DNA and Chromosomes: Identification of Noncoding Transcripts from within CENP-A Chromatin at Fission Yeast Centromeres

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Identification of Noncoding Transcripts from within CENP-A Chromatin at Fission Yeast Centromeres**

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The histone H3 variant CENP-A is the most favored candidate for an epigenetic mark that specifies the centromere. In fission yeast, adjacent heterochromatin can direct CENP-A^Cnp1 chromatin establishment, but the underlying features governing where CENP-A^Cnp1 chromatin assembles are unknown. We show that, in addition to centromeric regions, a low level of CENP-A^Cnp1 associates with gene promoters where histone H3 is depleted by the activity of the Hrp1/Chd1 chromatin-remodeling factor. Moreover, we demonstrate that noncoding RNAs are transcribed by RNA polymerase II (RNAPII) from CENP-A^Cnp1 chromatin at centromeres. These analyses reveal a similarity between centromeres and a subset of RNAPII genes and suggest a role for remodeling at RNAPII promoters within centromeres that influences the replacement of histone H3 with CENP-A^Cnp1.

Centromeres are the specific chromosomal loci at which kinetochore assembly occurs. Extensive investigation of centromeric DNA from a wide variety of eukaryotic cells indicates that the primary sequence is not conserved. Despite this, all functional centromeres, including neocentromeres formed at ectopic chromosomal loci, share a unique chromatin composition in which the evolutionarily conserved histone H3 variant CENP-A replaces canonical histone H3. At many centromeres, this CENP-A kinetochore chromatin is formed on repetitive arrays such as α-satellite on human chromosomes. However, the fact that this CENP-A chromatin is flanked by similar repeats assembled in heterochromatin (1–3) makes it difficult to distinguish repeats coated in heterochromatin from those assembled in CENP-A chromatin. In the fission yeast Schizosaccharomyces pombe, the heterochromatic outer repeats are distinct from the central domain over which CENP-A^Cnp1 and the kinetochore assemble (1). Although heterochromatin is required for the de novo assembly of CENP-A^Cnp1 chromatin on central domain DNA, it is dispensable for the subsequent maintenance of CENP-A^Cnp1 chromatin (4). Thus, kinetochore-associated DNAs may possess unidentified features that are not apparent from the primary sequences that make them favorable substrates for CENP-A^Cnp1 deposition and kinetochore assembly. Previous analyses have suggested that the acetylated state of histones may influence CENP-A assembly (5, 6). Moreover, transcripts homologous to centromere-associated DNAs have been detected in various organisms, and retrotransposon RNAs are implicated in centromere chromatin structure (7–10). In fission yeast, it has been shown that a GATA-like transcription factor is required for efficient CENP-A^Cnp1 deposition (11). Also in fission yeast, the ATP-dependent remodeling factor Hrp1 (orthologous to Saccharomyces cerevisiae Chd1 (chromo-helicase DNA-binding protein 1)) affects CENP-A^Cnp1 deposition (12). Chd1 is involved in transcriptional elongation by RNA polymerase II (RNAPII) (13) and has been shown to facilitate replication-independent histone H3 exchange (14). An attractive hypothesis is that transcription underlies chromatin remodeling in the centromeric DNAs, which in turn promotes CENP-A^Cnp1 deposition. Here, we investigated this possibility.

**EXPERIMENTAL PROCEDURES**

Standard procedures were used for growth and genetic manipulation. The details of PCR primers are listed in supplemental Table 1. The S. pombe strains used in this study are listed in supplemental Table 2. The procedures used are described under supplemental "Methods."

ChIP—Cells were fixed with 1% paraformaldehyde and lysed by bead beating. Chromatin was solubilized by shearing with a Bioruptor sonicator and immunoprecipitated using 10 μl of anti-CENP-A^Cnp1 antiserum with protein G-agarose beads. ChIPs were then analyzed by quantitative PCR.

ChIP-Chip—DNA was immunoprecipitated and hybridized to Affymetrix GeneChip S. pombe tiling 1.0FR arrays as described previously (15). 10 μl of anti-CENP-A^Cnp1 and 1.5 μg of anti-H3 (ab1791, ABCAM) antibodies were added to 100 μl

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**Supplemental Material**

The on-line version of this article (available at http://www.jbc.org) contains supplemental "Methods," Figs. S1–S5, and Tables 1 and 2.

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of chromatin extract. Hrp1<sup>Chd1</sup> binding data and histone H3 density maps are from a previous study (16).

Raw data from Affymetrix (.CEL format) were analyzed with Affymetrix TA5 (tiling analysis) version 1.1 software and visualized with Affymetrix IGB (integrated genome browser). The data were normalized using quantile normalization plus scaling and run with a bandwidth of 20. <i>p</i> values were calculated using hypergeometric probability distribution in R version 2.12.0.

RESULTS

Hrp1<sup>Chd1</sup> has previously been implicated in CENP-A<sup>Cnp1</sup> deposition but is not essential for cell viability (12). Expression of RNAPII-transcribed marker genes such as <i>arg3</i><sup>+</sup> is repressed when placed within the centromidal domain, and silencing of <i>cen1</i>: <i>arg3</i><sup>+</sup> is highly sensitive to defective CENP-A<sup>Cnp1</sup> deposition (17); however, this silencing is only partially impaired in <i>hrp1</i><sup>Δ</sup> cells (Fig. 1A). This suggests that redundant mechanisms operate to ensure CENP-A<sup>Cnp1</sup> deposition in the absence of Hrp1<sup>Chd1</sup>. In agreement with this and previous analyses (12), when <i>hrp1Δ</i> was combined with the <i>mis6</i>-302 temperature-sensitive mutation (defective in CENP-A<sup>Cnp1</sup> deposition), it reduced the restrictive temperature of <i>mis6</i>-302. Furthermore, <i>hrp1Δ</i> also reduced the restrictive temperature of <i>cnp1-87</i>, a weak temperature-sensitive allele (Fig. 1B) (18). Consistent with this, the levels of CENP-A<sup>Cnp1</sup> associated with the central domain were further reduced in <i>hrp1Δ cnp1-87</i> double mutants compared with either single mutant (Fig. 1C). We conclude that Hrp1 facilitates the assembly of CENP-A<sup>Cnp1</sup> chromatin, and it becomes essential when Mis6 or CENP-A<sup>Cnp1</sup> function is impaired.

Genome-wide analyses of CENP-A<sup>Cnp1</sup> and histone H3 localization by ChIP on tiling arrays with anti-CENP-A<sup>Cnp1</sup> and anti-H3 antibodies confirmed that Hrp1<sup>Chd1</sup> is required to maintain normal levels of CENP-A<sup>Cnp1</sup> and that, in <i>hrp1Δ</i> cells, H3 levels increase across the central domain (Fig. 1D and supplemental Fig. S1). Previously, we have shown that Hrp1<sup>Chd1</sup> acts at a subset of gene promoters to disassemble histone H3-containing nucleosomes close to the transcription start site (16). Further examination of the genome-wide analyses revealed that low but detectable levels of CENP-A<sup>Cnp1</sup> associate with a significant proportion of promoters in wild-type cells at which Hrp1<sup>Chd1</sup> acts to disassemble H3-containing nucleosomes (Fig. 2A). CENP-A<sup>Cnp1</sup> association is significantly reduced at some, but not all, of these promoters in <i>hrp1Δ</i> cells, and in agreement with this, we saw an increase in H3 (<i>p</i> = 2.7e<sup>-4</sup>, hypergeometric probability) (Fig. 2, B and C). Some promoters show an increase in CENP-A<sup>Cnp1</sup> association and a decrease in H3 in <i>hrp1Δ</i> cells; however, this is less significant (Fig. 2D). These analyses imply that Hrp1<sup>Chd1</sup> directly participates in a remodeling process that evicts H3 and allows CENP-A<sup>Cnp1</sup> deposition at the promoters of some genes. However, additional factors, such as Scm3, must also contribute to the replacement of H3 with CENP-A<sup>Cnp1</sup> and/or CENP-A<sup>Cnp1</sup> maintenance at centromeres (19, 20). This bears resemblance to the transcription-coupled replacement of H3.1 with H3.3 in metazoa (21) and suggests that remodeling at some promoters of RNAPII promoters in wild-type cells at which Hrp1Chd1 acts to disassemble histone H3-containing nucleosomes close to the transcription start site (16).

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which have reduced levels of CENP-AChn1 chromatin over the central domain (31, 32). Surprisingly, transcripts with discrete sizes (~0.5 kb) were identified in both mutants (Fig. 4A); these discrete transcripts (discrete TUKs) were highly enriched in poly(A)-selected RNA (Fig. 4B). A lower level of these specific transcripts was also enriched in the poly(A) fraction from dis3-54 (Fig. 4B, arrowhead). Dis3 is the key catalytic subunit of the exosome required to degrade aberrant transcripts, whereas Psr2 and Dhp1 are required for normal 3′-end formation/termination (27, 29, 30). Pfs2-11 and Dhp1-1 may generate transcripts with extended 3′-ends due to transcriptional read-through into downstream regions, resulting in the observed heterogeneous smear of transcripts (Fig. 4, A and B). The specific increase in discrete TUKs observed in mis6-302 and cnp1-1 cells could be interpreted to mean that intact CENP-AChn1 chromatin prevents expression of these transcripts; however, our analyses suggest that TUKs are constitutively produced and turned over.

FIGURE 1. Chromatin-remodeling factor Hrp1Chd1 contributes to CENP-AChn1 chromatin formation. A, silencing of cen1:arg3° in WT, cnp1-169, and hnrplΔ cells. The growth assay was performed on non-selective (N/S) or arginine-depleted (−Arg) plates at 25, 32, and 36 °C. B, viability of cells bearing hnrplΔ combined with mis6-302 (upper panels) or with cnp1-87 (lower panels) compared with wild-type and single mutants grown at 25, 32, or 36 °C. C, CENP-AChn1 ChIP analyses in WT, hnrplΔ, cnp1-87, and hnrplΔ cnp1-87 cells grown at 36 °C. The enrichment of the cnt1 product was compared with input DNA relative to the act1′ control by quantitative PCR. D, genome browser view of cen1 showing ChIP-chip binding profiles for CENP-AChn1 (purple) and H3 (green) in WT and hnrplΔ cells (as indicated) at 30 °C. The relative ratios of CENP-AChn1 and H3 (hnrplΔ/WT) are indicated (black). Data on the y axis are presented in log 2 scale, and the x axis shows genome positions in base pairs.
by the exosome in wild-type cells. The chromatin context in mis6-302 and cnp1-1 cells is dramatically altered from CENP-ACnp1 to H3 chromatin; CENP-ACnp1 chromatin may interfere with events required for the formation of specific transcripts, such as accurate 3'-termination, ensuring their turnover. Using RNA probes complementary to either reverse or forward strands of the cen1 central core (cc1/tm1) (supplemental Fig. S2), we detected other discrete transcripts in distinct central domain regions in poly(A) RNA from cnp1-1 cells (Fig. 4, C and D). To identify the 5'-ends of these central domain noncoding transcripts, we employed 5'-rapid amplification of cDNA ends (RACE)/PCR on 5'-capped poly(A) RNA extracted from wild-type and cnp1-1 cells. This demonstrated that these novel/unusual transcripts arose from within the central domain and do not arise by read-through from outer repeat transcripts (Fig. 4E and supplemental Fig. S3). The transcription start sites for these transcripts were identical in wild-type and cnp1-1 cells, suggesting that they are indeed produced from wild-type centromeres, albeit at a lower level. The fact that these transcripts are 5'-capped and polyadenylated indicates that TUKs are produced by RNAPII. Consistent with alterations in central domain chromatin affecting transcription (lower CENP-ACnp1 and higher H3 deposition) (Fig. 1D), increased levels of the central domain transcript were also detectable in hrp1Δ cells (supplemental Fig. S4).

On the basis of the 5'-RACE analyses above, we designed improved probes for Northern analyses to detect the forward and reverse strands of the cen1 central core (cc1/tm1) (supplemental Fig. S2) in additional mutants known to affect kinetochore integrity (mis12-537, mis16-53, and mis18-262 in addition to mis6-302 and cnp1-1) (6). At 36 °C, transcripts were detectable in all mutants apart from mis12-537 with this TM-forward 5 probe (Fig. 4F). Some transcripts were even detectable in mis6-302, cnp1-1, and mis16-53 at 25 °C, the permissive temperature. In all mutants, including mis12-537, this TM-reverse probe allowed detection of other transcripts (Fig. 4G) in addition to those observed with the original probe (Fig. 4D). Together, these analyses indicate that cryptic transcription is prevalent in the central kinetochore domain of fission yeast centromeres and revealed only in cells defective in RNA turnover or formation of subkinetochore chromatin. In support of this, low levels of H3K4 trimethylation (TM) represents a 3.3-kb element shared between the central domains of cen2 and cen3.

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methyltransferase, a modification associated with active transcription, have been shown to be enriched in the small amount of histone H3 that remains within subkinetochore chromatin in wild-type cells (33).

**DISCUSSION**

Centromere-associated DNA has been shown to be transcribed in plants and vertebrates (7, 10). 30 years ago, EM studies demonstrated the presence of RNase-sensitive material at the base of kinetochore microtubules in newt lung cells (34). Since then, centromeric transcripts have been found to associate with kinetochore proteins (9, 10). In this study, we demonstrated an analogy between the central domain-associated CENP-ACnp1 chromatin of centromeres and genes whose promoters are associated with Hrp1Chd1 in fission yeast. The fact that Hrp1Chd1 promotes eviction of histone H3 at a set of promoters suggests that similar remodeling processes may occur at RNAPII promoters within centromeres and may contribute to exchange of canonical H3-containing nucleosomes for CENP-ACnp1-containing nucleosomes (summarized in Fig. 5).

It is possible that transcription within centromeres occurs merely as a consequence of having RNAPII promoters whose presence or activation is critical to act as a seed for remodeling events that promote CENP-ACnp1 deposition. The resulting TUK transcripts are degraded by the exosome and thus may represent just nonfunctional by-products of transcription. However, we cannot rule out the possibility that these unstable transcripts might also have a dedicated function in guiding some components of the CENP-ACnp1 deposition machineries and/or kinetochore complex to their cognate DNA sequences (10). Alternatively, the production of TUKs might influence chromatin modification within centromeric chromatin by processes analogous to those associated with cryptic or antisense noncoding RNA production in S. cerevisiae (35, 36). However, we found that the accumulation of TUKs in pfs2-11, dhp1-1, or dis3-54 cells had no obvious effect on CENP-ACnp1 chromatin formation.

Intriguingly, it has recently been reported that S. cerevisiae CENP-ACse4 also tends to associate with a number of RNAPII promoters where RNAPII binding is high (37); however, it is not known if these promoters share any structural or mechanistically related features with S. cerevisiae centro-
meres that contribute to CENP-A_Ceu4 deposition. Interestingly, the *S. cerevisiae* centromeric protein Cbf1, which binds centromere DNA element I, functions as a transcrip-
tion factor at the *MET16* promoter (38). In this regard, it is possible that RNAPII transcription might also occur within or close to centromeres of *S. cerevisiae*. In fact, cryptic tran-
scription was detected close to CEN3 in *S. cerevisiae* exo-
some mutants (39); it is not known if this is a general feature of all *S. cerevisiae* centromeres. Recently, the human chromatin-remodeling factor FACT (facilitates chromatin tran-
scription), whose function is implicated in transcription, was
found to associate with affinity-purified CENP-A chromatin (40, 41) Moreover, depletion of FACT was found to impair incorporation of newly synthesized CENP-A in chicken cells (42). CHD1 was also found at centromeres in chicken cells and is required for centromeric localization of CENP-A in

**FIGURE 4.** Northern and 5’-RACE/PCR analyses of central domain transcripts. **A**, Northern analysis of total RNAs in RNA-processing and kinetochore mutants. An RNA probe complementary to cnt1 was used. Cells grown at the permissive temperature (25 °C for WT, pfs2-11, dhp1-1, mis6-302, and cpn1-1 cells and 36 °C for WT and dis3-54 cells) were shifted to the restrictive temperature (6 h at 36 °C for WT, pfs2-11, dhp1-1, mis6-302, and cpn1-1 cells and 9 h at 18 °C for WT and dis3-54 cells) before RNA extraction. act1* was used as a loading control. *, rRNA interference with hybridization. For additional EtBr images, see supplemental Fig. S5 A. **B**, the same cnt1 probe used on poly(A) RNA. For additional EtBr images, see supplemental Fig. S5 B. C and D, Northern analysis of total or poly(A) RNAs from WT and cpn1-1 cells with an RNA probe complementary to the reverse strand (forward probe; C) or the forward strand (reverse probe; D) of the ccl/tm1 sequence, which is shared by cnt1 and cnt3. *, nonspecific band. For additional EtBr images, see supplemental Fig. S5 (C and D). **E**, schematic representation of transcription start sites determined by 5’-RACE/PCR in WT and cpn1-1 cells. Black arrows, transcription start sites identified in WT and cpn1-1 cells; gray arrows, transcription start sites identified only in cpn1-1 cells. F and G, Northern blots showing transcripts complementary to the TM-forward (F) or TM-reverse (G) probes in WT, mis6-302, cpn1-1, mis12-537, mis16-53, and mis18-262 cells grown at permissive (25 °C) and restrictive (36 °C) temperatures. *, nonspecific band. EtBr staining confirmed equal loading.
human cells (42). These observations, together with the analyses presented here, implicate RNAPII transcription and the associated remodeling activities in the replacement of histone H3 with CENP-A.

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