Investigating the Role of RNA Polymerase II in RNAi-dependent Heterochromatin Assembly at Centromeric Repeats

Abstract

In *Schizosaccharomyces pombe*, a fission yeast, large domains of heterochromatin are found at telomeres, silent mating-type loci, and centromeric repeat regions of DNA (Bühler and Moazed, 2007). Much of the work done with *S. pombe* has shown that the assembly of heterochromatin around centromeric repeats depends on the coordination of two pathways: RNAi and histone modification. Current models suggest that initiation of heterochromatin formation begins with transcription of centromeric RNA by RNA polymerase II. These RNA transcripts are converted to dsRNA by RNA-dependent polymerase, cleaved to siRNA’s by the Dicer protein, and that it is these siRNA’s which are responsible for directing an RNA-induced transcriptional silencing (RITS) complex back to nascent centromeric RNA transcripts, from which it can recruit histone modification machinery (Grewal and Jia, 2007). It is not completely understood how targeting via siRNA is mediated, though mutations in several subunits of RNA polymerase II (RNAPII) result in phenotypes that mimic complete loss of the RNAi pathway and lead to a loss of heterochromatin formation. Our intention is to investigate RNAPII’s interaction with the RNAi proteins, and the status of siRNA binding of the RITS complex in RNAPII mutants, to generate a more complete understanding of RNAPII’s role in the RNAi silencing pathway.

Introduction

Chromatin states are an important part of gene regulation. In order to be packaged on chromosomes, DNA is wound about histones and this DNA/histone structure is called chromatin, of which there are two kinds. In eukaryotes, euchromatin is associated with transcriptionally
active regions of the genome, whereas heterochromatin is more compacted, dense, and correlates with silenced genes. For this reason, proper formation of heterochromatin in the appropriate areas is critical for normal regulation of gene expression.

A hallmark of heterochromatin is the presence of certain histone modifications, particularly H3K9 methylation, and methylation of associated DNA (Grewal and Jia, 2007). These two characteristics are connected: it is thought that certain histone modifications form epigenetic marks that can serve as targets for other proteins (DNA methyltransferases, for example) that either contribute to heterochromatin formation or methylate DNA and lead to silenced expression states (reviewed by Richards and Elgin, 2002). Because these modifications occur after DNA has been transcribed and histones translated, and does not change the sequence of DNA, particular attention has been focused on how this form of regulation can be maintained over time and in daughter cells after replication.

RNAi has been studied extensively in fission yeast and other organisms as one method of getting heterochromatin formation started. In *S. pombe*, heterochromatin forms around centromeric repeat regions, and the establishment of this heterochromatin is (paradoxically) dependent on transcription of RNA from centromeric repeats (Bühler and Moazed, 2007). This cenRNA is converted to dsRNA by RNA-dependent polymerase (Rdp1), and then processed to siRNA’s by Dicer protein (Dcr1) and an RNA-directed RNA polymerase Complex (RDRC, which also contains Rdp1). These siRNA’s bind an RNA-induced transcriptional silencing complex (RITS) via the Ago1 subunit, and presumably direct it to a region of the genome to silence via complementary base pairing with DNA or nascent RNA transcripts. The RITS complex can then recruit the Clr4-containing (CLRC) complex (Clr4 is a histone
methyltransferase that catalyzes addition of the H3K9 methyl mark), which leads to histone modification and ultimately gene silencing (Bühler and Moazed, 2007).

Much has been learned about this mechanism. Not surprisingly, deletion mutants for parts of the histone-modifying CLRC complex fail to exhibit H3K9me and show a lack of heterochromatin (Bayne, et al. 2010). Similar experiments with RNAi proteins, like Dcr1, show the RNAi components are necessary for establishment of heterochromatin but not for its maintenance. It is known that tethering either the CLRC or RITS complexes (the latter is also dependent on functional Dcr1) to a region of DNA will promote heterochromatin formation in that region, suggesting that normally the critical steps in the RNAi pathway are generation of siRNA’s and the targeting of RITS by those siRNA’s to genomic regions for silencing (Cam, et al., 2009). These steps are not understood, and the interest of our proposal lies in elucidating RNAPII’s contribution in light of 3 mutations in subunits of the polymerase that compromise RNAi in different ways.

RNAPII in *S. pombe* is a large protein, consisting of 12 different subunits (Spåhr, et al. 2009). There is a high degree of conservation in the subunits of RNAPII between *S. pombe* and humans, making yeast a good model organism in which to study its function. Mutations in the Rpb1, Rpb2, and Rpb7 subunits result in loss of heterochromatin formation around centromeric repeats. In Rpb7 mutants, transcription of RNA from the centromeric repeats is reduced, and no siRNA’s are detected (Djupedal, et al. 2005). This may simply indicate that a particular subunit is necessary for transcription from these repeat areas. However, Rpb2 mutants transcribe from the repeats normally, but do not produce siRNA’s (Kato, et al. 2005). And, perhaps most bizarrely, partial deletion of the Rpb1 subunit (a carboxyl terminal domain, CTD) of RNAPII results in a phenotype where centromeric RNA’s are transcribed and processed to siRNA’s, but
with no formation of heterochromatin (much like you might see in a Dcr1 mutant, where RNAi is lost completely) (Schramke, et al. 2005). Most importantly, all of these mutations were accompanied with normal transcription of other genes throughout the genome, demonstrating that the phenotypes are not due to loss of transcriptional function in RNA polymerase; rather, they imply that RNAPII plays some role in siRNA formation and targeting of RITS, aside from simple generation of cenRNA to used as a template for siRNA’s.

We propose experiments to test two hypotheses. The first is that RNAPII acts as a scaffold necessary for RNAi proteins to be able to generate siRNA’s. The second is that RNAPII interacts with RITS and Dcr1 in a way that allows siRNA to be attached to Ago1 and direct the RITS complex to nascent centromeric RNA’s. For these experiments, we intend to use WT strains of *S. pombe*, as well as strains containing mutations in the Rpb1, Rpb2, and Rpb7 subunits of RNAPII.

**Specific Aims and Experimental Design**

**Specific Aim #1**: Establish whether RNAPII associates with RNAi components.

**Experiment 1**: Coimmunoprecipitation assay

To perform Co-IP experiments with subunits of RNAPII, we will express FLAG-tagged versions of Rpb1, Rpb2, and Rpb7 in *S. pombe* via electroporation of constructs containing each gene with a selectable marker (hygromycin B, for example). Clones will be isolated on selective media, followed by PCR and Western blot analysis to verify successful integration and expression of the transgenes. For the Co-IP, antibodies to the FLAG-tag will be administered to cell lysates from *S. pombe* expressing either the Rpb1, Rpb2, or Rpb7 transgenes to pull down these parts of RNAPII. Western blot analysis will be used to check for association with RNAi
proteins, using antibodies against Dcr1, Ago1, Rdp1, and Clr 4 as a control. This procedure will also be used to assess binding of RNAi proteins in RNAPII mutants, by expressing FLAG-tagged mutant versions of the 3 RNAPII subunits of interest.

**Expected Results:** Because of the problems with siRNA generation and targeting in the Rpb1 and Rpb2 mutants, we expect to see an interaction between these subunits and Dcr1, Ago1 or Rdp1 under normal conditions, but not in the presence of mutations. The phenotype of the Rpb7 mutant is a lack of transcription of cenRNA, and this may or may not be due to a change in binding affinity for RNAi proteins, therefore a prediction cannot be made with regard to this RNAPII subunit.

**Experiment 2:** Screen for second-site suppression mutants

A screen for second-site suppression mutants will be conducted by introducing a *ura3* negative selection marker into the centromeric repeat region of a yeast strain with a deletion of *ura3* via electroporation and homologous recombination (confirm via PCR). Mutations for Rpb1, Rpb2, and Rpb7 will then be added to this background. Yeast will then be grown on 5FOA growth medium for negative selection. Sequencing of positive colonies will be done for Dcr1, Rdp1, Ago1, Rik1, and Clr4(control) to see if any of the surviving colonies have new mutations in any of these proteins.

**Expected Result:** Exposure to 5FOA will kill any yeast expressing the *ura3* gene. This will only be happening in yeast that fail to assemble heterochromatin in the centromeric repeat region (the yeast with mutations in RNAPII subunits, for example). If there is an interaction between any of the RNAi proteins and RNAPII, it is possible that one of them could develop a complementary mutation that restores function to the RNAi pathway, and indicate a direct interaction. A problem with this experiment is that a negative result won’t rule out direct
interactions, and could only mean that the proteins work together in a way that one of the mutations compromises too seriously to rescue.

Specific Aim #2: Determine if RNAPII and candidate RNAi proteins co-localize and interact in vivo.

Experiment 3: In vivo immunofluorescence assay.

This experiment will be run in two parts, both of which involve expression of fusion proteins in *S. pombe* that combine an RNAPII subunit of interest and an RNAi protein to be evaluated. In the first test, we will generate yeast that express Rpb1, Rpb2, and Rpb7 subunits fused to an eGFP reporter. Constructs with Dcr1, Ago1, and Rdp1 fused to mRFP will be electroporated into these cells. Both sets of constructs will contain selectable markers, and verification of integration will be done as before. Cells will then be visualized via confocal microscopy, using a DAPI stain to mark DNA and the green and red channels to label the RNAPII subunit and RNAi protein, respectively. The second test will use essentially the same method, however the constructs generated will fuse one half of a YFP reporter to each protein, and bimolecular fluorescence complementation will be used to gauge level of protein:protein interaction (method with appropriate controls described by Kerppola, 2006).

Expected Result: If any of the RNAi proteins interact with the Rpb1, Rpb2, or Rpb7 subunits of RNAPII, we expect to see strong colocalization of these elements in the cell in vivo. Similarly, we would expect to see strong reports from the YFP/BiFC reporters. The main problems in these tests are testing all the controls: making sure the reporters don’t interfere with normal function, interpreting when a signal actually means co-localization and not background, etc. But these experiments could provide good evidence of association under in vivo conditions.
**Experiment 4:** Yeast Two-hybrid assay.

In this experiment, we will generate yeast expressing Rpb1, Rpb2, and Rpb7 proteins fused to a Gal4 binding domain in combination with an RNAi protein (Dcr1, Ago1, or Rdp1) fused to a Gal4 activating domain through already-described methods. In addition, a *lacZ* reporter fused to a Gal4 binding sequence will be introduced to verify a successful interaction.

**Expected Result:** In conjunction with the above experiment, the presence of blue yeast colonies when grown on a medium containing X-gal will show if a direct interaction is occurring between the RNAPII subunits and one (or more) of the RNAi proteins tested.

**Specific Aim #3:** Determine if Rpb1 facilitates binding of RITS to siRNA.

**Experiment 5:** RNA-immunoprecipitation (RIP).

This experiment will be performed in WT yeast and the yeast with a mutation in Rdp1 (from experiment 1). Antibodies to Ago1 and Rdp1 (both in the RITS complex) will be used to pull down these proteins and any RNA associated with them. RT-PCR will be used to identify any RNA bound to the complex.

**Expected Results:** Because the Rpb1 mutant transcribes from centromeric repeats and produces detectable siRNA’s, I expect that the problem is in connecting the siRNA to RITS. Therefore, centromeric siRNA will not be detected in the RIP from Rpb1 mutant yeast, but will be present in WT.

**Discussion**

The RNAi pathway is critical for directing chromatin modification machinery to centromeric repeats. It depends on transcription of RNA from said repeats, followed by
formation of siRNA’s which can target RITS to nascent RNA transcripts. Mutations in subunits of the RNA polymerase II disrupt this portion of RNAi, and our experiments will shed more light on how RNAi proteins interact with RNAPII to facilitate RNAi-directed heterochromatin assembly.

References


