RNA Pol II subunit Rpb7 promotes centromeric transcription and RNAi-directed chromatin silencing

Ingela Djupedal,1,4 Manuela Portoso,2,4 Henrik Spåhr,3 Carolina Bonilla,1 Claes M. Gustafsson,2 Robin C. Allshire,2 and Karl Ekwall1,5

1Karolinska Institutet, Department of Biosciences/School of Life Sciences, University College Sodertorn, S-141 89 Huddinge, Sweden; 2Wellcome Trust Centre for Cell Biology, Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh EH9 3JR, United Kingdom; 3Karolinska Institutet, Department of Medical Nutrition, Novum, S-141 86 Huddinge, Sweden

Fission yeast centromeric repeats are transcribed into small interfering RNA (siRNA) precursors (pre-siRNAs), which are processed by Dicer to direct heterochromatin formation. Recently, Rpb1 and Rpb2 subunits of RNA polymerase II (RNA Pol II) were shown to mediate RNA interference (RNAi)-directed chromatin modification but did not affect pre-siRNA levels. Here we show that another Pol II subunit, Rpb7 has a specific role in pre-siRNA transcription. We define a centromeric pre-siRNA promoter from which initiation is exquisitely sensitive to the rpb7-G150D mutation. In contrast to other Pol II subunits, Rpb7 promotes pre-siRNA transcription required for RNAi-directed chromatin silencing.

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In RNA interference (RNAi), Dicer processes double-stranded RNA to produce short small interfering RNAs (siRNAs) that act to silence gene expression post-transcriptionally [Fire et al. 1998; Zamore et al. 2000; Bernstein et al. 2001]. RNAi also mediates transcriptional silencing via formation of heterochromatin. Centromeres in many organisms are composed of repetitive DNAs, which act as a substrate for the formation of heterochromatin (Cleveland et al. 2003). In fission yeast [Schizosaccharomyces pombe] genes inserted within this heterochromatin are transcriptionally silenced (Allshire et al. 1995). Paradoxically, the centromeric repeats themselves are transcribed to form long siRNA precursors (pre-siRNAs), which are required for the RNAi-mediated chromatin modifications resulting in transcriptional silencing [Provost et al. 2002; Volpe et al. 2002]. Defective RNAi leads to loss of silencing and defective heterochromatin formation in several organisms (Mochizuki et al. 2002; Pal-Bhadra et al. 2002; Zilberman et al. 2003) and transcripts homologous to centromere repeats have been detected in chicken cells as well as mice [Fukagawa et al. 2004; Kanellopoulou et al. 2005].

Interestingly, in plants, specialized RNA polymerase IV (RNA Pol IV) subunits have been implicated in RNAi-directed silencing (Herr et al. 2005; Onodera et al. 2005). Animal and fungal genomes lack such Pol IV subunits. Very recently, however, two subunits of RNA pol II (Rpb1 and Rpb2) were shown to have specific roles in chromatin modification and siRNA production, respectively, but did not affect pre-siRNA levels (Kato et al. 2005; Schramke et al. 2005).

Here, we have characterized the temperature sensitive csp3 [centromere: suppressor of position effect] mutant isolated in a screen for trans-acting mutants that are required for centromeric silencing [Ekwall et al. 1999]. csp3 is a G150D missense mutation in the rpb7 gene, a subunit of Pol II. We show that Rpb7 has a specific defect in centromeric pre-siRNA transcription. We define a centromeric pre-siRNA promoter from which initiation is exquisitely sensitive to the rpb7-G150D mutation. Thus, in contrast to the previously characterized silencing defective Pol II subunits, which affect siRNA production and/or downstream events, Rpb7 has a distinct role in generating centromeric pre-siRNAs needed for RNAi-directed chromatin silencing.

Results and Discussion

The csp3 gene was isolated by complementation of its temperature sensitivity. Complementing plasmids contained the rpb7 gene, and the corresponding open reading frame bears a missense mutation (rpb7-G150D) in csp3 mutant cells. The rpb7 gene was previously reported to encode an essential component of Pol II in S. pombe (Sakurai and Ishihama 1997; Mitsuzawa et al. 2003). The rpb7-G150D mutation lies within an RNA-binding motif [Fig. 1A]. To biochemically characterize the rpb7-G150D mutation, we purified Pol II from wild-type and rpb7-G150D cells [Spahr et al. 2003]. The subunit composition of wild-type and mutant Pol II was identical, as demonstrated by SDS-PAGE analysis, Coomassie Brilliant Blue staining, and protein identification by mass spectrometry [Fig. 1B]. Western blotting showed that Rpb7 remained associated with the rpb7-G150D mutant polymerase [Fig. 1C]. The phenotype associated with the rpb7-G150D mutation was therefore not due to gross alterations in the Pol II subunit composition. We analyzed mutant Pol II isolated from the rpb7-G150D strain in a reconstituted in vitro system [Spahr et al. 2003]. At the permissive temperature (23°C), basal transcription with mutant Pol II was functional, albeit reduced, as compared with wild type Pol II. At 36°C, however, the activity of the wild type Pol II was unaffected, whereas the activity of Pol II from the rpb7-G150D strain was almost completely abolished [Fig. 1D]. Interestingly, Pol...
II from the rpb7-G150D strain had higher affinity for binding single-stranded RNA (ssRNA) than wild type Pol II at 23°C–25°C and 36°C [Supplementary Fig. S1]. The basic biochemical properties of Pol II, however, seem to be unaffected in rpb7-G150D at 23°C–25°C.

In order to assay the general effects on transcription of the rpb7-G150D mutation, cDNA expression profiling microarray analyses were performed [Xue et al. 2004]. To compare the drastic reduction at the restrictive temperature (36°C) with 25°C, the data were normalized using luciferase RNA spiking controls. At 25°C, only 106 genes were more than twofold down-regulated in rpb7-G150D as compared with wild type, and expression of genes with known functions in centromeric silencing were not significantly altered [Supplementary Table S2]. In contrast, only after 1 h at the growth restrictive temperature (36°C) a substantial part of the genome (>1803 genes) were down-regulated in rpb7-G150D as compared with wild type [Supplementary Fig. S2]. Thus, the rpb7-G150D strain is generally defective for transcription at the restrictive temperature but displays a specific defect in centromeric silencing at 25°C.

Next, we tested whether rpb7-G150D had any defects in the RNAi pathway that directs heterochromatin formation and transcriptional silencing over the centromeric dg–dh repeats. RT–PCR was used to investigate silencing of a cen1R:ade6+ marker [Fig. 2A]. At 25°C in wild type, rpb7-G150D, and swi6A, a lower level of ade6+ transcript was observed as compared with wild type. In contrast, only after 1 h at the restrictive temperature, rpb7-G150D strains had any defects as compared with the wild type, as compared with all three mutant strains (Supplementary Table S2).

The relative fold enrichment was calculated by comparing the cDNA from the immunoprecipitated chromatin [IP] with input material [T]. The ChIP method involves shearing of DNA that will cause shorter genes, such as urad-4-D/E and ade6 An/N allele at the endogenous locus. Arrows, B11 and B12, mark the location of primers. RT–PCR analysis performed on total RNA from wild-type, dcr1Δ, dcr1Δ rpb7-G150D double-mutant, and rpb7-G150D strains. Expression of the centromeric marker gene was undetectable in wild type, as compared with all three mutant strains where a distinct band was visible. Control reactions, without reverse transcriptase, were run to check for DNA contamination. [Right panels] ChIP analysis of cen1R:ura4+ marker gene at the centromeric dg–dh repeat and the truncated urad-4-D/E allele at the endogenous locus. Competitive PCR was used in which one set of primers amplify two products: cen1R:ura4+ and urad-4-D/E. The relative fold enrichment was calculated by comparing the cDNA from the immunoprecipitated chromatin [IP] with input material [T]. The ChIP method involves shearing of DNA. The ChIP method involves shearing of DNA that will cause shorter genes, such as urad-4-D/E, to give rise to stronger signals. [Right panels] ChIP analysis of dg–dh chromatin in wild type, rpb7-G150D, and swi6A. [C] ChIP with Rad21-HA antibodies at 25°C in wild type, rpb7-G150D, and swi6A. [D] ChIP with Swi6 antibodies at 25°C in wild type, rpb7-G150D, and swi6A. The numbers indicate the mean and standard deviations of three experiments.
cen1R/ade6+ expression was undetectable in wild-type cells, but detectable in rpb7-G150D, dcr1Δ, and dcr1Δrpb7-G150D double-mutant cells. Dicer and RITS mutants show reduced dimethylation of histone H3 at Lys 9 [H3K9me2] and reduced binding of Swi6 and cohesin (Volpe et al. 2002, 2003; Verdel et al. 2004). We therefore tested if this was also defective in rpb7-G150D at the permissive temperature (25°C). Chromatin immunoprecipitation (ChIP) analyses with rpb7-G150D strains showed strongly reduced levels of H3K9me2, Swi6, and Rad21 cohesin associated with a ura4 marker gene inserted in the centromere repeats and at endogenous dg–dh sequences (Fig. 2B–D, Supplementary Table S4). Loss of centromeric cohesin causes a typical lagging chromosome defect in anaphase cells [Pidoux et al. 2000; Bernard et al. 2001]. Dicer and RITS mutant cells show this phenotype [Provost et al. 2002, Hall et al. 2003; Volpe et al. 2003]. We found that <0.1% of wild-type control and 16% of rpb7-G150D anaphase cells displayed lagging chromosomes at 25°C. Thus, rpb7-G150D cells show defects in transcriptional silencing, heterochromatin assembly at centromeres, and chromosome segregation. siRNAs homologous to dg–dh repeats have been detected in wild-type cells (Reinhart and Barrell 2002) and transcripts from the reverse strand of dg–dh are produced (Volpe et al. 2002). These transcripts are rapidly processed in wild-type cells and their levels are very low but they accumulate to high levels in dcr1Δ cells. To determine if the production of forward and reverse strand transcripts from the dg–dh repeats is defective in rpb7-G150D cells, a dcr1Δrpb7-G150D double mutant was created, because such transcripts were expected to be undetectable in a Dicer context. The double mutant was similar to both dcr1Δ and rpb7-G150D regarding centromeric silencing defects (Fig. 2A). Accumulation of transcripts originating from centromeric repeats was assayed with strand specific RT–PCR, real-time PCR, and Northern analysis [Fig. 3B,C]. Both reverse [real-time PCR, 276 times wild-type levels] and forward [596 times wild-type levels] dg–dh transcripts were readily detectable in dcr1Δ cells [Fig. 3B,C]. In rpb7-G150D cells, forward dg–dh transcripts were increased [real-time PCR, 16 times wild-type levels] and reverse transcripts were found by real-time PCR to be 25% reduced as compared with wild-type cells. It is known that forward strand dg–dh transcripts are repressed by Swi6. Therefore, the increase of forward transcript levels in rpb7-G150D is likely to be a secondary effect, caused by the compromised heterochromatin, which allows more transcription. Importantly, transcript levels, particularly that of the reverse, remained low even in a dcr1Δrpb7-G150D double mutant at 25°C, whereas both transcripts accumulated to high levels in dcr1Δ [Fig. 3B,C]. The fact that reverse transcript levels remained low in the dcr1Δrpb7-G150D double mutant indicated that Rpb7 functions upstream of Dcr1 in the RNAi pathway and may be required for the generation of centromeric pre-siRNAs. Recently, two other Pol II subunits, Bpf1 and Rpb2, were implicated in centromeric silencing [Kato et al. 2005; Schramke et al. 2005]. Importantly dcr1Δrpb1-11 and dcr1Δrpb2-m203 mutants show no reduction of reverse dg–dh transcripts as compared with dcr1Δ controls.

3’-RACE mapping of centromeric dg–dh transcripts was performed and the resulting sequences were aligned with genomic DNA sequences revealing that the reverse transcripts are polyadenylated [Fig. 3D] supporting the idea that transcripts originating from centromeres indeed are transcribed by Pol II. We were also able to ChIP the dg–dh region in wild-type and rpb7-G150D cells with antibody against Pol II [Supplementary Fig. S1]. Thus, Pol II is associated with centromeric transcripts at 25°C of forward and reverse dg–dh repeat transcripts using Pol I transcribed rRNA probes as controls [Fig. 3E]. These analyses show a reproducible twofold reduction of reverse synthesis and a 1.5-fold increase in forward synthesis in rpb7-G150D versus wild type. This finding is consistent with RT–PCR/Northern data [Fig. 3B,C] and indicates that initiation or elongation rates of reverse dg–dh transcription are reduced in rpb7-G150D cells.

Previous studies have indicated a role for Rpb7 in ini-
tiation of transcription. Together with Rpb4, Rpb7 forms a dissociable substructure of Pol II (Choder 2004) and it was found that Pol II lacking Rpb4/7 was inactive in promoter-directed initiation of transcription whereas addition of Rpb4/7 restored activity (Edwards et al. 1991). To test if Rpb4 has a role together with Rpb7 in RNAi-directed centromeric functions, we expressed rpb4+ with a high copy expression vector [pREP3x-rpb4+] and, interestingly, we were able to partially suppress both the microtubule-poison (TBZ) sensitivity and cen1R->ade6 silencing defects of rpb7-G150D by overexpression of Rpb4 (Supplementary Fig. S3). This suggested that Rpb4 and Rpb7 share a special role at fission yeast centromeres.

Rpb7 has been previously shown to affect transcriptional initiation of some genes in budding yeast (Choder 2004). Rpb7 interacts strongly in protein–DNA photo-cross-linking experiments with promoter DNA (Chen et al. 2004). Therefore to test if transcriptional initiation was defective in rpb7-G150D cells, we first mapped the 5′ end of the dg-dh transcripts by 5′-RACE. The putative forward [For] and reverse [Rev] transcript promoters in the dg-dh region were then cloned into a lacZ reporter plasmid (Forbury 1998). Truncated versions of the Rev promoter were also constructed [Fig. 4A]. The plasmids were introduced into wild-type, rpb7-G150D, and rpb1-11 cells, and β-galactosidase assays were performed [Fig. 4B]. Importantly, elongation, processing, and stability of the lacZ transcript were not affected since expression of β-galactosidase from the positive control nmt1 promoter–lacZ construct was not reduced in any of the mutants. It was clear from these data that the Rev promoter activity is strongly reduced at 25°C in rpb7-G150D but relatively unaffected in rpb1-11 as compared with wild type. In expression profiling microarray analysis rpb1-11 reduced transcription of 26 genes genome wide as compared with wild type (Schramke et al. 2005). Using the same microarray platform, normalizations, and cut-off value, we found that rpb7-G150D reduces expression of 51 genes as compared with wild type at 25°C (Supplementary Table S3). Therefore the two mutant Pol II enzymes are both only mildly compromised for transcription. Thus, the two mutant enzymes have a similar degree of genome-wide transcriptional defects, but only rpb7-G150D shows a defect in initiation of transcription from the Rev promoter.

As a consequence of reducing siRNA precursor synthesis, centromeric siRNA levels are expected to be reduced in the rpb7-G150D strain compared with wild type. In order to test this, centromeric siRNA levels were probed by Northern analysis [Fig. 4C] and, although readily detected in wild-type cells, they were undetectable in rpb7-G150D and dcr1Δ cells.

Taken together, the data presented represents the first direct evidence that pre-siRNA’s are transcribed by Pol II. Pol II has previously been reported to transcribe microRNAs (Lee et al. 2004). However, microRNAs, which work in post-transcriptional gene silencing, are very different from centromeric pre-siRNAs, which are transcribed from heterochromatin and act in transcriptional gene silencing. Furthermore, our data suggest that a particular subunit of Pol II, Rpb7, is required for initiation of centromeric siRNA precursor transcription and that this reverse dg–dh transcript is required to drive centromeric silencing. We speculate that the increased ssRNA-binding property of rpb7-G150D may inhibit initiation of transcription by stalling the transition from Pol II initiation to elongation complex. The effect is specific to rpb7-G150D, since two other fission yeast Pol II mutants do not have the same phenotype [Kato et al. 2005; Schramke et al. 2005]. The CTD deleted mutant rpb1-11 has normal synthesis of pre-siRNAs, as well as siRNAs corresponding to centromeres [Schramke et al. 2005]. A point mutation in the second largest Pol II subunit rpb2-m203 produces normal levels of pre-siRNAs but no centromeric siRNAs [Kato et al. 2005]. Hence, both mutants are different than rpb7-G150D, which has reduced levels of pre-siRNA and no centromeric siRNAs. Thus, remarkably, the three different subunits Rpb7, Rpb2, and Rpb1 affect distinct steps of the same pathway [pre-siRNA synthesis, generation of siRNA, and chromatin modifications]. This may reflect an adaption of the RNAi
machinery to the multisubunit structure of RNA pol II, to coordinate different mechanistic steps of the RNAi-directed chromatin silencing pathway.

Materials and methods  

Strains and media  
Cells were grown in yeast extract supplemented with adenine [YES]. Standard genetic techniques were used for crossing of strains. *S. pombe* strains used are listed in Supplementary Table S1.

Expression profiling DNA microarrays  
Microarrays were purchased from Eurogentec and experiments were carried out as described [Xue et al. 2004]. The microarray covers up to 99.5% of the annotated fission yeast genes. RNA was extracted from log phase cells using a standardized acid phenol method. cDNA was generated with a combination of *S. pombe*-specific primers and random hexamers and labeled with Cy3 and Cy5, respectively. Dye-swaps were done for all experiments. Hybridized slides were scanned using a Bio-Rad scanner and spots were quantified using ImageQuant version 4.2 (ImageGene). The gene expression analysis was done with Genespring (Silicon Genetics). Normalization: To directly compare the rpb7-G150D 25°C and 36°C data sets, each gene’s measured intensity was divided by the median of a list of positive control genes [nucleic acid splicing controls]. To compare rpb7-G150D 25°C with rpb7-111 expression profiles [Schramke et al. 2005] the Lowess normalization was used [Xue et al. 2004].

RT–PCR  
Log phase cells were subjected to RNA extraction by standard acid phenol extraction or with RNaseasy Mini kit for Preparation (QIAGEN). Before RT–PCR, all RNA samples were DNase treated (DNaseI, Invitrogen). Superscript first-strand synthesis system [Invitrogen] or OneStep RT–PCR Kit (Qiagen) was used for RT–PCR. In strand-specific RT–PCR reactions only one primer, forward or reverse, was added to the RT reaction. Control reactions lacking RT were run for all RNA samples. Real-time PCR for cen forward, reverse and actin controls was carried out using a MyQ (Bio-Rad) single-color apparatus.

ChIP  
ChIP was performed as previously described except for the following modification: Cells were spheroplasted at 108 cells/mL in PEMS (100 mM Pipes at pH 7, 1 mM EDTA, 1 mM MgCl2, 1.2 M sorbitol) with 0.4 mg/mL Zymolyase-100T for 25 min at 36°C. Cells were washed twice in PEMS, and cell pellets were frozen at −20°C. Thereafter the standard ChIP procedure was followed. Quantitation of bands was performed using the Eastman Kodak Co. EDAS 290 system and ID Image Analysis software.

Immuno-fluorescence microscopy  
Cell growth, fixation in 3.8% paraformaldehyde, staining, detection of α-tubulin, collection of images, and spindle length measurements have been described [Mitsuzawa et al. 2003].

Northern blots  
Ten micrograms of total RNA, prepared as described above, was resolved on 6.3% formaldehyde gels containing 1% agarose and blotted overnight to a Hybond- XL membrane (Amersham Biosciences). DNA probes, complementary to centromeric *dg-dh* repeats and actin, were generated with Random Primed DNA Labeling Kit [Roche]. The probes were hybridized to the membranes overnight at 65°C in a rotating oven and washed according to the protocol provided by the manufacturer of the membrane.

3′ and 5′-RACE  
3′- and 5′-RACE mapping of RNA transcripts from strain 978 was done according to protocol from the manufacturer, RACE System for Rapid Amplification of cDNA Ends version 2.0 (Invitrogen). Sequencing was done with ABI Prism BigDye Terminator Cycle Sequencing Ready Reactions Kits, Original and version 2.0 (AB Applied Biosystems).

TRO assay  
TRO assays were carried out as described [Allen et al. 2002]. Seven-hundred-nucleotide single-stranded MAXIscript probes for rRNA and *dg-dh* region were used.

Small RNA preparations and detection  
Log phase cells were subjected to RNA extraction by standard acid phenol extraction followed by PEG precipitation. Samples were resuspended in 50 mM formamide and separated on a 17.5% Urea-denaturing polyacrylamide gel. A 32P-DNA probe, complementary to centromeric *dg-dh* repeats, was generated with Random Primed DNA Labeling Kit (Roche). The probe was hybridized to the membranes overnight at 42°C in a rotating oven and washed twice with 2× SSC 2% SDS at 50°C. Phosphor screen or films were exposed for a minimum of 3 h up to 3 d. DNA Oligonucleotides 22 nucleotides (nt) in length were used as a marker.

RNA filter assay  
Centromeric *dg-dh* repeats PCR-fragments were generated with T7 promoters at both ends. Radioactively labeled RNA was produced from these templates with the MAXIscript in vitro transcription Kit from Ambion. Pol II was purified from strains 303 and 801 and subjected to transcription assays as described previously (Spahr et al. 2003). Pol II binding to RNA was assayed by nitrocellulose filter binding in a 25 μL reaction volume. Proteins were incubated with in vitro transcribed RNA [40,000 counts per minute] in RNA-binding buffer [5 mM magnesium acetate, 2.5 mM magnesium sulfate, 3.7 mM EDTA, 37 mM Hepes-KOH at pH 7.6, 100 mM potassium acetate, 1.25 mM diithiothreitol, and RNase inhibitors] for 30 min at 36°C. The mix was transferred to a nitrocellulose membrane (Schleicher and Schuell, Protran 0.45 μM), washed twice with 1 mL RNA-binding buffer without RNase inhibitor, and measured in a liquid scintillation counter (Walla 14100).

Purification of RNA Pol II  
Purification of RNA Pol II was carried out according to Spahr et al. (2003). Western blotting with Rpb7-specific [Santa Cruz] and Rpb1-specific [Neocline] antibodies was performed according to the manufacturers instructions.

β-Galactosidase activity assay  
Cells containing the pREP–lacZ constructs (Fig. 4B) were grown in PMG medium lacking leucine and thiamine to mid-logarithmic phase at 25°C. Units were defined as equal to (1000 × OD414)×( min×mL×OD595)−1.

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