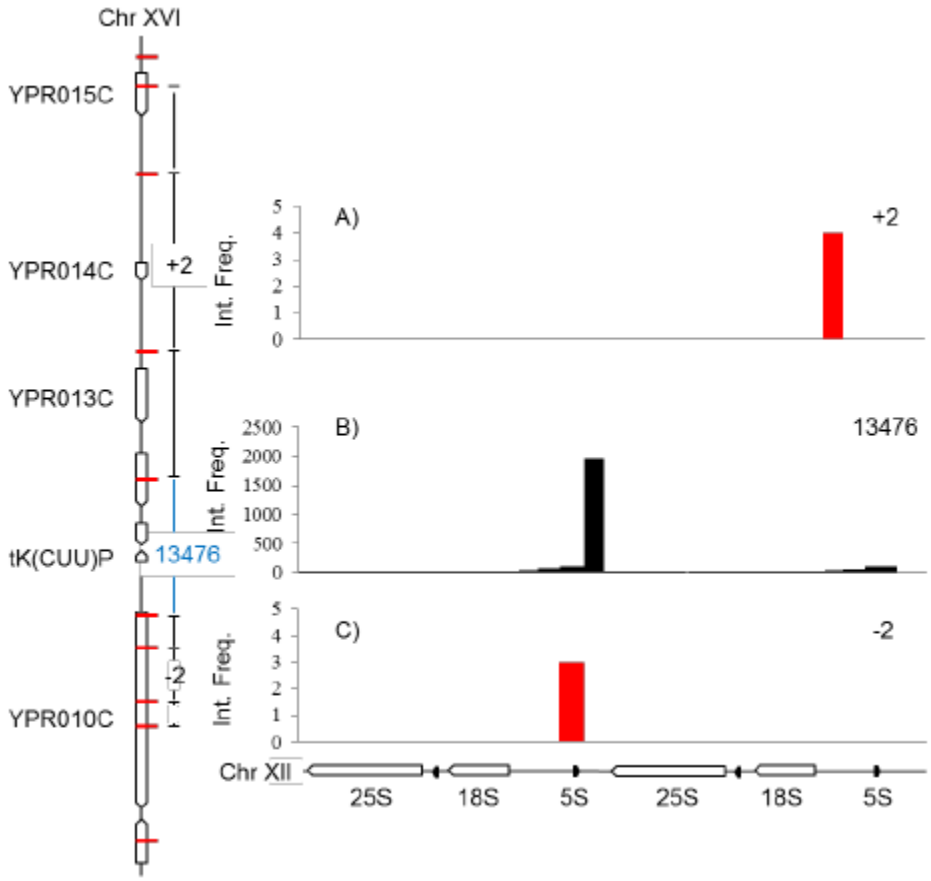


Figure 2.2. The high frequency interactions between the *tK(CUU)P*-containing fragment and the NTS1 sequence is isolated and not mirrored at adjacent sites. GCC was performed on unsynchronized exponentially growing *S. cerevisiae* cells, and interactions that occurred above the experimental false detection rate were counted and mapped between restriction fragments surrounding Fragment #13476 on Chr XVI (relevant portion of linear chromosome map illustrated on left) and restriction fragments across the entire rDNA repeat (illustrated below the graphs). Of the six fragments which immediately flank the *tK(CUU)P*-containing fragment (#13476) (Chr XVI: 581,025-583,522), only the "-2" and "+2" fragments interact with the rDNA. However, neither interacts with the NTS1 fragment, and the maximum number of interactions observed was three orders of magnitude lower. A) Map of interaction frequencies between Fragment "+2" (Chr XVI: 585884-589137) and the rDNA locus. B) Map of interaction frequencies between Fragment #13476 and the rDNA locus. C) Map of interaction frequencies between Fragment "-2" (Chr XVI: 549477-580469) and the rDNA locus.

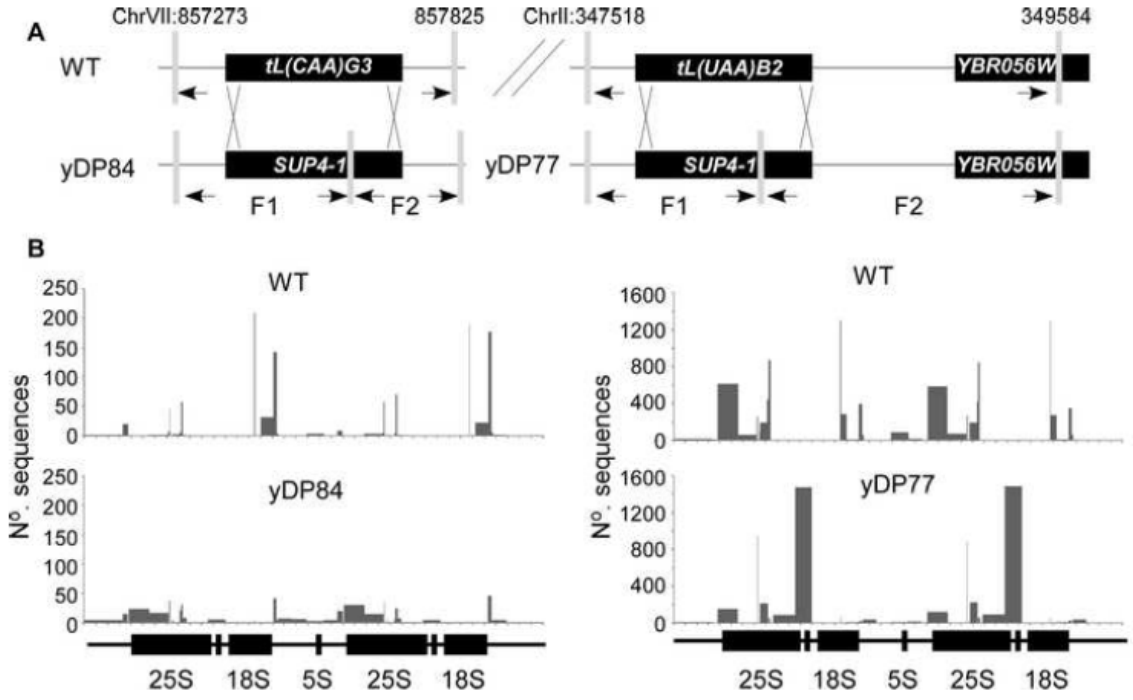


Figure 2.3. tRNA gene identity dictates interaction patterns with the rDNA repeats. A) Two $tRNA^{Leu}$ genes [*tL(CAA)G3* and *tL(UAA)B2*], which are located on different chromosomes, were replaced with the $tRNA^{Tyr}$ variant *SUP4*-1 suppressor gene to generate strains yDP84 and yDP77, respectively. Gray bars denote *Msp*I restriction sites; arrows denote 4C primer positions. B) Interactions with the rDNA locus were identified by 4C coupled to high throughput sequencing. Raw interaction frequencies have been plotted across a tandem rDNA repeat for clarity. Note, only patterns of interaction along the rDNA locus should be compared, as inter-experiment variation has not been corrected for.

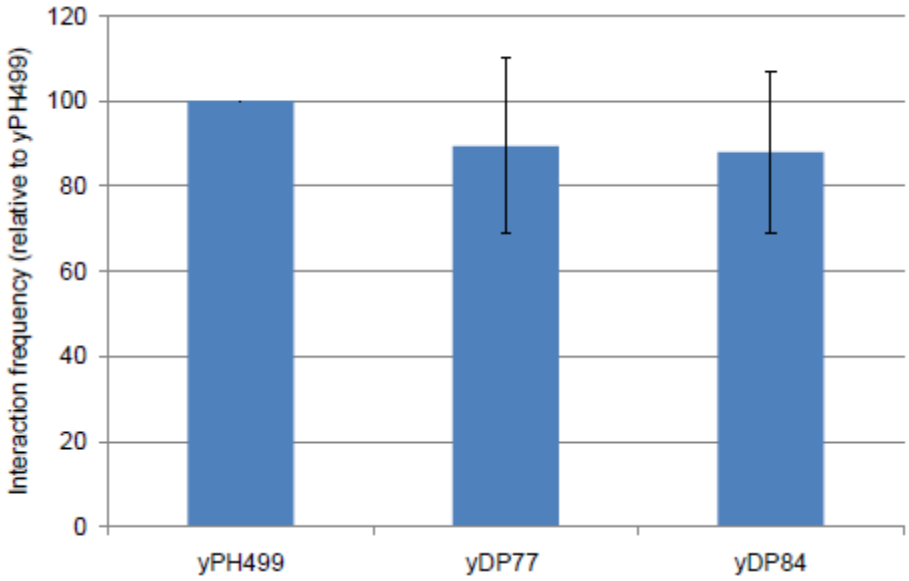


Figure 2.4. Genetic background does not affect interaction frequency between *tY(GUA)J2* and the 25S rDNA. 3C was performed using *Msp*I on crosslinked chromatin isolated from yPH499, yDP77, and yDP84. Interaction frequencies were determined by quantitative 3C analyses using fluorescent probes and have been corrected for nuclear genome copy number to facilitate inter-strain comparisons (see Methods). Interaction values are expressed as percentages of the yPH499 sample (set at 100%) +/- standard error of the mean (n=3)

Line	*tL(UAA)B2*		*tL(CAA)G3*		Chr	Chromosomal coordinates		Genes contained within fragment
	WT	SUP4-1	WT	SUP4-1		Start	Stop	
1	0	153	0	11	I	57456	57688	*YAL044W*
2	0	74	0	2	II	168544	170155	*RPL19B*
3	0	1	0	99	IV	30590	30807	*LRC1*
4	0	174	0	6	IV	946425	946524	between *SUP2* and *AMD2*
5	0	1	0	26	IV	1108925	1109604	*ASP1*
6	0	139	0	53	IV	1198674	1198905	*TFC6*
7	0	4	0	570	IV	1251279	1251371	*RVS167*
8	0	315	0	2	IV	1268016	1268117	*UTP5*
9	0	1	0	110	IV	1367585	1367804	*NHX1*
10	0	7	0	8	V	171720	171958	*SEC3*
11	0	96	0	2	VI	209975	210610	*CDC14, SUP6*
12	0	481	0	2	VII	730784	731066	*COG2*
13	0	1	0	39	VII	798801	798859	*CYS4*
14	0	2	0	3770	VII	901128	901626	*ELP2*
15	0	386	0	16190	VIII	189377	189488	*SRB2*
16	0	123	0	30471	VIII	219090	219185	*MED6*
17	0	484	0	253	X	542361	542952	*YJR056C, SUP4*
18	0	2	0	16	XI	386088	386154	*TFA1*
19	0	66	0	3	XI	514497	515593	*GAP1*
20	0	40	0	4	XIII	168876	169176	*SUP5, YML053C*
21	0	2	0	5	XIII	398049	398171	*AEP1*
22	0	16	0	2	XIII	592567	592857	*PAH1*
23	0	1875	0	1	XIII	837399	838007	*YMRWdelta21, SUP8*
24	0	239	0	3	XIV	413951	414121	*DBP2*
25	0	1	0	11	XV	956789	956927	*YOR338W*
26	0	2690	0	8	XV	986036	986929	*PYK2, PUT4*
27	0	14	0	398	XVI	115139	115441	*USV1*
28	0	117	0	1	XVI	204335	205567	*CTI6, TCO89*

Table 2.1. Gain of interactions upon tRNA gene replacement. This table outlines the interaction frequencies captured by 4C between the “bait” fragments (which contain either the *tL(UAA)B2* or *tL(CAA)G3* gene in the WT strain, or these genes replaced with the *SUP4-1* sequence) and the captured "prey" fragments, the chromosomal location and genetic makeup of which are described.

Chapter III

Condensin binds specifically to tRNA genes by recognizing TFIIIC

Abstract

The multi-subunit condensin complex is required for the proper compaction of genetic material in the nucleus and is conserved throughout eukaryotes. In budding yeast condensin is responsible for the spatial clustering of the tRNA genes at the nucleolus, mediated via direct interactions between condensin and the transcription factors bound to the tRNA gene. Here we show that the specificity of interaction between condensin and the tRNA gene transcription complex is mediated by the RNA polymerase III (Pol III) transcription factor TFIIIC. This study demonstrates a specific mechanism for recognizing tRNA genes by condensin for the purpose of their spatial organization.

Introduction

Condensin is a five-subunit member of the Structural Maintenance of Chromosomes (SMC) family of protein complexes found in organisms from bacteria to eukaryotes. Condensin in yeast is composed of the Smc2p-Smc4p coiled-coil heterodimer along with the associated proteins Ycg1p, Ycs4p, and Brn1p (Hirano, 2005, 2006; Losada and Hirano, 2005). Along with the other two SMC protein complexes, cohesin and the Smc5/6 complex, condensin serves a variety of functions in the nucleus. The primary role of condensin is to compact and individualize chromatin into discrete chromosomes in preparation for mitosis or meiosis. However, it is now clear that condensin is not just important for cell division but that it has many roles in the interphase nucleus as well, particularly in genome maintenance, gene regulation, and DNA repair (Hudson et al.,

2009; Wood et al., 2010). For example, although it has been known for some time that condensin is required for proper condensation and faithful transmission of the ribosomal DNA (rDNA) cluster during mitosis (Freeman et al., 2000), it is now evident that condensin is required for maintenance of rDNA integrity even in interphase (Tsang et al., 2007a; Tsang et al., 2007b).

It was recently shown that condensin is bound to every tRNA gene in the yeast genome (D'Ambrosio et al., 2008). Further studies indicated that nucleolar clustering of tRNA genes (Thompson et al., 2003; Wang et al., 2005) requires condensin, and mutations in any one of the five subunits of condensin alleviate tgm silencing (Haeusler et al., 2008). Condensin also mediates the centromeric localization of tRNA genes in fission yeast (Iwasaki et al., 2010). Additionally, condensin interacts in vivo in *S. cerevisiae* with a subset of the Pol III transcription machinery (i.e., TFIIIC and TFIIIB) (Haeusler et al., 2008) and is also found bound to the nine extra-TFIIIC (ETC) sites in the yeast genome that only bind TFIIIC but are not Pol III genes (D'Ambrosio et al., 2008; Moqtaderi and Struhl, 2004). Therefore, we hypothesized that TFIIIC may be the recognition factor for condensin association with tRNA genes.

In this work we explore the interaction of purified condensin with tRNA gene complexes in vitro. We demonstrate that the Pol III transcription factor TFIIIC is necessary and sufficient for condensin to specifically recognize a tRNA gene, though TFIIIC-TFIIIB complexes can also support condensin binding. As expected, in the absence of TFIIIC, condensin does not specifically recognize the tRNA gene, instead binding nonspecifically to the DNA. Further work is necessary to determine the details of this interaction.

Results and Discussion

To test the hypothesis that TFIIIC is required for condensin to recognize a tRNA gene, we used an electrophoretic mobility shift assay (EMSA) to demonstrate the ability of purified condensin to bind stably to a tRNA gene-TFIIIC complex. We first verified that purified TFIIIC can bind stably to a tRNA gene in vitro. A radiolabeled DNA fragment

containing the *SUP4* $tRNA^{Tyr}$ gene variant (Figure 3.1A) was mixed with molar excess amount of pUC19 supercoiled carrier plasmid as a nonspecific DNA competitor for protein binding. This mixture was incubated with TFIIIC, and complexes were separated on a native gel. Addition of TFIIIC yields a discrete shifted band, indicating specific recognition of the tRNA gene-containing fragment by TFIIIC (Figure 3.1B, third lane). The complexes appear to be partially unstable under electrophoresis conditions, evidenced by smearing in the shifted lanes.

Having confirmed that TFIIIC binds to the expected site on a tRNA gene in vitro, we tested whether purified condensin alone could bind selectively to the tRNA gene. When condensin was incubated in large molar excess over labeled tRNA gene in the absence of nonspecific DNA competitor, it caused aggregation consistent with both binding of condensin to the DNA and aggregation due to protein-protein interaction between condensin-DNA complexes (Figure 3.1C, middle lane). Adding the competitor plasmid DNA back into the reaction mixture inhibited the aggregation and formation of stable complexes between the labeled tRNA gene fragment and the condensin (Figure 3.1B, left lane, and Figure 3.1C, left lane). Condensin is a DNA-binding protein and has been shown to bind non-specifically along the length of random DNA (Stray et al., 2005). Our results are consistent with both non-specific DNA binding by condensin and condensin-condensin-mediated aggregation of the DNA-condensin complexes, the proposed method of DNA compaction by condensin (Losada and Hirano, 2005). Our results also confirm lack of DNA sequence specificity in the binding of condensin alone to tRNA genes.

We next tested the ability of condensin to recognize a tRNA gene-TFIIIC complex. After incubation with TFIIIC, the DNA fragment was then incubated with condensin, and the assembled complex was separated on a native gel. Addition of condensin to the TFIIIC-DNA complex yields a discrete super-shifted band in addition to the original shifted band (Figure 3.1B, second lane, and Figure 3.1C, third lane), indicating that condensin is recognizing the TFIIIC-DNA complex. This result is consistent with in vivo data showing that condensin is bound to the ETC sites where only TFIIIC but no other factor is bound and supports the hypothesis that condensin recognizes tRNA genes via TFIIIC. The condensin is present in the reaction mixtures at

1.5-fold molar excess to the TFIIIC, yet only a fraction of the TFIIIC-shifted band is super-shifted upon addition of condensin. The reason for this incomplete occupancy of condensin on the tRNA gene complex is unclear but could be due to instability of the complexes. We also verified that condensin is able to bind to a more fully assembled Pol III transcription complex, consisting of TFIIIC and TFIIIB bound to a tRNA gene. Before addition of condensin, we added purified TFIIIB onto the DNA-TFIIIC complex and then added condensin. The DNA-TFIIIC-TFIIIB complexes, while very unstable through electrophoresis conditions, are able to accommodate condensin binding (not shown).

Our results indicate overall that condensin is bound to tRNA genes in the yeast genome via direct recognition of the Pol III transcription factor TFIIIC. However, while we have determined what the interaction is, our data leave unanswered exactly how that interaction occurs. Early studies used DNA footprinting to demonstrate that TFIIIC binds stably to the internal promoter regions of a tRNA gene, and that TFIIIB then binds upstream of the tRNA gene (Kassavetis et al., 1989). We performed the footprinting assays as described (Kassavetis et al., 1990; Kassavetis et al., 1989) to determine where along the DNA fragment condensin is binding. While we were able to reproduce the TFIIIC footprint, we were unable to demonstrate an additional footprint upon the addition of condensin (Figure 3.2). It is possible that the lack of an additional footprint means that there is something intrinsic to this assay that is not allowing us to visualize the condensin protection of DNA from DNase I digestion. However, another possible explanation is that the condensin may be binding directly on top of the TFIIIC, making minimal or no contacts directly with the DNA (Figure 3.3). Because condensin is a DNA-binding protein complex, one would expect there to be at least minimal DNA contacts, so perhaps the contacts are not sufficiently stable or position-specific to observe beyond the existing TFIIIC footprint. Were condensin to bind directly to TFIIIC without extensive DNA contacts, this would also be consistent with in vivo co-IP data showing that the TFIIIC-condensin association persists even when the DNA is digested (Haeusler et al., 2008). Binding on top of TFIIIC would also allow the upstream region of the tRNA gene to be free of bulky protein complexes, so that TFIIIB may bind in preparation for Pol III recruitment.

Since the footprint was unable to yield a definite result, direct visualization would be useful in examining the structure of these complexes. Further assessment of the TFIIIC-condensin complex on a tRNA gene is theoretically possible via electron microscopy (EM). However, preliminary attempts at visualization have been difficult because the condensin appears too heterogeneous when placed on a charged EM grid (Figure 3.4), even though all five individual subunits of the condensin complex appear intact via silver stain ((St-Pierre et al., 2009) and communicated by Damien D'Amours, Université de Montreal). In spite of this setback, it may still be possible to visualize condensin bound to tRNA genes in the future, and work is being done to improve the EM procedure.

While many functions for condensin have been revealed in the past decade, very little is still known about its mechanism of action. No crystal structure has yet been determined, perhaps because the heterogeneity seen in our EM studies also makes producing crystals difficult. It has been proposed that condensin compacts DNA with a DNA-binding domain that binds DNA, and then condensin-condensin interaction domains bring condensin-bound regions of DNA together (Hirano et al., 2001; Losada and Hirano, 2005). In the case of tRNA genes, though, there may be a TFIIIC-recognition domain in condensin, along with the DNA-binding domain, that specifically allows tRNA genes to cluster together. Once bound to TFIIIC-bound DNA, condensin may recognize other TFIIIC-bound condensin molecules and thus specifically cluster tRNA genes together. Condensin-bound tRNA gene complexes would then be able to cluster with the condensin-bound rDNA repeats, somehow based on the identity of the individual tRNA genes (see Chapter II). Therefore, while many questions about the mechanism of condensin function are still unanswered, the results of this study begin to assess the molecular mechanisms by which condensin clusters tRNA genes.

Methods and Materials

Electrophoretic mobility shift assays. Radiolabeled DNA was generated by end-labeling DNA oligonucleotide oDP115 (5'-GAATTCCTTCGGAGGGCTGT-3') with

gamma-^{32}P-ATP. 50 pmol of end-labeled oDP115 was then paired with 50 pmol of unlabeled DNA oligonucleotide oDP118 (5'-CACACCCGTCCTGTGGAT-3') in a PCR reaction using plasmid template pSUP4o (Hull et al., 1994) to generate an end-labeled 288-bp PCR product containing the *SUP4* tRNA^{Tyr} gene (Figure 3.1A). The PCR product was purified away from free oligonucleotides by passive elution out of a nondenaturing polyacrylamide gel and precipitated.

All binding reactions were done in 20 µL of modified EMSA buffer (40 mM Tris pH 8; 7.5% glycerol; 7 mM MgCl2; 3 mM DTT; 80 mM NaCl; 0.3 mg/mL bovine serum albumin). Where appropriate, the probe was first mixed with 20 ng of pUC19 plasmid and then incubated with 300 fmol of purified TFIIIC (Kassavetis et al., 1990; Kassavetis et al., 1989) for 30 minutes at 25°C. Where condensin was added, the indicated amount of purified condensin (St-Pierre et al., 2009) was added and incubated for an additional 30 minutes at 25°C. (Total reaction time for all complexes was 1 hour.) Complexes were separated on a 4% nondenaturing polyacrylamide gel (40:1 acrylamide:bis), supplemented with 5% glycerol and 5 mM $MgCl_2$ and run in Tris-borate buffer supplemented with 5 mM $MgCl_2$. The gel was dried and exposed onto a Typhoon Trio+ cassette (Molecular Dynamics), and total radioactive signal per lane was quantitated with Image J (NIH) and plotted with GraphPad Prism.

DNase I Footprinting. Radiolabeled DNA (generated as for the EMSAs) was mixed with 20 ng of pUC19 and Footprint Buffer (40 mM Tris pH 8; 7 mM $MgCl_2$; 80 mM NaCl; 5% glycerol; 3 mM DTT). 300 fmol of TFIIIC were added to the reaction to a final volume of 20 µL and incubated for 30 minutes at 25°C. Where condensin was added, 450 fmol of condensin were added to the reaction to a final volume of 20 µL and incubated for an additional 30 minutes at 25°C. (Total reaction time for all complexes was 1 hour.) DNase I (Sigma-Aldrich) was then added to a final concentration of 7.8 units/nL and allowed to incubate for exactly 15 minutes at 25°C. Stop Mix (final concentrations of 0.2% SDS; 10 mM EDTA; 100 µg/mL Proteinase K) was added to each reaction and incubated for 1 hour at 37°C. Samples were precipitated, resuspended in loading buffer (10 mM Tris pH 8; 10 mM EDTA; 0.0166% SDS; and 65% formamide), boiled, and

loaded onto a 6% denaturing gel that had been pre-run for 1 hour. Gel was dried and exposed as above.

Electron microscopy. Complexes were mixed in EM buffer (10 mM Tris pH8, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) before being stained with uranyl formate and placed on a charged grid via the conventional negative staining protocol (Ohi et al., 2004). Complexes were visualized on a Morgagni 288 electron microscope.

Acknowledgements

Purified TFIIIC and TFIIIB were provided by Dr. George Kassavetis at University of California San Diego. Purified condensin was provided by Dr. Damien D'Amours at Université de Montreal. I would like to thank Drs. Jeffrey Herbstman and Giorgios Skiniotis of the University of Michigan for collaboration in acquiring preliminary EM data.

References

D'Ambrosio, C., Schmidt, C.K., Katou, Y., Kelly, G., Itoh, T., Shirahige, K., and Uhlmann, F. (2008). Identification of cis-acting sites for condensin loading onto budding yeast chromosomes. Genes Dev *22*, 2215-2227.

Freeman, L., Aragon-Alcaide, L., and Strunnikov, A. (2000). The condensin complex governs chromosome condensation and mitotic transmission of rDNA. J Cell Biol *149*, 811-824.

Haeusler, R.A., Pratt-Hyatt, M., Good, P.D., Gipson, T.A., and Engelke, D.R. (2008). Clustering of yeast tRNA genes is mediated by specific association of condensin with tRNA gene transcription complexes. Genes Dev *22*, 2204-2214.

Hirano, M., Anderson, D.E., Erickson, H.P., and Hirano, T. (2001). Bimodal activation of SMC ATPase by intra- and inter-molecular interactions. The EMBO journal *20*, 3238-3250.

Hirano, T. (2005). Condensins: organizing and segregating the genome. Curr Biol *15*, R265-275.

Hirano, T. (2006). At the heart of the chromosome: SMC proteins in action. Nat Rev Mol Cell Biol *7*, 311-322.

Hudson, D.F., Marshall, K.M., and Earnshaw, W.C. (2009). Condensin: Architect of mitotic chromosomes. Chromosome Res *17*, 131-144.

Hull, M.W., Erickson, J., Johnston, M., and Engelke, D.R. (1994). tRNA genes as transcriptional repressor elements. Mol Cell Biol *14*, 1266-1277.

Iwasaki, O., Tanaka, A., Tanizawa, H., Grewal, S.I., and Noma, K. Centromeric localization of dispersed Pol III genes in fission yeast. Molecular biology of the cell *21*, 254-265.

Kassavetis, G.A., Braun, B.R., Nguyen, L.H., and Geiduschek, E.P. (1990). S. cerevisiae TFIIIB is the transcription initiation factor proper of RNA polymerase III, while TFIIIA and TFIIIC are assembly factors. Cell *60*, 235-245.

Kassavetis, G.A., Riggs, D.L., Negri, R., Nguyen, L.H., and Geiduschek, E.P. (1989). Transcription factor IIIB generates extended DNA interactions in RNA polymerase III transcription complexes on tRNA genes. Molecular and cellular biology *9*, 2551-2566.

Losada, A., and Hirano, T. (2005). Dynamic molecular linkers of the genome: the first decade of SMC proteins. Genes & development *19*, 1269-1287.

Moqtaderi, Z., and Struhl, K. (2004). Genome-wide occupancy profile of the RNA polymerase III machinery in Saccharomyces cerevisiae reveals loci with incomplete transcription complexes. Molecular and cellular biology *24*, 4118-4127.

Ohi, M., Li, Y., Cheng, Y., and Walz, T. (2004). Negative Staining and Image Classification - Powerful Tools in Modern Electron Microscopy. Biological procedures online *6*, 23-34.

St-Pierre, J., Douziech, M., Bazile, F., Pascariu, M., Bonneil, E., Sauve, V., Ratsima, H., and D'Amours, D. (2009). Polo kinase regulates mitotic chromosome condensation by hyperactivation of condensin DNA supercoiling activity. Molecular cell *34*, 416-426.

Stray, J.E., Crisona, N.J., Belotserkovskii, B.P., Lindsley, J.E., and Cozzarelli, N.R. (2005). The Saccharomyces cerevisiae Smc2/4 condensin compacts DNA into (+) chiral structures without net supercoiling. The Journal of biological chemistry *280*, 34723-34734.

Thompson, M., Haeusler, R.A., Good, P.D., and Engelke, D.R. (2003). Nucleolar clustering of dispersed tRNA genes. Science *302*, 1399-1401.

Tsang, C.K., Li, H., and Zheng, X.S. (2007a). Nutrient starvation promotes condensin loading to maintain rDNA stability. Embo J *26*, 448-458.

Tsang, C.K., Wei, Y., and Zheng, X.F. (2007b). Compacting DNA during the interphase: condensin maintains rDNA integrity. Cell Cycle *6*, 2213-2218.

Wang, L., Haeusler, R.A., Good, P.D., Thompson, M., Nagar, S., and Engelke, D.R. (2005). Silencing near tRNA genes requires nucleolar localization. J Biol Chem *280*, 8637-8639.

Wood, A.J., Severson, A.F., and Meyer, B.J. Condensin and cohesin complexity: the expanding repertoire of functions. Nature reviews *11*, 391-404.

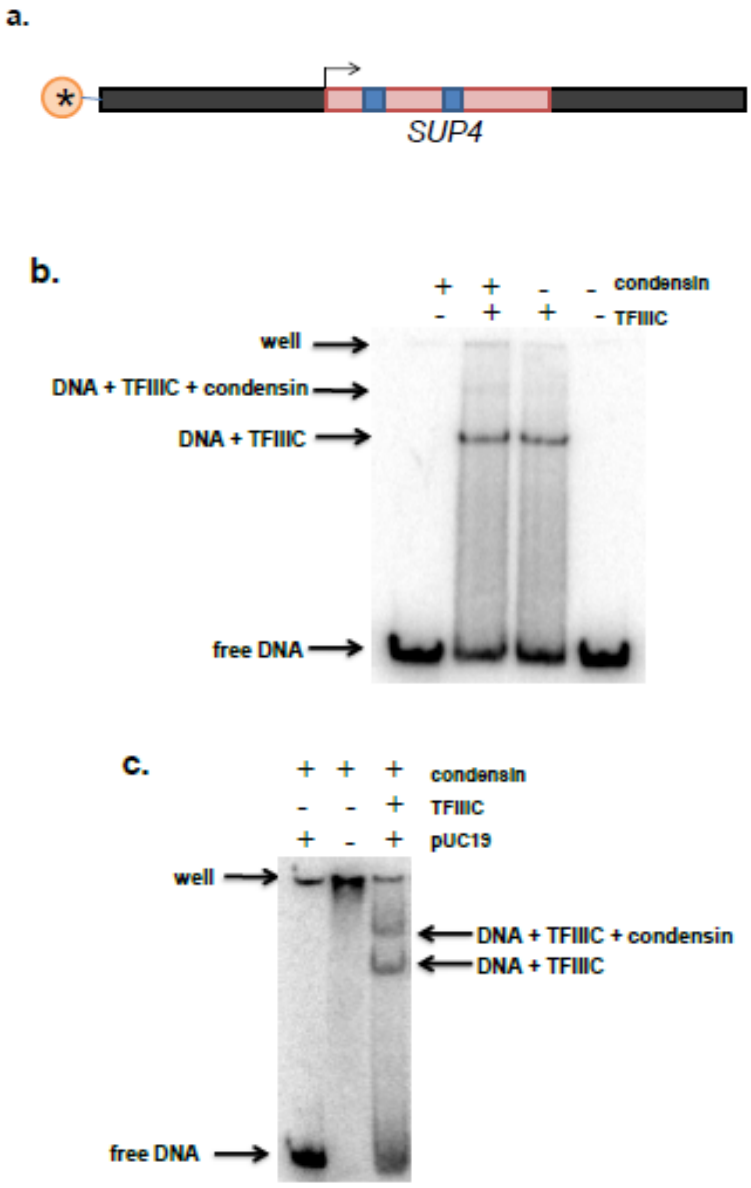


Figure 3.1. TFIIIC mediates specific binding between condensin and a tRNA gene. A. DNA fragment used for in vitro EMSA and DNA footprinting assays. tRNA gene shown in red; the internal promoters to which TFIIIC binds are shown in blue; direction of transcription indicated by arrow; radiolabel as indicated is on the upstream end of the fragment. B. Binding of condensin to a tRNA gene-TFIIIC complex. Radiolabeled DNA containing the *SUP4* tRNA gene was incubated with TFIIIC or condensin as indicated and separated on a 5% native polyacrylamide gel. C. Effect of competitor DNA on condensin binding. Complexes were assembled with TFIIIC and/or plasmid competitor before addition of condensin and separation on a native gel.

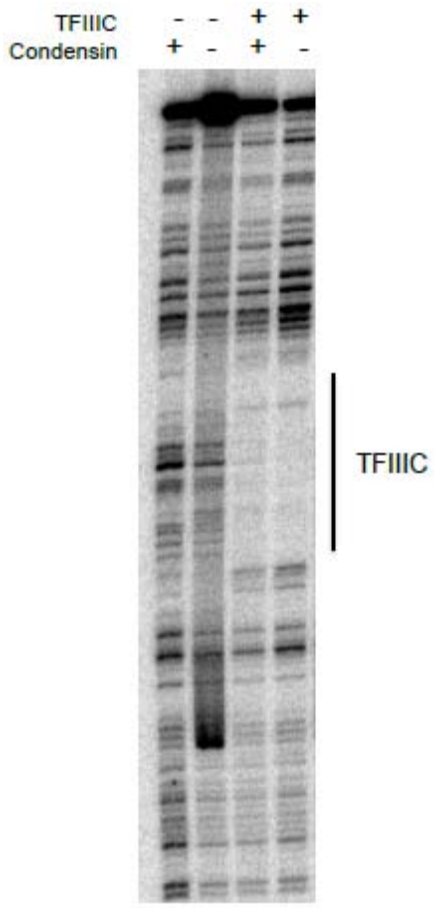


Figure 3.2. DNA footprint of a tRNA gene with bound factors. End-labeled DNA fragment containing the *SUP4* tRNA gene was incubated with TFIIIC where indicated for 30 minutes and with condensin where indicated for an additional 30 minutes. Complexes were digested with DNase I, samples were treated with Proteinase K, and the remaining DNA was precipitated and run out on a 6% denaturing gel. The TFIIIC footprint is indicated.

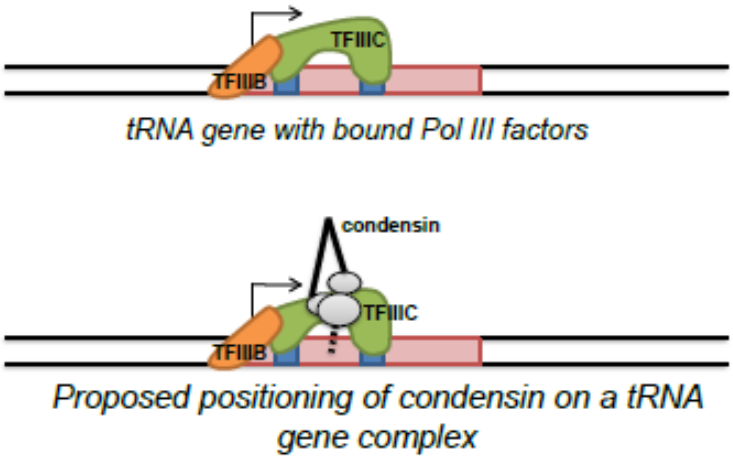


Figure 3.3. Proposed model of condensin binding to a tRNA gene complex. The top panel shows a tRNA gene with bound factors TFIIIC and TFIIIB. Our results indicate that condensin directly interacts with TFIIIC. Based on lack of DNA footprint, we propose that condensin binds directly on top of TFIIIC, making little or no contacts with the DNA (dashed line indicates weak or no interaction between condensin and DNA). Presence of TFIIIB on the complex does not prevent condensin binding.

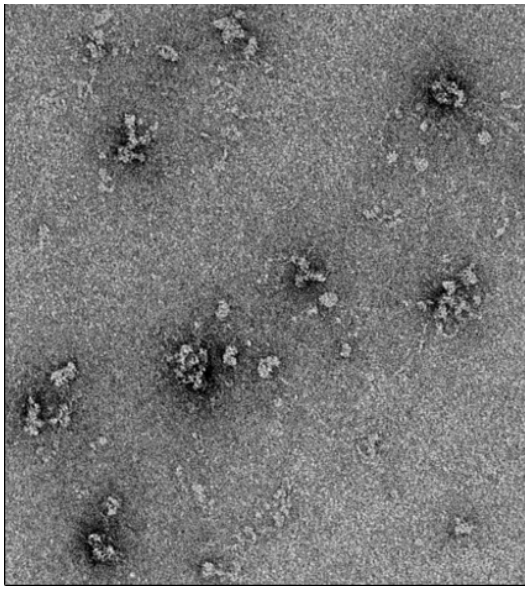


Figure 3.4. Electron micrograph of purified condensin. Condensin appears heterogeneous when visualized.

Chapter IV

RNAs inhibit Pol II transcription by preventing Pol II binding to DNA template

Abstract

Many RNAs are known to act as regulators of transcription in eukaryotes, including certain small RNAs that directly inhibit RNA polymerases both in prokaryotes and in eukaryotes. We have examined the potential for a variety of RNAs to directly inhibit transcription by yeast Pol II. We find that several structured and unstructured RNAs are able to bind Pol II purified from yeast, and can subsequently block its transcriptional activity. The inhibition is achieved by the RNAs binding in or near the active site of the enzyme and blocking the enzyme's ability to bind a DNA template. Unstructured RNAs are more potent at this activity than structured ones, suggesting flexibility of the RNA is necessary for efficient blocking of DNA binding. The RNA must find the enzyme before the DNA template does, as RNA is not able to invade into and displace DNA template that is already bound to Pol II, nor can RNA inhibit elongating Pol II. These results suggest a need for co-transcriptional removal of nascent RNAs from the vicinity of transcription initiation, either by packaging the RNAs into ribonucleoprotein complexes or by efficiently transporting them away from the site of synthesis.

Introduction

Noncoding RNAs (ncRNA) can be potent regulators of eukaryotic gene expression, both through modulation of RNA function and stability and through directed modification of chromatin [reviewed in (Hawkins and Morris, 2008; Kurokawa et al., 2009; Storz et al., 2005)]. There is emerging evidence that some RNAs are able to directly inhibit RNA

polymerases. The bacterial 6S RNA inhibits transcription by binding stably in the active site of RNA polymerase (Wassarman and Saecker, 2006). In addition there is evidence that certain RNAs in mammalian cells might directly inhibit RNA polymerase II (Pol II). The RNA transcripts of B2 and Alu small interspersed elements (SINEs) inhibit transcription initiation by purified Pol II in vitro, by binding directly to mammalian Pol II (Allen et al., 2004; Espinoza et al., 2004; Mariner et al., 2008; Yakovchuk et al., 2009). SINEs can be actively transcribed in vitro by RNA polymerase III (Pol III), as expected since the various families of these high copy DNA elements in vertebrates are mostly derived by retrotransposition from tRNA, 7SL RNA, or 5S rRNA genes [reviewed in (Batzer and Deininger, 2002; Hasler and Strub, 2006; Okada, 1991; Price et al., 2004; Weiner, 2002)]. Some SINE elements can also be transcribed by Pol II in vivo, and although SINEs are not normally found stably expressed at significant levels in vivo, they can be expressed under certain conditions of cell stress, viral infection, or in specific cell types (reviewed in Lunyak and Atallah, 2011). It is not clear whether the relative paucity of RNA from these high copy elements is entirely due to lack of transcription in vivo, or whether rapid turnover of the RNA transcripts also takes place.

Direct inhibition of Pol II by RNAs has not been tested extensively in a yeast system, although an early report suggested that tRNA can inhibit Pol II (Sawadogo, 1981). The hypothesis that tRNA transcripts might directly inhibit Pol II transcription is particularly interesting, given that Pol II transcription is antagonized in the immediate vicinity of tRNA genes (Bolton and Boeke, 2003; Hull et al., 1994; Kinsey and Sandmeyer, 1991). This local silencing of Pol II transcription by tRNA genes, termed tRNA gene-mediated (tgm) silencing, involves subnuclear clustering of the tRNA genes to the nucleolus (Wang et al., 2005) and requires condensin (Haeusler et al., 2008), but other aspects of the molecular mechanism are uncharacterized. Since tRNA and 5S rRNA genes are the only repetitive Pol III transcription units in yeast, there being no SINE elements in these small genomes, we set out to investigate the ability of tRNAs to bind directly to purified Pol II from *Saccharomyces cerevisiae* and inhibit transcription. Surprisingly, we show that a variety of RNAs inhibit Pol II transcription by binding directly to Pol II and preventing Pol II from binding to a DNA template. Unstructured RNAs are in fact more effective inhibitors than highly structured tRNAs or 5S rRNA.

These results are discussed in terms of the possible need to remove nascent transcripts from the site of synthesis through ribonucleoprotein assembly and transport.

Results

We first asked whether pre-incubation of Pol II with RNAs could inhibit Pol II transcription in a non-specific in vitro transcription assay. To start, we assessed inhibitory activity of the Pol III-transcribed pre-tRNA^{Tyr}. Pol II purified from *Saccharomyces cerevisiae* was pre-bound to unlabeled pre-tRNA^{Tyr}, followed by addition of plasmid template, unlabeled nucleotide triphosphate (NTP) substrates, and α-^{32}P-UTP. Pol II initiates non-specifically off the plasmid, creating a random size distribution of radioactive products that were quantitated (Figure 4.1A, right-most lane). With increasing amounts of pre-tRNA^{Tyr}, Pol II transcripts are severely reduced in number (Figure 4.1A, right to left), indicative of the pre-tRNA inhibiting Pol II transcription (Figure 4.1B, top left panel).

One hypothesis for the tgm silencing phenomenon is that the nascent transcripts from *S. cerevisiae* tRNA genes act locally as repressors of Pol II; therefore, our result that tRNA directly inhibits Pol II was intriguing. Yet it was not clear whether this inhibition would actually be specific to tRNAs. Specificity would not be unprecedented, since, for example, certain SINE transcripts have been shown to inhibit mammalian Pol II significantly better than others (Mariner et al., 2008). To determine whether Pol II inhibition by RNA was limited to tRNAs, we tested other RNAs with varied degrees of intrinsic structure for their ability to inhibit Pol II transcription in vitro, from tightly structured RNAs (5S rRNA) (Garrett and Olesen, 1982) to ones predicted to be completely unstructured in solution (polyU RNA). We also tested a half-tRNA molecule, containing the first half of the pre-tRNA^{Tyr} sequence but truncated just past the anticodon loop and lacking the tertiary structure of the full tRNA. Another RNA tested is a 250 nt region of the transcribed *PHO84* ORF of the yeast genome that is expected to have various local structures in aqueous solution, characteristic of mixed sequence RNAs, but to not be tightly structured overall. The transcription inhibition assay was repeated as for

the pre-tRNA^{Tyr} and plotted (Figure 4.1B). As with the pre-tRNA, the other three RNAs tested all inhibited transcription, but to different degrees. Contrary to the hypothesis that tRNAs might have some specific ability to inhibit Pol II, the half-tRNA and the 250 nt ORF RNA inhibited better than the pre-tRNA and the 5S rRNA. For all RNAs, the levels of inhibition approach zero but never reach it at the concentrations tested, particularly for the more structured RNAs, suggesting that the intrinsic structure of those RNAs somehow hampers its ability to inhibit the enzyme. Thus, while a variety of RNAs can inhibit Pol II transcription, unstructured RNAs seem to be better at this activity than structured ones.

To determine whether Pol II inhibition is due to a direct physical interaction between RNA and Pol II, the ability of purified Pol II to bind stably to RNA was demonstrated by electrophoretic mobility shift assays (EMSA). Incubation of the polymerase with RNA resulted in a complex that was stable to conditions of native electrophoresis (Figure 4.2A, shown for the pre-tRNA^{Tyr}). We next asked whether RNA binding was interfering with template binding, transcription initiation, or transcript elongation. To do this, we performed a number of assays. For template binding, purified Pol II binding to purely double stranded DNA templates ("closed complexes") proved too unstable for reliable analysis by EMSA, so we directly tested the ability of RNA pre-bound to Pol II to interfere with the ability of Pol II to bind an "open complex," where the two DNA strands have been separated in a pre-initiation bubble. RNA was pre-bound to Pol II, as in the transcription assays, then added to a radiolabeled 50 bp DNA template constructed to have a pre-existing 15 nt unpaired region. The pre-bound RNA-Pol II complex was incubated with this open DNA, and then complexes were separated on a native gel EMSA. Pol II stably binds the open DNA complex in the absence of RNA (Figure 4.2B, second lane from right), though the Pol II-DNA complexes appear to be slightly unstable through the electrophoresis conditions, as evidenced by the slight smearing in the lane. As increasing amounts of RNA are pre-incubated with the Pol II (Figure 4.2B, right to left), less of the DNA is bound to Pol II. Shifted signal was quantitated in triplicate and compared to total signal per lane. This ratio of shifted DNA to total DNA was plotted for pre-tRNA, half-tRNA, 5S rRNA, and the 250 nt ORF RNA competitions (Figure 4.2C). Once again, the unstructured RNAs prevent binding to the

template more potently than the structured pre-tRNA and 5S rRNA. Together, these results indicate that RNAs bind to Pol II and inhibit transcription by preventing the Pol II from accessing the DNA template.

We next assessed what length of the unstructured RNA was required to bind Pol II to achieve inhibition. Poly(U) RNA was used for this purpose, to avoid issues of structure or sequence specificity as the length increases. Binding and inhibition assays were carried out as for the other RNAs, quantitated in triplicate, and plotted (Figure 4.2D). The results using increasing poly(U) lengths demonstrate clearly that as the length of the RNA increases, binding of Pol II to the DNA template is inhibited more potently. Of note is an apparent break point between 25 and 30 nt, suggesting RNA of this length or greater fits into the structure of Pol II in such a way that is desired for optimum inhibition. This 25-30 nt length is considerably longer than the 10 nt active site-to-exit channel length of Pol II (Gnatt et al., 2001), suggesting RNA is contacting the polymerase at sites not normally accessible during transcription. One possibility is that sites normally used for DNA template contacts are in use, which would be compatible with the observed inhibition of DNA binding. Less intrinsic structure appears to facilitate positioning the RNA properly within the polymerase and more effectively block binding to DNA.

This model predicts that pre-formation of a stable open complex between Pol II and the DNA template might preclude inhibition by RNA, as long as the RNA was not able to invade the complex to displace the DNA or to inhibit some other aspect of transcription (e.g., nucleotide acquisition). To test the first prediction, we first pre-bound the radiolabeled open DNA to Pol II, and then added increasing levels of RNA. We used the pre-tRNA as an example of a structured RNA and the half-tRNA as an unstructured RNA. Analysis was done via EMSA as in Figure 4.2. RNAs did not cause displacement of Pol II from the open DNA to any observable degree (Figure 4.3A). We further tested the ability of RNAs to inhibit an elongation complex. Elongation inhibition assays were performed with the more unstructured RNAs, half tRNA and the 250 nt ORF RNA, to test optimal inhibitory conditions. In both cases, the RNAs were not able to significantly inhibit elongation by Pol II (Figure 4.3B). Taken together, these results show that the

RNA needs to pre-bind the polymerase in order to prevent binding of the polymerase to DNA.

In the course of our experiments, we observed that Pol II was able to transfer radiolabel to the inhibitory RNAs. Such activity is consistent with the RNA-dependent RNA polymerase (RdRP) activity observed for Pol II previously (Johnson and Chamberlin, 1994; Lehmann et al., 2007). To test whether Pol II directly labels RNAs in this system, the structured and unstructured RNAs were incubated with Pol II and α-^{32}P-UTP, in the absence of DNA template (Figure 4.4A). All four RNAs were labeled, albeit inefficiently. We determined that this labeling was likely not due to self-templating by the RNA, since all four α-^{32}P-labeled NTPs can be added to the RNA substrate (Figure 4.4B), when used alone in the absence of other nucleotides. The assumption that reactions were catalyzed by the Pol II active site was confirmed by inhibition at low levels of the Pol II-specific inhibitor, α-amanitin (Figure 4.4C). This non-templated addition of nucleotides to RNA appears to be highly inefficient, as only ~0.1% of the RNA molecules become labeled. Digestion with RNase T_1 confirms that the radiolabel is being added onto the 3' end of the RNA (data not shown), consistent with previously observed RDRP activity of Pol II. The size heterogeneity of labeled RNA is likely due to heterogeneity at the 3' end of the RNA substrate that results from T7 transcription. Assaying for increasing times (2 minutes to 2 hours) shows that the RNA doesn't processively increase in size, though the number of labeled molecules does increase with time (Figure 4.4D). We conclude that inhibition of Pol II by RNAs is due to this binding near the active site in such a way as to exclude DNA binding and allow at least inefficient incorporation of nucleotides into the RNA.

Discussion

It has been known for some time now that the presence of a tRNA gene adjacent to a Pol II gene in yeast reduces expression of the Pol II gene. Yet the mechanism of this tgm silencing of Pol II genes is to date unknown and proceeds by a mechanism distinct from other forms of transcriptional silencing in yeast [(Hull et al., 1994; Wang et al., 2005),

and reviewed in (Perrod and Gasser, 2003; Rusche et al., 2003)]. The tRNA genes causing the silencing require an active tRNA gene transcription complex, including condensin (Haeusler et al., 2008; Hull et al., 1994), and all tRNA genes genome-wide appear capable of forming such complexes (D'Ambrosio et al., 2008; Haeusler et al., 2008; Kassavetis et al., 1990; Moqtaderi and Struhl, 2004; Roberts et al., 2003). Thus, the hypothesis for this study was that nascent pre-tRNAs might bind to Pol II in the immediate vicinity and inhibit transcription.

The results of this study show that tRNAs do indeed inhibit transcription by Pol II, though the inhibition is not specific to tRNA transcripts. Thus, if this process is involved in tgm silencing in vivo, interaction of the pre-tRNA with Pol II would likely need to be either facilitated by a directed mechanism or due to a very high buildup of the local pre-tRNA concentration at the spatially clustered tRNA genes (Bertrand et al., 1998; Thompson et al., 2003). We had not anticipated that many different RNAs would inhibit purified Pol II. There is no stringent sequence requirement for inhibition, since poly(U) homopolymers are able to inhibit at least as well as mixed sequence RNAs lacking polyU stretches, consistent with RNAs interacting with surfaces of Pol II that form non-specific nucleic acid interactions. In addition, the fact that the unstructured RNAs that we tested (half-tRNA, ORF RNA, and poly(U) RNA) inhibit much better than the more tightly structured RNAs suggests pre-existing tight structure interferes with inhibition, rather than fitting into an evolved binding site. Eukaryotes might possess this general RNA inhibition property for functional reasons and may have simply evolved away from this function.

The apparent break point between a 25 and 30 nt RNA in the ability to displace DNA template (Figure 4.2D) suggests something in this length range is important for efficient intrusion into the Pol II structure. This length requirement is significantly longer than the 10 nt distance between the active site and the exit tunnel of Pol II. This suggests that the RNA might be making contacts in the Pol II that RNAs do not normally make in the presence of DNA template. One hypothesis is that the RNA is making contacts in the enzyme that normally are in contact with template DNA. This would be consistent with the fact that the RNA pre-bound to the Pol II blocks the DNA from binding the enzyme. Additional support for this idea comes from the result that pre-bound DNA is unable to

be disrupted by RNA (Figure 4.3A), suggesting that the region where the RNA would enter or bind Pol II for inhibition is already occupied by the DNA template.

Unstructured RNAs prevent the binding of Pol II to DNA more potently than structured ones, and in light of the hypothesis that the RNAs are making contacts in the enzyme that RNAs normally do not make, one might suspect that greater conformational possibilities of the more flexibly structured half-tRNA, ORF RNA, and the poly(U) RNAs would help make the required contacts in the enzyme needed for optimal binding. The apparent lack of strong sequence dependence is consistent with Pol II not having position-specific contacts with the nucleotide bases, but rather binding the sugar-phosphate backbone as it does with both the DNA template and RNA product during transcription. The nucleotide addition activity, though extremely inefficient, suggests the 3' end of the RNA is positioned for at least part of the time at the active site of the enzyme.

This phenomenon that RNAs, particularly less structured RNAs, can tightly bind and inhibit Pol II poses an interesting set of questions. In retrospect, nuclear processes are largely arranged in a way that does not allow substantial concentrations of naked RNAs to accumulate in the vicinity of Pol II transcription. Co-transcriptional assembly of pre-mRNA into ribonucleoprotein complexes has been studied in detail, and the massive synthesis of ribosomal RNAs is resolved by not only assembling and processing them co-transcriptionally, but generally segregating them into specific subnuclear areas, the nucleoli. The timing and spatial organization of assembly of the small RNAs into RNPs is somewhat less understood. All acquire some number of protein partners early in their biosynthesis. In yeast, the most abundant small RNAs made by Pol III, pre-tRNA and 5S rRNA, not only bind La and Lsm proteins (Maraia and Intine, 2002; Phizicky and Hopper, 2010; Wilusz and Wilusz, 2005), but also have their genes clustered at the nucleolus (Bertrand et al., 1998; Thompson et al., 2003; Wang et al., 2005), thus sequestering them away from most of the centers of Pol II transcription.

Overall, this study demonstrates that RNAs can bind to Pol II at or near its active site, preventing binding of Pol II to a DNA template. While flexible RNA structure is necessary for efficient inhibition, both structured and unstructured RNAs do prevent Pol II transcription, suggesting cellular mechanisms to segregate the highly transcribed

structured RNAs away from Pol II are likely necessary. In light of the many RNAs that have been shown to regulate eukaryotic gene expression, our results are consistent with the possibility that transcribed RNAs can be direct *trans*-acting repressors of transcription.

Materials and Methods

RNA preparation. Yeast precursor $tRNA^{Tyr}$ ($pre\text{-}tRNA^{Tyr}$) (Goodman et al., 1977), a half-$pre\text{-}tRNA^{Tyr}$ generated by inserting a poly(T) terminator in the coding sequence of $pre\text{-}tRNA^{Tyr}$ just past the anticodon loop (5'-CUCUCGGUAGCCAAGUUGGUUUAAGGCGCAAGACUGUAAUUUUUUU-3'), yeast 5S ribosomal RNA (rRNA), and a 250 nt mixed-sequence RNA from the *PHO84* gene (Chromosome XIII, coordinates to 24237 to 25486) were in vitro transcribed from DNA templates using T7 RNA polymerase (Milligan and Uhlenbeck, 1989). Radiolabeled $pre\text{-}tRNA^{Tyr}$ was transcribed using T7 RNA polymerase in reactions containing 1 µM α-^{32}P-UTP (Milligan and Uhlenbeck, 1989). The RNAs were then purified by passive elution from denaturing polyacrylamide gels. $Poly(U)_{50}$ RNA was purchased from Integrated DNA Technology. The smaller sizes of poly(U) RNA were generated by alkaline hydrolysis and subsequent purification by passive elution from denaturing polyacrylamide gels (Marvin et al., 2011).

Pol II transcription assays. In vitro-transcribed RNAs to be used in Pol II transcription inhibitions were serially diluted in water. 300 fmol of purified 12-subunit Pol II (Elmlund et al., 2010) were added to desired amount of RNA in 5 µL of transcription buffer (20 mM Tris pH 8.0, 40 mM KCl, 5 mM $MgCl_2$, and 1 mM DTT). Pol II-RNA complexes were allowed to bind for 15 minutes at 30°C. 400 ng of supercoiled plasmid pSUP4o (Hull et al., 1994), containing no Pol II promoter sequences, were added to the reaction, incubated briefly (2-3 minutes) at 30°C, before nucleotide triphosphates (NTPs) were added (ATP, CTP, and GTP at 500 µM final; UTP at 9 µM final; and α-^{32}P-UTP at 1 µM final) to a final reaction volume of 10 µL. Transcription by Pol II from non-specific

initiation sites was allowed to proceed for 25 minutes at 30°C. Samples were boiled in formamide and analyzed on a 10% denaturing gel. The gel was dried and exposed onto a Typhoon Trio+ cassette (Molecular Dynamics), and total radioactive signal per lane was quantitated with Image J (NIH) and plotted with GraphPad Prism.

Electrophoretic mobility shift assays. For the Pol II-RNA binding assay, amounts of Pol II as shown were added to 7 nmol of radiolabeled pre-$tRNA^{Tyr}$ in EMSA buffer (20 mM Tris pH 8.0, 10% glycerol, 1 mM DTT, 5 mM $MgCl_2$, 40 mM KCl, and 100 µg/mL bovine serum albumin) to a final reaction volume of 10 µL. Pol II-tRNA complexes were allowed to bind for 15 minutes at 30°C and then separated on a 5% nondenaturing polyacrylamide gel (40:1 acrylamide:bis, supplemented with 5% glycerol and 5 mM $MgCl_2$ in Tris-Borate buffer supplemented with 5 mM $MgCl_2$). The gel was dried, exposed, and scanned as above.

For Pol II-DNA binding inhibition assays, radiolabeled DNA was generated by end-labeling DNA oligonucleotide CKO433 (5'-gggttggcttttcgccGTGTCCCTCTCGATGgctgtaagtaaggctatgg-3') with γ-^{32}P-ATP. The end-labeled oligonucleotide was then annealed to DNA oligonucleotide CKO432 (5'-ccatagccttacttacagcGTAGCTCTCCCTGTGggcgaaaagccaaccc-3') by slow cooling from 65°C to 25°C to generate a "bubbled" DNA fragment (the sequences in capital letters denote the mismatched, "bubbled" region) used to create open pre-initiation complexes with Pol II. After annealing the two strands, the double stranded DNA was purified away from free oligonucleotide by passive elution out of a 6% nondenaturing polyacrylamide gel and precipitated. For inhibition assays, Pol II was pre-incubated with RNA for 15 minutes at 30°C in 5 µL of EMSA buffer. The labeled CKO433-CKO432 open DNA complex was then added to Pol II-RNA complexes in a final volume of 10 µL EMSA buffer. Complexes were allowed to bind for 15 minutes at 30°C. Formation of Pol II-DNA complexes was analyzed by EMSA as above. Where pre-binding of Pol II-DNA was tested, Pol II was first added to end-labeled DNA, allowed to bind for 15 minutes at 30°C, and then RNA was added for 15 minutes. Reactions proceeded otherwise the same.

Elongation inhibition assays. Transcription on annealed oligonucleotide elongation scaffolds was performed basically as described in (Kaplan et al., 2008) with the following modifications. Elongation scaffolds were formed using partially mismatched DNA oligonucleotides, allowing single-step annealing of template, non-template and 5′-radiolabeled RNA primer. 2 µM of template DNA (CKO433), non-template DNA (CKO432) and nine nucleotide RNA primer (RNA9) in 10 µl in vitro transcription buffer (IVT buffer) (5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.0, 40 mM KCl, 2 mM DTT) were annealed by slow cooling from 65°C to 25°C to form elongation scaffolds. 5 µl of scaffold were mixed with 5 µl purified Pol II (~ 2 µM) (Kaplan et al., 2008) and incubated for 5 minutes at room temperature. Complexes were diluted 20-fold in IVT buffer and aliquoted for addition of NTPs with or without inhibitory RNAs. Productive scaffolds were advanced one nucleotide by the addition of 10 µM GTP (in IVT buffer) to a final concentration of 1 µM for 5 minutes at room temperature. Run-off transcription was initiated by the addition of 10 µM of all four NTPs with or without differing concentrations of inhibitory RNA species. Aliquots of each reaction at time points from 10 seconds to 5 minutes were quenched and analyzed by polyacrylamide gel electrophoresis as previously described (Kaplan et al., 2008).

Non-templated labeling of RNAs by Pol II. 0.5 µg of Pol II was pre-incubated with 1 µg of desired RNA for 15 minutes at 30°C in 5 µL of transcription buffer. α-^{32}P-UTP was added in to 1 µM in a final volume of 10 µL of transcription buffer, and the reaction was allowed to proceed for 30 minutes at 30°C. Reactions were then run out on a 10% denaturing gel. The gel was dried, exposed, and scanned as above. RNA labeling efficiency was estimated by measuring α-^{32}P incorporation into otherwise unlabeled RNA. For treatment with α-amanitin, the inhibitor was added to a 50 ng/µL final concentration and allowed to bind for 5 minutes prior to adding RNA.

Acknowledgements

This manuscript is currently in revision at Nucleic Acids Research. I would like to thank Dr. Roger Kornberg at Stanford University for providing purified yeast Pol II. I would like to thank Dr. Craig Kaplan at Texas A&M University for performing the elongation inhibition assays, useful discussion of the experiments, and critical reading of the manuscript.

References

Allen, T.A., Von Kaenel, S., Goodrich, J.A., and Kugel, J.F. (2004). The SINE-encoded mouse B2 RNA represses mRNA transcription in response to heat shock. Nature structural & molecular biology *11*, 816-821.

Batzer, M.A., and Deininger, P.L. (2002). Alu repeats and human genomic diversity. Nature reviews *3*, 370-379.

Bertrand, E., Houser-Scott, F., Kendall, A., Singer, R.H., and Engelke, D.R. (1998). Nucleolar localization of early tRNA processing. Genes Dev *12*, 2463-2468.

Bolton, E.C., and Boeke, J.D. (2003). Transcriptional interactions between yeast tRNA genes, flanking genes and Ty elements: a genomic point of view. Genome Res *13*, 254-263.

D'Ambrosio, C., Schmidt, C.K., Katou, Y., Kelly, G., Itoh, T., Shirahige, K., and Uhlmann, F. (2008). Identification of cis-acting sites for condensin loading onto budding yeast chromosomes. Genes & development *22*, 2215-2227.

Elmlund, D., Davis, R., and Elmlund, H. Ab initio structure determination from electron microscopic images of single molecules coexisting in different functional states. Structure *18*, 777-786.

Espinoza, C.A., Allen, T.A., Hieb, A.R., Kugel, J.F., and Goodrich, J.A. (2004). B2 RNA binds directly to RNA polymerase II to repress transcript synthesis. Nature structural & molecular biology *11*, 822-829.

Garrett, R.A., and Olesen, S.O. (1982). Structure of eukaryotic 5S ribonucleic acid: a study of Saccharomyces cerevisiae 5S ribonucleic acid with ribonucleases. Biochemistry *21*, 4823-4830.

Gnatt, A.L., Cramer, P., Fu, J., Bushnell, D.A., and Kornberg, R.D. (2001). Structural basis of transcription: an RNA polymerase II elongation complex at 3.3 A resolution. Science (New York, NY *292*, 1876-1882.

Goodman, H.M., Olson, M.V., and Hall, B.D. (1977). Nucleotide sequence of a mutant eukaryotic gene: the yeast tyrosine-inserting ochre suppressor SUP4-o. Proceedings of the National Academy of Sciences of the United States of America *74*, 5453-5457.

Haeusler, R.A., Pratt-Hyatt, M., Good, P.D., Gipson, T.A., and Engelke, D.R. (2008). Clustering of yeast tRNA genes is mediated by specific association of condensin with tRNA gene transcription complexes. Genes & development *22*, 2204-2214.

Hasler, J., and Strub, K. (2006). Alu elements as regulators of gene expression. Nucleic acids research *34*, 5491-5497.

Hawkins, P.G., and Morris, K.V. (2008). RNA and transcriptional modulation of gene expression. Cell cycle (Georgetown, Tex *7*, 602-607.

Hull, M.W., Erickson, J., Johnston, M., and Engelke, D.R. (1994). tRNA genes as transcriptional repressor elements. Molecular and cellular biology *14*, 1266-1277.

Johnson, T.L., and Chamberlin, M.J. (1994). Complexes of yeast RNA polymerase II and RNA are substrates for TFIIS-induced RNA cleavage. Cell *77*, 217-224.

Kaplan, C.D., Larsson, K.M., and Kornberg, R.D. (2008). The RNA polymerase II trigger loop functions in substrate selection and is directly targeted by alpha-amanitin. Molecular cell *30*, 547-556.

Kassavetis, G.A., Braun, B.R., Nguyen, L.H., and Geiduschek, E.P. (1990). S. cerevisiae TFIIIB is the transcription initiation factor proper of RNA polymerase III, while TFIIIA and TFIIIC are assembly factors. Cell *60*, 235-245.

Kinsey, P.T., and Sandmeyer, S.B. (1991). Adjacent pol II and pol III promoters: transcription of the yeast retrotransposon Ty3 and a target tRNA gene. Nucleic acids research *19*, 1317-1324.

Kurokawa, R., Rosenfeld, M.G., and Glass, C.K. (2009). Transcriptional regulation through noncoding RNAs and epigenetic modifications. RNA biology *6*, 233-236.

Lehmann, E., Brueckner, F., and Cramer, P. (2007). Molecular basis of RNA-dependent RNA polymerase II activity. Nature *450*, 445-449.

Lunyak, V.V., and Atallah, M. Genomic relationship between SINE retrotransposons, Pol III-Pol II transcription, and chromatin organization: the journey from junk to jewel. Biochemistry and cell biology = Biochimie et biologie cellulaire *89*, 495-504.

Maraia, R.J., and Intine, R.V. (2002). La protein and its associated small nuclear and nucleolar precursor RNAs. Gene expression *10*, 41-57.

Mariner, P.D., Walters, R.D., Espinoza, C.A., Drullinger, L.F., Wagner, S.D., Kugel, J.F., and Goodrich, J.A. (2008). Human Alu RNA is a modular transacting repressor of mRNA transcription during heat shock. Molecular cell *29*, 499-509.

Marvin, M.C., Walker, S.C., Fierke, C.A., and Engelke, D.R. Binding and cleavage of unstructured RNA by nuclear RNase P. RNA (New York, NY *17*, 1429-1440.

Milligan, J.F., and Uhlenbeck, O.C. (1989). Synthesis of small RNAs using T7 RNA polymerase. Methods in enzymology *180*, 51-62.

Moqtaderi, Z., and Struhl, K. (2004). Genome-wide occupancy profile of the RNA polymerase III machinery in Saccharomyces cerevisiae reveals loci with incomplete transcription complexes. Molecular and cellular biology *24*, 4118-4127.

Okada, N. (1991). SINEs. Current opinion in genetics & development *1*, 498-504.

Perrod, S., and Gasser, S.M. (2003). Long-range silencing and position effects at telomeres and centromeres: parallels and differences. Cell Mol Life Sci *60*, 2303-2318.

Phizicky, E.M., and Hopper, A.K. tRNA biology charges to the front. Genes & development *24*, 1832-1860.

Price, A.L., Eskin, E., and Pevzner, P.A. (2004). Whole-genome analysis of Alu repeat elements reveals complex evolutionary history. Genome research *14*, 2245-2252.

Roberts, D.N., Stewart, A.J., Huff, J.T., and Cairns, B.R. (2003). The RNA polymerase III transcriptome revealed by genome-wide localization and activity-occupancy relationships. Proceedings of the National Academy of Sciences of the United States of America *100*, 14695-14700.

Rusche, L.N., Kirchmaier, A.L., and Rine, J. (2003). The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annual review of biochemistry *72*, 481-516.

Sawadogo, M. (1981). On the inhibition of yeast RNA polymerases A and B by tRNA and alpha-amanitin. Biochemical and biophysical research communications *98*, 261-267.

Storz, G., Altuvia, S., and Wassarman, K.M. (2005). An abundance of RNA regulators. Annual review of biochemistry *74*, 199-217.

Thompson, M., Haeusler, R.A., Good, P.D., and Engelke, D.R. (2003). Nucleolar clustering of dispersed tRNA genes. Science (New York, NY *302*, 1399-1401.

Wang, L., Haeusler, R.A., Good, P.D., Thompson, M., Nagar, S., and Engelke, D.R. (2005). Silencing near tRNA genes requires nucleolar localization. The Journal of biological chemistry *280*, 8637-8639.

Wassarman, K.M., and Saecker, R.M. (2006). Synthesis-mediated release of a small RNA inhibitor of RNA polymerase. Science (New York, NY *314*, 1601-1603.

Weiner, A.M. (2002). SINEs and LINEs: the art of biting the hand that feeds you. Current opinion in cell biology *14*, 343-350.

Wilusz, C.J., and Wilusz, J. (2005). Eukaryotic Lsm proteins: lessons from bacteria. Nature structural & molecular biology *12*, 1031-1036.

Yakovchuk, P., Goodrich, J.A., and Kugel, J.F. (2009). B2 RNA and Alu RNA repress transcription by disrupting contacts between RNA polymerase II and promoter DNA within assembled complexes. Proceedings of the National Academy of Sciences of the United States of America *106*, 5569-5574.

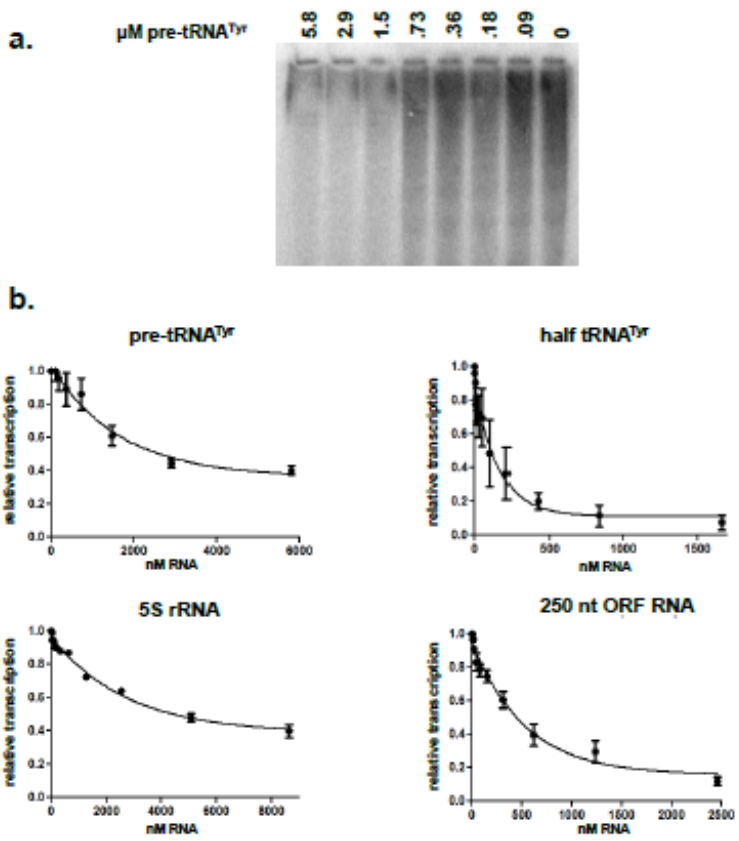


Figure 4.1. Pre-incubation of Pol II with RNAs inhibits transcript formation. A. Inhibition of Pol II transcription by pre-tRNA^{Tyr}. Serially diluted pre-tRNA^{Tyr} was incubated with constant amount of Pol II for 15 min at 30°C. The Pol II-RNA complex was then incubated with pSUP4o plasmid template, and then added to NTPs. Reactions proceeded for 25 mins, then boiled in formamide and loaded onto a 10% denaturing gel. B. The reaction in panel A was repeated in triplicate for four different RNAs. Total radioactive signal per lane was quantitated and plotted.

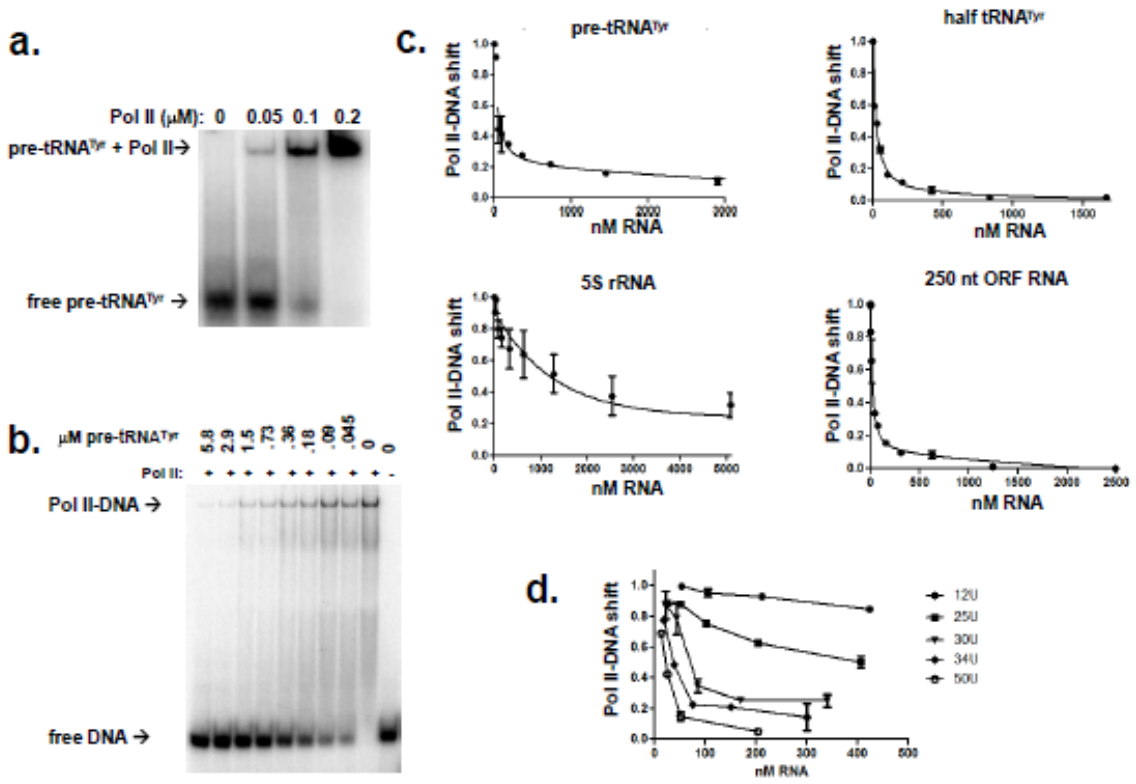


Figure 4.2. RNAs bind directly to Pol II and prevent the binding of Pol II to an open DNA complex. A. Binding of Pol II to a pre-tRNA^{Tyr} transcript. Constant amount of α-^{32}P pre-tRNA^{Tyr} was incubated with titrated amounts of purified yeast Pol II for 15 minutes and separated on a 5% native polyacrylamide gel. B. Inhibition by RNA of Pol II-DNA complex formation. tRNA^{Tyr} was serially diluted and incubated with constant amount of Pol II for 15 min at 30°C. The Pol II-RNA complexes were then incubated with the CKO432-CKO433 pre-formed open DNA complex for 15 minutes to a final volume of 10 µL. Complexes were separated on a 5% native gel. Pol II-DNA band separated from free DNA. The reaction in Panel B was repeated in triplicate for four different RNAs. Total radioactive signal per lane was quantitated and plotted. D. Inhibition of Pol II by RNA is not sequence-specific but is size-dependent. Varying lengths of poly(U) RNA were incubated with Pol II for 15 minutes at 30°C. The Pol II-RNA complex was then incubated with radiolabeled CKO432-CKO433 open DNA for 15 minutes, and complexes were separated on a 5% native gel. Shifted bands were quantitated relative to total signal, in triplicate for each RNA.

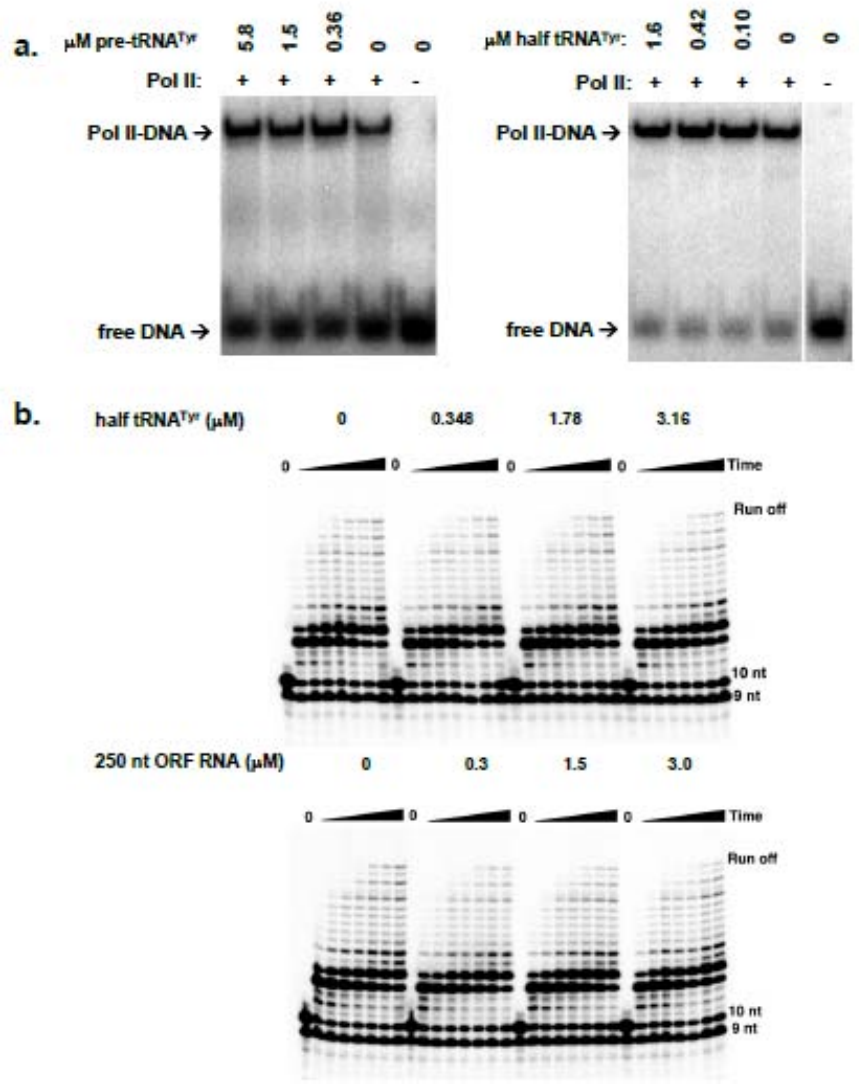


Figure 4.3. Pre-formed Pol II-DNA open complexes and pre-formed elongation scaffolds are not disrupted by RNAs. A. Pol II is first incubated with the radiolabeled open DNA template for 15 minutes at 30°C. Serial dilutions of RNA are then added into the complex and incubated for 15 minutes at 30°C. Complexes are separated on a 5% native gel. Increasing RNA does not displace Pol II off the DNA. B. Template oligonucleotide DNA, RNA primer and partially mismatched non-template DNA were annealed to form oligonucleotide elongation scaffolds. Purified Pol II was pre-bound to the scaffold, and active elongation complexes were advanced from nine nucleotides to ten by the addition of GTP (1 μM). Complexes were then allowed to transcribe to the end of the template by the addition of all four NTPs (10 μM each) in the presence or absence of different concentrations of RNA (half tRNA, top; 250 nt ORF RNA, bottom).

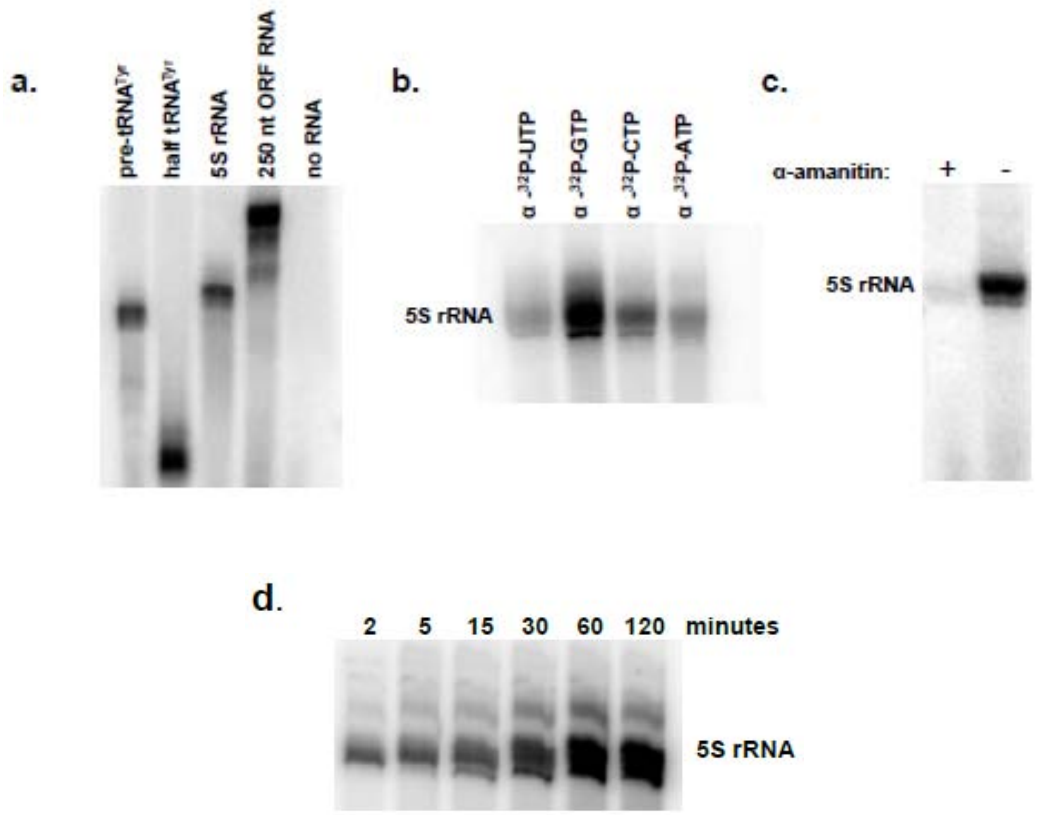


Figure 4.4. Template-independent labeling of RNAs by Pol II. A. Pol II was incubated for 15 minutes at 30°C with 1 µg each of pre-tRNA^{Tyr}, half tRNA^{Tyr}, 5S rRNA, and the 250 nt ORF RNA. α-^{32}P-UTP was added to the Pol II-RNA complexes, with no DNA present in the reactions, and reactions proceeded for 30 minutes at 30°C. Samples were analyzed on a 10% denaturing gel. All RNAs tested get radiolabel added in the absence of template DNA. B. All 4 α-^{32}P-labeled NTPs add label on to the RNAs (labeled 5S rRNA shown here). C. Treatment with the Pol II-specific inhibitor α-amanitin prior to addition of RNA abolishes this labeling activity (5S rRNA shown here). D. Time course of Pol II labeling 5S rRNA. Labeling reactions were stopped at the times indicated by heating samples to 95°C for 5 minutes. More label is added with time, but the labeled species do not get larger with time.

Chapter V

Conclusions and Future Directions

It has been fifty years since Jacob and Monot described operons in *E. coli*. Much has since been revealed about genomic organization, yet the study of how eukaryotic genomes are packaged and arranged in three dimensions is still a relatively new field. The work presented here addressed several questions concerning the spatial organization of tRNA genes in yeast. The results of this study continue our understanding of how this large family of co-regulated genes is organized and how this organization is coordinated with function. Still, the findings presented here, while answering some questions, leave many others unanswered and raise some interesting new ones.

We have known for nearly a decade that tRNA genes in yeast cluster and co-localize to the nucleolus. This phenomenon was shown at the time by designing oligonucleotide probes to tRNA genes that are present in multiple copies and visualizing clusters of these families. Interactions between the individual tRNA genes, and interactions between a single tRNA gene and the rDNA repeats, were at the time not discernible due to the low resolution of microscopy and the small size of yeast nuclei. In Chapter II we employed newer technologies to investigate the interaction of individual tRNA genes. Since all tRNA genes bind to the same protein complexes (TFIIIC, TFIIIB, and condensin), it was compelling to think that the identity of the tRNA gene would not matter in its positioning at the rDNA repeats. However, results of this work indicate otherwise, that the identity of the gene does indeed affect binding partner selection. In particular, it affects where exactly along the rDNA repeats the interaction occurs, but also appears to strongly affect many non-tRNA gene binding partners of tRNA genes (e.g., *SRB2* and *MED6*). This confers a level of complexity to organization of tRNA genes previously unsuspected.

However, contrary to the idea that tRNA genes might be global drivers of genome organization, our results indicate that deletion of a tRNA gene does not specifically dissociate the genomic locus away from its site. This result does not necessarily mean that tRNA genes are not strong localization signals to the nucleolus, because it is possible that other signals are stronger in retaining the locus there. One way to test the relative strength or importance of tRNA gene localization signals would be to place adjacent to a tRNA gene a known strong localization signal, for example, one of the "DNA zip codes" that direct regions of the genome to the nuclear pore complex (see Chapter I). These localization signals can be activated under nutrient conditions and, provided that signal is stronger than the imperative for tRNA genes to remain associated, the hypothesis is that the tRNA gene adjacent to the signal would now get localized to the nuclear pore rather than remaining at the nucleolus.

The GCC and/or 4C experiments can also be repeated in strains with condensin mutations, since it has been shown in both budding yeast and in fission yeast that condensin mutants have defects in tRNA gene organization. While the results of this experiment would mostly be simple confirmation of the microscopy showing dissociation of tRNA gene clusters in condensin mutants (Haeusler et al., 2008), it might reveal unexpected tRNA gene localization patterns in the absence of a proper mechanism for compaction. The microscopy shows that the clusters disperse, but the limits of the microscopy prevent visualizing the dispersal pattern of the tRNA genes. The temperature-sensitive mutants of condensin used in microscopy were viable at permissive temperatures, suggesting that nucleolar localization of tRNA genes is not essential for growth. As discussed in Chapter II, it likely provides for efficiency in transcription and processing of tRNAs to co-localize them, along with potential co-regulation with Pol I transcription of the 35S rRNA, even if these functions are not essential.

The experiments in Chapter III demonstrate an aspect of the mechanism of tRNA gene organization that had not previously been tested, that the Pol III transcription factor TFIIIC provides the specificity needed for condensin to recognize a tRNA gene. Very little is still known about the structure and mechanism of the condensin complex. The Smc2/4 coiled-coil heterodimer of condensin has been shown to indiscriminately coat DNA (Stray et al., 2005); in our system, aggregation of DNA by purified condensin was

observed in the absence of TFIIIC bound to a tRNA gene. Therefore, condensin appears to be able to bind DNA nonspecifically, unless specificity is provided. In the course of the experiments determining TFIIIC-mediated condensin binding, we consistently noticed incomplete occupancy of condensin on a TFIIIC-tRNA gene complex (Figure 3.1). It is possible that simply the conditions of the electrophoresis destabilize this large complex (over 1 megadalton in total size) to such an extent that only a small amount of the condensin remains bound to the DNA. However, it is also possible that binding of condensin to the TFIIIC-tRNA gene is inefficient in our in vitro system without other cellular factors. It has been shown that the cohesin loader Scc2/4 co-localizes with condensin binding in vivo (D'Ambrosio et al., 2008). One could investigate whether the cohesin loader is additionally needed for efficient loading of condensin onto the tRNA gene complex by repeating our experiments with purified or partially purified Scc2/4.

Direct visualization of tRNA gene-condensin complexes would greatly advance our understanding of the mechanism of tRNA gene clustering and of general condensin function. Although electron microscopy experiments so far have been unsuccessful at visualizing condensin complexes on DNA with TFIIIC, were this protocol to be optimized, there are additional experiments to perform in order to understand the mechanism of how condensin brings two pieces of DNA together. Ideally one would like to be able to visualize condensins bound to two regions of DNA and bringing them together. One way to do this would be to design a DNA fragment containing two tRNA genes, with sufficient length of DNA between them so that one could observe whether intramolecular looping can be caused by condensin-condensin interaction, or whether additional components would be necessary to mimic tRNA gene “clustering” in the reconstituted system. We also do not know whether condensin binding to a tRNA gene complex is directional. The looping experiment would be done with the tRNA genes either in the same or in opposite orientation to see whether that affects condensin binding and/or condensin-condensin interactions.

While TFIIIC is the recognition factor for condensin, in vivo interaction data indicate that a complex consisting of TFIIIC, TFIIIB, and condensin associates together at tRNA genes, and remains intact even when the associated DNA is digested (Haeusler et al., 2008). While initiation of Pol III transcription is dependent simply on the proper

loading of TFIIIC and TFIIIB, it is becoming clear that the presence of other protein complexes is important for other cellular imperatives, such as maintaining genome structure. Additionally, the finding of proteins other than Pol III transcription factors at tRNA genes, such as condensin and Ctf18 (see Appendix A), suggests other proteins yet to be identified may also be at tRNA genes. A technique called proteomics of isolated chromatin segments (PICh) has been developed to allow one to purify proteins from genomic regions of interest and then to identify these proteins by mass spectrometry (Dejardin and Kingston, 2009). Although it would not be possible to isolate individual tRNA genes due to their repetitive nature, because the technique employs hybridization of oligonucleotide probes of 20-25 nt, this is of sufficient length to capture families of tRNA genes. Using this technique, one could rather readily identify any other protein complexes associated with tRNA genes.

Assembled Pol III transcription factors and nucleolar localization of tRNA genes are required for silencing of Pol II genes adjacent to tRNA genes (tgm silencing). The work in Chapter IV initially started as an investigation of the tgm silencing phenomenon, with the hypothesis that the nascent pre-tRNA transcripts inhibit nearby Pol II. The idea was that there is something intrinsic about the structure of the tRNA that allows it to specifically recognize Pol II, bind to it, and prevent transcription. This would have been consistent with data that certain mammalian SINE RNAs directly bind to and inhibit Pol II (mouse B2 RNA, e.g.), but other SINE RNAs are incapable of this activity (mouse B1 RNA) (Mariner et al., 2008). Contrary to this hypothesis, though, the results presented in Chapter IV indicate that not only is the binding and inhibition not specific to tRNAs, but that the tight structure of tRNAs actually makes them less potent inhibitors than fully unstructured RNAs. This does not rule out local Pol II inhibition by pre-tRNA transcripts, particularly because the nucleolar clustering of tRNA genes required for tgm silencing (Wang et al., 2005) would provide locally high concentration of nascent pre-tRNA transcripts needed for strong silencing of Pol II at the nucleolus. Pre-tRNAs might additionally possess a means of mediating its direct interaction with Pol II that is not available to other RNAs in the nucleus. The tRNA modifiying enzyme Mod5 has been shown to interact in vivo with the tRNA gene complex, and deletions of *MOD5* alleviate tgm silencing (M. Pratt-Hyatt, unpublished data). While the role of Mod5 in the cytosol is

understood, the function of Mod5 at tRNA genes is unclear; it could bind to nascent transcripts and chaperone them to Pol II. In vitro binding assays to determine the ability of tRNA to directly bind Mod5 will be needed to address this question. Were Mod5 shown to not possess this ability, the PICh experiments proposed above might reveal additional tRNA binding proteins that are present at the site of transcription.

Inhibition of Pol II is dependent upon the RNA being able to gain access to the enzyme prior to template engagement, consistent with the mechanism of 6S RNA inhibiting bacterial RNA polymerase by preventing template binding (Wassarman and Saecker, 2006). In contrast to that study, in our Pol II inhibition experiments, the least structured RNAs that we tested were the best inhibitors, suggesting that rather than needing an evolved inhibitory structure, "floppiness" of RNAs might facilitate the contacts in the polymerase required for inhibition of template. Based upon this reasoning, our data suggest that multiple contacts are being made between the RNA and the polymerase. To test this further, one could crosslink either chemically or using ultraviolet light a pre-bound Pol II-RNA complex and then identify the contact positions on the RNA by primer extension.

The finding that RNAs can directly inhibit polymerase defines an interesting problem for cells: how to eliminate the possibility of nascent transcripts binding either to a nearby polymerase, or perhaps even binding to the very polymerase that is synthesizing it, once the template is released. Pol II-transcribed mRNA is co-transcriptionally packaged into ribonucleoprotein complexes, thus providing a way to prevent Pol II from being inhibited by its own transcripts. Furthermore, spatial organization of tRNA genes at the nucleolus would serve to keep centers of Pol II transcription away from the high levels of synthesis both of tRNA genes and of rRNA genes. In this way, clustering of tRNA genes would not only confer the benefit of coordination of their own transcription and processing, but also would benefit Pol II genes by sequestering naked transcripts away from those sites.

The goal of this work was to examine the mechanism and consequences of spatial organization of tRNA genes in yeast. We have shown that the identity of tRNA genes matters in binding partner selection but that other nuclear signals seem to be stronger than tRNA gene localization in driving overall genome arrangement. In addition, we have

shown that these gene interactions are specified by condensin recognizing TFIIIC bound to the tRNA gene. Finally, we have shown that RNAs directly inhibit Pol II transcription, consistent with clustering of tRNA genes at the nucleolus segregating these highly transcribed RNAs away from Pol II genes.

References

D'Ambrosio, C., Schmidt, C.K., Katou, Y., Kelly, G., Itoh, T., Shirahige, K., and Uhlmann, F. (2008). Identification of cis-acting sites for condensin loading onto budding yeast chromosomes. Genes & development *22*, 2215-2227.

Dejardin, J., and Kingston, R.E. (2009). Purification of proteins associated with specific genomic Loci. Cell *136*, 175-186.

Haeusler, R.A., Pratt-Hyatt, M., Good, P.D., Gipson, T.A., and Engelke, D.R. (2008). Clustering of yeast tRNA genes is mediated by specific association of condensin with tRNA gene transcription complexes. Genes & development *22*, 2204-2214.

Mariner, P.D., Walters, R.D., Espinoza, C.A., Drullinger, L.F., Wagner, S.D., Kugel, J.F., and Goodrich, J.A. (2008). Human Alu RNA is a modular transacting repressor of mRNA transcription during heat shock. Mol Cell *29*, 499-509.

Stray, J.E., Crisona, N.J., Belotserkovskii, B.P., Lindsley, J.E., and Cozzarelli, N.R. (2005). The Saccharomyces cerevisiae Smc2/4 condensin compacts DNA into (+) chiral structures without net supercoiling. The Journal of biological chemistry *280*, 34723-34734.

Wang, L., Haeusler, R.A., Good, P.D., Thompson, M., Nagar, S., and Engelke, D.R. (2005). Silencing near tRNA genes requires nucleolar localization. J Biol Chem *280*, 8637-8639.

Wassarman, K.M., and Saecker, R.M. (2006). Synthesis-mediated release of a small RNA inhibitor of RNA polymerase. Science (New York, NY *314*, 1601-1603.

Appendix A

Genes tested for alleviation of tgm silencing

Rationale

tRNA gene-mediated (tgm) silencing occurs when expression of a Pol II gene next to a tRNA gene is silenced. Many genes have been previously tested for the ability to alleviate tgm silencing when the gene is either deleted or, for essential genes, replaced with a conditional mutation. In this work a number of previously untested genes were tested for alleviation of tgm silencing, using either a deletion strain or a temperature-sensitive mutant strain. In the case of one result (*CTF18*), a positive result in this assay was used to do further studies (see Appendix B).

Results

This table shows results of the tgm silencing screen for a number of yeast genes:

Gene	**Allele tested**	**Gene product description**	**Alleviates tgm silencing?**
SMC1	*smc1-3*	Essential subunit of the cohesin complex	no
SMC3	*smc3-2*	Essential subunit of the cohesin complex	no
MCD1	*mcd1-1*	Essential subunit of the cohesin complex	no
PDS5	*pds5-3*	Co-localizes with the cohesin complex	no
SMC5	*smc5-6*	Essential subunit of the Smc5/6 complex	no
SMC6	*smc6-9*	Essential subunit of the Smc5/6 complex	no
CTF4	*ctf4Δ*	Chromatin-associated protein required for sister chromatid cohesion	no
CTF18	*ctf18Δ*	Subunit of the Ctf18-Replication Factor C (RFC) complex	yes
CTF8	*ctf8Δ*	Subunit of the Ctf18-RFC complex	no
DCC1	*dcc1Δ*	Subunit of the Ctf18-RFC complex	yes (weak)

RAD24	*rad24Δ*	Subunit of the Rad24-RFC clamp loader complex, may have redundant function with Ctf18-RFC	no
ELG1	*elg1Δ*	Subunit of the alternative Elg1-RFC complex	no
TOF2	*tof2Δ*	Required for rDNA silencing and mitotic rDNA condensation. Rationale for testing, along with next two, is from (Johzuka and Horiuchi, 2009)	no
CSM1	*csm1Δ*	Nucleolar protein required for condensin recruitment to replication fork barrier site	no
LRS4	*lrs4Δ*	Forms complex with Csm1	no
ULS1	*uls1Δ*	Involved in sumoylation; antagonizes silencing during mating-type switching	yes

Materials and Methods

Yeast strains. The *smc1-3* and *smc3-2* alleles were provided by Rohinton Kamakaka of University of California Santa Cruz; *mcd1-1* and *pds5-3* alleles were provided by Doug Koshland of University of California Berkeley; *smc5-6* and *smc6-9* alleles were provided by Luis Aragon of the Medical Research Council (UK); all deletion strains come from the yeast deletion library (Open Biosystems).

tgm silencing assay. Plasmid reporter assays to test for tgm silencing are as described (Hull et al., 1994; Wang et al., 2005).

Appendix B

Ctf18 is present at tRNA genes

Rationale

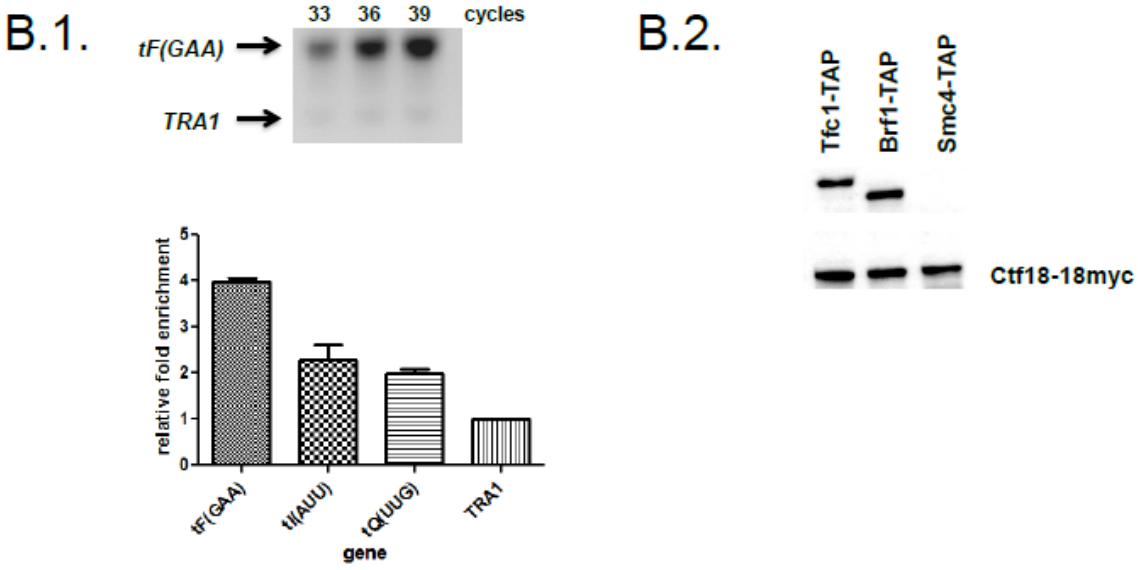
Ctf18 is a subunit (along with Ctf8 and Dcc1) of the Ctf18-Replication factor complex (Ctf18-RFC), one of three alternate clamp loaders that load proliferating cell nuclear antigen onto unwinding DNA during replication (Rad24-RFC and Elg1-RFC being the other two). Defects in Ctf18 function have numerous defects in genome stability, particularly in the DNA replication checkpoint. In budding yeast tRNA genes have been demonstrated to transiently pause replication forks. Therefore, we hypothesized that certain protein complexes involved in replication, particularly those involved in the early stages, may be present at tRNA genes. Based on results of tgm silencing screen (see Appendix A), where *ctf18Δ* strongly alleviated silencing, and *dcc1Δ* weakly alleviated silencing, we decided to test for the presence of Ctf18 at tRNA genes.

Results

To test whether Ctf18 is preferentially bound to tRNA genes, we performed chromatin immunoprecipitations with myc-tagged Ctf18. Ctf18-associated chromatin was used in semi-quantitative PCRs to detect the presence of several tRNA genes relative to a non-tRNA gene control (Figure B.1). Results indicate four-fold enrichment of the $tRNA^{Phe}$ gene *tF(GAA)*, and slightly lower yet still significant levels of enrichment of the $tRNA^{Ile}$ gene *tI(AUU)* and the $tRNA^{Gln}$ gene *tQ(UUG)*.

Condensin was shown to both bind to tRNA genes by ChIP and to associate with TFIIIC and TFIIIB by co-IP. We therefore performed co-IP experiments to determine

association of Ctf18 with TFIIIC, TFIIIB, and condensin. Myc-tagged Ctf18 was immunoprecipitated from cell extracts and blotted to detect the presence of the TAP tag on Tfc1, Brf1, or Smc4, respectively. Tfc1 and Brf1 both associate with Ctf18, yet curiously we did not see association with Smc4 (Figure B.2). It is possible that while both Ctf18 and condensin associate with TFIIIC and TFIIIB, they associate in different places so that they do not associate with each other. An alternative hypothesis is that they both transiently associate and so their association with each other is at too low of a level to be detected. Further work is necessary to investigate these interactions.



Materials and Methods

Yeast strains. *S. cerevisiae* strain yDP120 was derived from BY4741 (*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). PCR fragments generated from plasmid template (Knop et al., 1999) contained 18 tandemly repeated copies of the myc gene (18x myc) fused to a LEU selection marker. PCR fragments were recombined exactly at the 3' end of the *CTF18* coding sequence, eliminating the stop codon to allow the 18x myc gene to be expressed from the promoter of *CTF18*. Transformants were selected on medium lacking leucine,

and presence of the myc tag was verified by PCR and sequencing to check for proper integration and by Western Blot to check for expression of the tagged protein.

Strains yDP126, yDP128, and yDP130 were derived from *TFC1*-TAP, *BRF1*-TAP, and *SMC4*-TAP, respectively (Open Biosystems). CTF18 was tagged in these strains exactly as above with the 18x myc gene.

Chromatin immunoprecipitation assay (ChIP). Cell prep was done essentially as described (Ren et al., 2000) with the following modifications. 100 mL of yDP120 were grown in YPD with 2% glucose to mid-logarithmic phase at 30°C. Cells were crosslinked by adding paraformaldehyde (Electron Microscopy Sciences) to 1% final for 30 minutes at 25°C and then quenched in 125 mM glycine for 10 minutes at 25°C. Glass bead lysis was done for 4 minutes in a FastPrep 24 (MP Biomedicals). After separating cell suspension away from the glass beads, sonication was done with a Branson Sonifier 250, 10 times for 10 seconds each at Hold and Constant power setting, yielding 30% output; this allowed DNA to be sheared to an average size of 700 bp, the majority of it being no greater than 1200 bp. Samples were centrifuged and the supernatant was added to Magnabind Protein A magnetic beads (Thermo Scientific) that had been pre-incubated overnight with myc antibody (1 µg total antibody added per immunoprecipitation; 9E10, Santa Cruz). Complexes were allowed to bind for four hours. Samples were washed as described and eluted in elution buffer for 15 mins at 65°C with frequent mixing. Crosslinks were reversed overnight at 65°C, and samples were purified and precipitated as described and resuspended in 100 µL water. 1 uL of this was used per PCR reaction.

Semi-quantitative PCR analysis was done in 50 µL volumes with the following tRNA gene-specific primers: for the $tRNA^{Gln}$ gene *tQ(UUG)*, 5'-GTGGTTATCACTTTCGGTTTTGATCC-3' and 5'-GAAAGCGGGTGTTTCTCCAATAAAT-3'; for the $tRNA^{Ile}$ gene *tI(AUU)*, 5'-GCGCTTCCACCACTTAGTATGATTC-3' and 5'-TTATTAGCACGGTGCCTTAACCAACT-3'; and for the $tRNA^{Phe}$ gene *tF(GAA)* as described (Haeusler et al., 2008). To the *TRA1* gene, the primers 5'-CCAATTTTTGATAAGCCACCCTAG-3' and 5'-CGTAATTTCTAAGGTCTTGTTCTCCCA-3' were positioned at least 5 kb away from

any promoter region, repetitive sequence, or autonomous replicating sequence. PCR conditions were as described (Haeusler et al., 2008) for the indicated number of cycles.

Co-immunoprecipitation assay (co-IP). 50 mL each of yDP126, yDP128, and yDP130 were grown in YPD with 2% glucose to mid-logarithmic phase at 30°C (without crosslinking). Extract prep and immunoprecipitation were done as for the ChIP assay. Beads were washed four times in ChIP lysis buffer and once in TE, and eluted in ChIP elution buffer as above. Eluted samples were assayed by Western blot to detect presence of either the myc tag (1:400 dilution of the 9E10 antibody) or of the TAP tag (1:2000 dilution of TAP antibody; A00683, GenScript).

Appendix C

Smc5/6 does not associate strongly with condensin in vivo

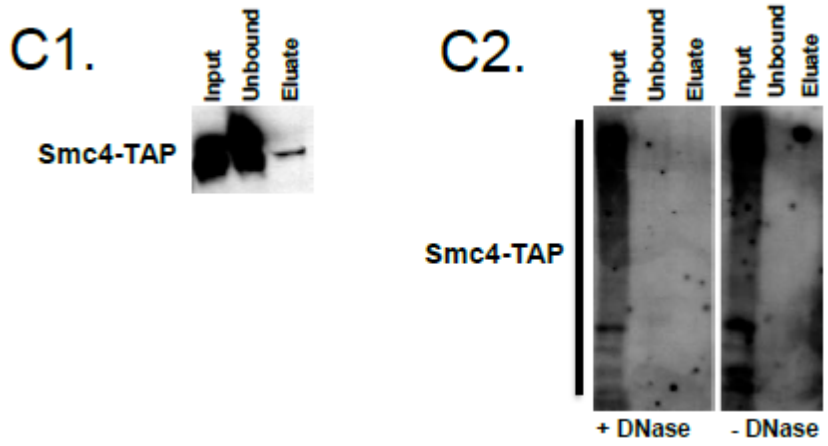
Rationale

The three members of the structural maintenance of chromosomes (SMC) family of protein complexes (condensin, cohesin, and Smc5/6) are evolutionarily conserved throughout eukaryotes. The condensin complex is responsible, outside of its well-studied roles in mitosis and meiosis, for maintaining proper compaction of the tandemly repeated ribosomal DNA (rDNA) cluster during interphase. Similarly, it has also been shown that the Smc5/6 complex is required for maintaining rDNA integrity during interphase. Since condensin and Smc5/6 are both present at the rDNA locus, we hypothesized that the Smc5/6 complex might be present along with condensin at or near tRNA genes as well.

Results

To test for the presence of the Smc5/6 complex at tRNA gene transcription complexes, we performed co-IP assays to condensin. Myc-tagged Smc5 was immunoprecipitated from cell extracts and blotted to detect the presence of the TAP tag on Smc4 (Figure C.1). There is no clear association between Smc5 and Smc4 (the faint band seen in the elution lane is likely due to spill-over from the neighboring well; this is confirmed by the results of Figure C.2). This experiment was repeated by crosslinking growing cells before extract preparation, in order to stabilize potentially low-level interactions; no association was seen under crosslinked conditions either (Figure C.2). This does not, however, preclude the possibility that Smc5/6 is present at tRNA genes, since Ctf18 associated with TFIIIC

and TFIIIB but not with condensin. Co-IP assays between Smc5/6 and both TFIIIC and TFIIIB should be performed to verify these results.



Materials and Methods

Yeast strains. The *S. cerevisiae* strain yDP57 was derived from the *SMC4*-TAP strain (Open Biosystems). PCR fragments generated from a plasmid template (Longtine et al., 1998) contained 13 tandemly repeated copies of the myc gene (13x myc) fused to the *kanMX6* expression cassette. PCR fragments were recombined exactly at the 3' end of the coding sequence of *SMC4*, removing the stop codon, so the 13x myc gene is expressed from the promoter of *SMC4*. Transformation and screening of transformants were performed essentially as in Appendix B except selection was done on medium containing G418.

Co-immunoprecipitation assay. 50 mL of yDP57 were grown in YPD with 2% glucose to mid-logarithmic phase. Where crosslinking was tested, it was done as for the ChIP assay in Appendix B. Cells were harvested and samples were immunoprecipitated with the myc antibody as in Appendix B. Where DNase treatment was tested, extracts were treated with 50 units of DNase I (Sigma-Aldrich) for 30 minutes at 4°C prior to IP. Samples were assayed by Western blot to detect presence of the TAP tag (1:2000 dilution of TAP antibody; A00683, GenScript).

Appendix D

RNase P is not directly bound to tRNA genes

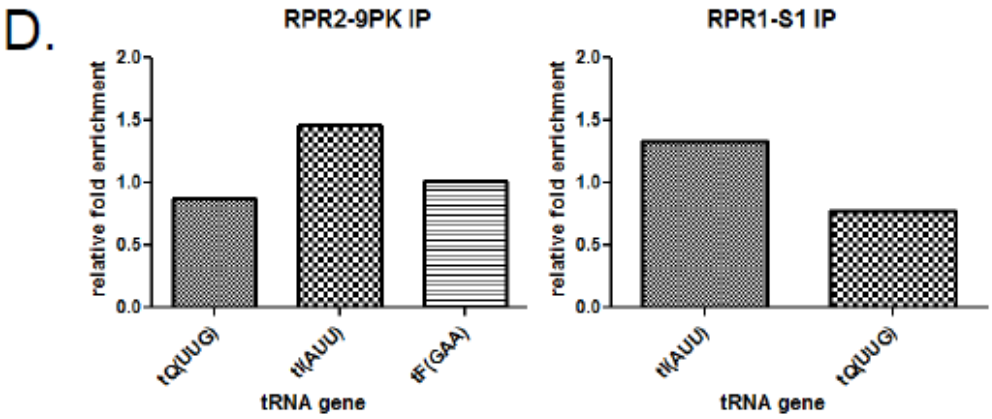
Rationale

Ribonuclease P (RNase P) is a multi-subunit ribozyme that catalyzes the cleavage of the 5' leader sequence of precursor tRNAs. This activity is thought to occur either co-transcriptionally or very soon after transcription, and the enzyme has been localized in vivo to the nucleolus coincident with the clusters of tRNA genes. Co-localization of the enzyme at the site of transcription allows for efficiency in the process, since all tRNA genes are transcribed with a 5' leader that needs to be cleaved. Direct physical association of the enzyme to the site of transcription could help in this spatial coordination. Therefore, it was hypothesized that RNase P might be bound directly at or near tRNA genes in order to facilitate efficient activity.

Results

We performed ChIP assays to determine association of Pk-tagged Rpr2 (the only unique subunit of RNase P that is not also shared with the mitochondrial RNase MRP). Rpr2-associated chromatin was used in semi-quantitative PCRs to detect the presence of several tRNA genes relative to a non-tRNA gene control (Figure D). While the experiments were not repeated to give quantitative data, initial results do not show enrichment of Rpr2 at tRNA genes above the control (1.4-fold enrichment maximum with the $tRNA^{Ile}$ gene). These experiments were also repeated by performing the immunoprecipitations using streptavidin binding to the S1 RNA aptamer tag to RPR1, the

RNA subunit of RNase P; no enrichment of the two tRNA genes tested was detected with the RPR1 IPs either. We conclude that RNase P is not strongly bound to tRNA genes.



Materials and Methods

Yeast strains. RPR2-9Pk strain was derived from W303-1a (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*). PCR fragments generated from plasmid template (Knop et al., 1999) contained 9 copies of the tandemly repeated Pk tag derived from the paramyxovirus Simian Virus 5 (Southern et al., 1991) conjugated to a TRP selection marker. Transformation and screening of transformants were performed essentially as in Appendix B except they were selected on medium lacking tryptophan. The RPR1-S1 strain is as described (Srisawat and Engelke, 2001).

ChIP Assay. ChIP assays were performed with the Pk tag essentially as in Appendix B. 5 µg of the Pk antibody (abcam) were added per immunoprecipitation. For the S1 tag, streptavidin-conjugated beads were pre-blocked with egg white avidin to remove nonspecific binding, incubated with extract and washed as above, and eluted with 5 mM biotin as described (Srisawat and Engelke, 2001). For the PCRs, the same primers to the tRNA genes were used as in Appendix B, but for the baseline control, primers were designed to the *ATG22* gene (Haeusler et al., 2008), positioned at least 2 kb away from any tRNA genes or other repetitive sequences.

References

Haeusler, R.A., Pratt-Hyatt, M., Good, P.D., Gipson, T.A., and Engelke, D.R. (2008). Clustering of yeast tRNA genes is mediated by specific association of condensin with tRNA gene transcription complexes. Genes Dev *22*, 2204-2214.

Hull, M.W., Erickson, J., Johnston, M., and Engelke, D.R. (1994). tRNA genes as transcriptional repressor elements. Mol Cell Biol *14*, 1266-1277.

Johzuka, K., and Horiuchi, T. (2009). The cis element and factors required for condensin recruitment to chromosomes. Molecular cell *34*, 26-35.

Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K., and Schiebel, E. (1999). Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast (Chichester, England) *15*, 963-972.

Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast (Chichester, England) *14*, 953-961.

Ren, B., Robert, F., Wyrick, J.J., Aparicio, O., Jennings, E.G., Simon, I., Zeitlinger, J., Schreiber, J., Hannett, N., Kanin, E., *et al.* (2000). Genome-wide location and function of DNA binding proteins. Science (New York, NY *290*, 2306-2309.

Southern, J.A., Young, D.F., Heaney, F., Baumgartner, W.K., and Randall, R.E. (1991). Identification of an epitope on the P and V proteins of simian virus 5 that distinguishes between two isolates with different biological characteristics. The Journal of general virology *72 (Pt 7)*, 1551-1557.

Srisawat, C., and Engelke, D.R. (2001). Streptavidin aptamers: affinity tags for the study of RNAs and ribonucleoproteins. RNA (New York, NY *7*, 632-641.

Wang, L., Haeusler, R.A., Good, P.D., Thompson, M., Nagar, S., and Engelke, D.R. (2005). Silencing near tRNA genes requires nucleolar localization. J Biol Chem *280*, 8637-8639.