The CCCTC-Binding Factor (CTCF) of *Drosophila* Contributes to the Regulation of the Ribosomal DNA and Nucleolar Stability

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Abstract

In the repeat array of ribosomal DNA (rDNA), only about half of the genes are actively transcribed while the others are silenced. In arthropods, transposable elements interrupt a subset of genes, often inactivating transcription of those genes. Little is known about the establishment or separation of juxtaposed active and inactive chromatin domains, or preferential inactivation of transposable element interrupted genes, despite identity in promoter sequences. CTCF is a sequence-specific DNA binding protein which is thought to act as a transcriptional repressor, block enhancer-promoter communication, and delimit juxtaposed domains of active and inactive chromatin; one or more of these activities might contribute to the regulation of this repeated gene cluster. In support of this hypothesis, we show that the *Drosophila* nucleolus contains CTCF, which is bound to transposable element sequences within the rDNA. Reduction in CTCF gene activity results in nucleolar fragmentation and reduced rDNA silencing, as does disruption of poly-ADP-ribosylation thought to be necessary for CTCF nucleolar localization. Our data establish a role for CTCF as a component necessary for proper control of transposable element-laden rDNA transcription and nucleolar stability.

Introduction

Electron micrographs of transcribing rDNA loci by O. L. Miller, Jr. and colleagues have provided a cytological foundation to subsequent studies showing histone modification-, DNA methylatation-, and regulatory RNA-mediated epigenetic regulation of the rDNA loci across kingdoms [1,2]. Many studies have led to the prevailing view that only about one-half of the rDNA cistrons are active, while the remainder are kept silent through epigenetic modification of chromatin structure. Although much is now known about rDNA chromatin structure, relatively little is known about the decisions of how many and which cistrons are inactive, whether all cell types make this decision, and once made how active and inactive chromatin domains are kept separate. Balance between activating and repressive factors may control the ratio [3], although such a model does not account for the preferential inactivation of the subset of cistrons that might be interrupted by transposable element. To account for this, a simple model suggests a sequence-specific repressor might inactivate some rDNA cistrons, and a boundary element may maintain separation of active and inactive domains [1,4]. Although RNA Polymerase III, RNA Polymerase I regulators, or DNA-replication proteins may serve to separate domains in yeasts [5,6,7,8], little is known of how similar regulation may be accomplished in animals and plants.

In arthropods, the R1 and R2 non-long-terminal-repeat (non-LTR) retrotransposable elements interrupt a high proportion of 33S rDNA cistrons (17%–67% rDNA copies are interrupted by R1, 2%–28% by R2, and up to 16% by both) [9], and molecular and cytological evidence show that these are almost always inactivated [10,11]. These elements are inserted in a conserved site within the 28S subunit and are colinearly transcribed with the 33S rDNA [12,13], showing that transcriptional silencing due to their presence affects the rDNA promoter approximately four kilobases away.

CTCF is a protein with complex roles in gene regulation, having been shown to act as both transcriptional activator and repressor, and be responsible for two features of genomic “boundary elements,” namely the abilities to separate chromatin with activating and repressing histone modifications and to block enhancer-promoter interactions (recently reviewed in [14]). CTCF plays regulatory roles in the large Homeotic gene complexes of flies and mammals [15,16], is thought to be necessary to maintain monoallelic expression of genomic imprinted loci in mouse and humans [17], and binds the inactive (dosage compensated) female mammalian X chromosome [18]. Hence, it possess the properties expected for a protein that might regulate and separate interspersed active and inactive rDNA cistrons. Unraveling the overlapping and separate properties of CTCF has been difficult, since consensus DNA binding sites, interaction partners, and genetic properties have proven difficult to exhaustively enumerate [15,19].

Torrano and colleagues noted that CTCF moves to the nucleoli of terminally-differentiated mammalian (human and rat) cells [20]. It has been suggested that the localization might be a necessary
step for CTCF to regulate the euchromatin [21], implying that it has no active role in the nucleus. This view is perhaps appealing because of the example of p53 and ARF, whose regulation includes facultative nucleolar retention as means of gene product regulation [22,23]. However, Torrano and colleagues showed over-expression of CTCF resulted in reduced nascent nucleolar transcription and argued for a direct role in transcriptional regulation, and recently van de Nobelen and colleagues showed CTCF at the tDNA promoter [24].

We directly addressed whether CTCF is also found in the nucleolus of Drosophila, binds to the tDNA, regulates its expression, and influences the stability of the nucleolus. In the course of our work, we discovered that CTCF is not solely a marker for terminal differentiation since it is nuclear in many non-differentiated cell types. We found that CTCF binds to at least one specific site within the transposable elements of the repeated tDNA cistron, which contributes to a model for regional regulation of tDNA expression. We used RNAi-mediated reduction of gene activity, nucleolar instability.

Results

To determine if CTCF plays a role in tDNA regulation in Drosophila, we first had to ascertain whether it could be detected in nuclei of numerous different cell types, and moreover to determine if it is generally used to regulate tDNA. We observed strong immunofluorescence signal for CTCF in the nuclei of differentiated salivary glands of third instar Drosophila larvae, showing that it is cytologically associated with the tDNA, and supporting our belief that the biology of Drosophila CTCF may be similar to that of mammals. Even in occasional nuclei with multiple nuclei, CTCF was found to overlap with all focal localization of fibrillarin, a marker for the fibrillar component of the nucleus. Nucleolar localization of CTCF was in addition to a focal nucleoplasmic staining (Figure 1A) [16,21,25,26]. At higher magnification, we observed that CTCF did not conform to any obvious landmarks of DNA within the nucleus, although it was largely excluded from the visible DNA threads and foci (Figure 1B).

Unlike mammalian tissue culture and nervous tissue, nuclear CTCF did not require terminal differentiation and cessation of division in Drosophila since we observed CTCF in the nuclei of undifferentiated cycling interphase S2 tissue culture and larval neuroblast cells (Figures 1C, D). The amount of nuclear CTCF differed in those cell types, in the former it was neither enriched nor excluded but appeared similar to levels in the non-nuclear chromatin, while in the latter it was moderately enriched over the amount found in the chromatin. Many non-nuclear nuclear proteins are seen to be excluded from the nucleolus, and so the lack of CTCF exclusion is indicative of some localization even if it is not enriched in this compartment; this is especially true given the thousands of euchromatic binding sites to which it is being found in the chromatin. Many non-nucleolar nuclear proteins are seen to be excluded from the nucleolus, and so the lack of CTCF exclusion is indicative of some localization even if it is not enriched in this compartment; this is especially true given the thousands of euchromatic binding sites to which it is being compared [27].

Nonetheless, we wished to observe clear CTCF nuclear localization, and so used a stage of the cell cycle when binding to nucleolar DNA is cytologically distinct. We therefore detected CTCF in the secondary constrictions (locations of the nucleolar organizing ribosomal DNA) on neuroblast sex chromosomes (Figure 1E, F). tDNA localization is the only heterochromatin binding that we could detect, however the strong signal from the euchromatic compartments of the genome limited our ability to detect CTCF in distal heterochromatic blocks that juxtapose euchromatin. This localization at a time when nuclei are disassembled and transcriptionally silent suggests localization is not due solely to protein-protein or protein-rRNA interactions in a mature nucleus, but instead is due to direct DNA binding by CTCF.

CTCF is a sequence-specific Zinc-Finger DNA binding protein [28], and we thought it was likely to bind the tDNA directly. To confirm this, we predicted potential CTCF binding sites in the entire tDNA sequence including the non-transcribed spacer (NTS) that separates the 35S primary transcription units, and the 28S-interrupting R1 and R2 arthropod transposable elements [29], using the Patser algorithm informed by two different published Drosophila CTCF consensus sequences (Figure 2A) [15,19,30]. We identified six potential sites (sites 18, 21, 28–31, data in gray), and manually scanned the R1 and R2 sequences for similar potential consensus sites that differed in only one nucleotide of the core conserved consensus, which identified an additional 15 sites which served as an expected “negative” out-group (asterisks). In addition to potential binding sites identified by Patser, we designed primers to amplify sequences approximately every 350 base pairs to test potential non-consensus binding across the entire 35S tDNA transcription unit (sites 5–17, 19–20, 22–27). We did not identify any sequences in the NTS that were similar to the CTCF consensus, although sites have been reported in the human tDNA NTS [24,31], so we designed primers for chromatin immunoprecipitation of the NTS (sites 1–4) regardless of lack of obvious consensus. Of the 46 tested sites, only one showed robust binding using chromatin immunoprecipitation with antibodies raised against CTCF (Figure 2B, site 28). This site was in the DNA that corresponds to the R1 element, near the beginning of this transposable element. Although another site (site 29) showed moderate but statistically significant immunoprecipitation, its close linkage to site 29 makes ancillary immunoprecipitation from incomplete DNA shearing a likely explanation. Even for site 28, the relative enrichment by chromatin immunoprecipitation was modest relative to the positive control of the Fab-8 element (which had approximately 40-fold enrichment over background, data not shown) [15], but the biology of the tDNA locus makes chromatin immunoprecipitation potentially insensitive to occupancy at this locus, since we must consider that of the hundreds of copies of the cistron, only a fraction are silent and thus possess corresponding histone modifications or regulatory protein binding. For these reasons, we expect our chromatin immunoprecipitation signals to be lower than expected occupancy at any subset of tDNA cistrons.

The location of CTCF binding relative to R1 is consistent with CTCF-mediated silencing of inserted 35S cistrons, either by direct repression or by separating active (expressed) from inactive (“heterochromatic”) compartments with R1-inserted cistrons as boundaries. A recent model proposed by Eickbush and colleagues is based on data suggesting proximity to R1-inserted elements is causal in tDNA cistron silencing, and this corresponds to cytological evidence of silenced inserted rRNA genes [10,32]. Based on this hypothesis, and prior reports showing that CTCF may act as a transcriptional repressor [20,28], we predicted that CTCF acts as a direct transcriptional repressor of nearby tDNA.

The ribosomal RNAs are the most abundant RNA species in the cell, and have very long half-life once assembled into ribosomes [33]. Our experiments are able to detect small changes in rRNA expression by measuring steady-state rRNA levels [34], however the pool of stable rRNAs is vast, and so changes due to alteration of transcriptional activity was expected to be small or...
Figure 1. Indirect immunofluorescence reveals CTCF as a component of the nucleoli of Drosophila cells. (A) Confocal microscopy of whole mount third instar larval salivary gland nucleus. CTCF and fibrillarin (fib) shown separately, and merged with DNA (blue). (B) Higher magnification of nucleolus showing CTCF, DNA, and color merge. The DNA-only separation has been inverted and non-linearly adjusted for bright and contrast to reveal the filamentous structure of the DNA within the nucleolus. Inset in merged image shows a different nucleus with the CTCF-
containing nucleus in the context of CTCF-banded chromosome arms. (C) Epifluorescence microscopy of S2 tissue culture cell nucleus. CTCF and fibrillarin shown separately, and merged with DNA. (D) Confocal microscopy of third instar larval diploid interphase neuroblast nucleus. CTCF and fibrillarin shown separately, and merged with DNA (blue). (E) Epifluorescence microscopy of condenscd mitotic X chromosome (arrow points to rDNA locus) from a third instar larval diploid metaphase neuroblast. The panoply of CTCF sites in the euchromatin are visible as immunofluorescence on the chromosomes. (F) Epifluorescence microscopy of condensed mitotic Y chromosome from a third instar larval metaphase diploid neuroblast. For (E) and (F), CTCF is shown separately from a color merge with DNA. Scale bars 5 μm.

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Three day double-stranded RNA treatment resulted in a large fraction of cells with reduction of CTCF detectable by immunofluorescence (Figure 3A–F). Prior to treatment, CTCF was found in the nucleolus and foci throughout the nucleus. After treatment, CTCF was overall reduced, undetectable in many cells, and absent from foci. Integration of fluorescence showed that treatment reduced CTCF to 31.7% (±19.1% S.D.) of wild-type levels. What little CTCF remained appeared equally reduced in both the chromatin and the nucleolus, which argues against CTCF recruitment to the nucleolus as a means of sequestration of excess protein. Cells with reduced CTCF exhibited clear and reproducible disruption to the fibrillary component of the nucleoli as fibrillarin immunolocalization was either reduced or appeared more diffuse and fragmented (Figure 3B, D, E, F), in severe cases vesiculating into small foci. Fluorescence intensity co-varied with CTCF fluorescence (Figure 3C) with regression R^2 = 0.32 in wild-type and 0.39 in cells with RNA-mediated CTCF reduction. This disruption shows that CTCF is necessary for the proper structure of the nucleolus, a common feature of regulators of rDNA expression (e.g., chromatin modifying enzymes, RNA Polymerase I) [20,34,35,36,37]. Such disruption was not observed when cells were treated with double-stranded RNA directed at LacZ, GFP, or Rho1, or in untreated cells. In the population of S2 cells with RNAi-mediated reduction of CTCF (Figure 4G), we observed

Figure 2. Chromatin Immunoprecipitation of CTCF identifies binding to the rDNA locus. (A) Map showing structure of typical rDNA repeat unit. NTS = non-transcribed spacer, ETS = external transcribed spacer, ITS = internal transcribed spacers, 18S, 5.8S, 25, and 28S are final rRNA products, R1 and R2 are transposable element insertions (dotted lines indicate insertion sites in the 28S). Numbers indicate location of potential or predicted CTCF binding sites - all sites are shown, indicated either by numbers or by vertical hash marks. Asterisks indicate location of near-consensus sites within R1 and R2, collectively used as an "out-group." Blow-out shows detail around R1 and R2 insertion sites in the 28S sequence; primers "a"–"f" are used for R1-, R2-, and uninserted 35S specific transcript detection. (B) Real-Time PCR quantification of amplification using DNA purified from chromatin immunoprecipitated by anti-CTCF antibodies. Data are presented as average boxed by pooled standard deviations of triplicate samples from three independent experiments. White data are from sites that do not match Drosophila CTCF consensus, gray data (18, 21, 28–31) match the Drosophila consensus. All data are normalized to the pooled average of the outgroup data (*), which was then defined as 100% (dashed line). Sequences show CTCF consensus sites (18, 21, 28–31).
CTCF Regulates Drosophila rDNA

A. CTCF
B. CTCF
C. CTCF (fluorescence/sec) vs. Fib (fluorescence/sec)
D. Fib
E. Fib
F. Fib
G. CTCF
H. 28S rRNA, R1 mRNA, R2 mRNA, pre-rRNA junctions
Figure 3. RNAi-mediated or mutational reduction of CTCF gene activity disrupts nucleolar structure and increases rDNA expression. (A) Indirect immunofluorescence detection of CTCF in S2 cell culture nuclei. (B) CTCF immunodetection after three-day treatment of double-stranded RNA directed at CTCF. Images from (A) and (B) are presented with the same exposure/bright/contrast conditions. (C) Quantification of all data from untreated (circles) and double-stranded RNA treated (crosses) cells. X-axis shows CTCF intensity (fluorescence per unit time, corrected to DNA), y-axis shows fibrillarin intensity, and regression lines are for separate datasets. (D) Higher magnification of fibrillarin-containing nucleolus from cell treated with double-stranded RNA directed at LacZ. (E) Higher magnification of fibrillarin-containing nucleolus from cell treated with double-stranded RNA directed at CTCF. (F) As in (E), but a more pronounced nucleolar vesiculation/disruption phenotype. (G) Salivary gland nuclei derived from third instar larvae mutant for CTCF. In images from (A), (B), and (G), CTCF and fibrillarin (fib) are shown separately, and merged with DNA (blue). (H) In S2 cell culture, no double-stranded RNA treatment, or treatment with double-stranded RNAs directed at LacZ have no effect on 28S rRNA (Figure 2, primers a–d), R1 (Figure 2, primers b–c) or R2 (Figure 2, primers e–f) mRNA levels, or pre-rRNA unprocessed junctions (2S-ITS2 and ITS2-28S) but treatment with double-stranded RNAs directed at CTCF increases 28S, R1, R2, and pre-RNA junction RNA species. Whole intact animals bearing homozygous mutation of CTCF35.2 results in increased 28S, R1, R2, and pre-RNA junction RNA species compared to heterozygous CTCF35.2/+ controls. Note discontinuity in ordinate for final R2 datum (end-hashed dashed line). All data are normalized (100%, dashed lines) to untreated cells (“no dsRNA”) and heterozygous animals (“CTCF/+”). Scale bars 5 μm.
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Figure 4. Disruption of poly-ADP-ribosylation decreases nucleolar CTCF, disrupts nucleolar structure, and increases rDNA expression. (A) Indirect immunofluorescence detection of CTCF and fibrillarin (fib) in S2 cell culture, and (B) after treatment with double stranded RNA directed at LacZ. (C) Structure of nucleoli after treatment with control double-stranded RNA directed at Poly-ADP-Ribose Polymerase (PARP), or (D) Poly-ADP-Ribose Glycohydrolase (PARG). (E) Confocal microscopy of whole mount third instar larval salivary gland nuclei derived from PARG mutants. In all preceding images, CTCF and fibrillarin (fib) are shown separately, and merged with DNA (blue). (F) Squashed chromosomes from whole mount third instar larval salivary gland nuclei show retention of CTCF at euchromatic bands but loss from the nucleolus (arrowheads). (G) Double-
increased R1 and R2 expression after three days (Figure 3H), in contrast to untreated cells or cells treated with double-stranded RNA directed at lacZ. Expression of R1-inserted tDNA increased 4.5-fold, while R2 increased 2.5-fold. Although we did not detect any binding of CTCF to the R2 element sequence, the R1 and R2 inserts are thought to cluster in the repeat arrays, and so may share co-regulation [11]. Additionally, we detected an increase in steady-state levels of the uninterrupted 28S rRNA; although significant, the increase was small possibly due to the already considerable pool of stable (ribosome-bound) rRNAs.

It is possible to address CTCF function in intact animals using mutations that eliminate detectable CTCF protein. We used a previously characterized hypomorph, CTCF[35.2] [16]. Mutants are lethal as pharate adults, the late stage presumably owing to perdurance or maternal mRNA and protein [16]. We could therefore generate and analyze third instar larvae with reduced CTCF activity. In salivary glands obtained from CTCF[35.2] homozygotes, we again observed dramatic vesiculation of nucleolar structure (Figure 3G), similar to the disruption we observed in S2 cell culture. These nucleoli were also reminiscent of those seen upon mutation of chromatin modifying proteins known to regulate the rDNA, or upon reduction of the tDNA arrays [34,35]. In both of those cases, copies of the rRNA genes were lost, resulting in a decrease in array size. To determine if the same tDNA array size reduction was an effect of CTCF mutation, we outcrossed flies bearing CTCF alleles derived from two independent sources to females of genotype G[1]/DX, y b w b / L[BP]. Female offspring have a paternal X chromosome linked tDNA array as the sole source of rDNA and nucleolar organizer since the G[1]/DX compound-X chromosome is entirely devoid of the tDNA [34,38,39]. All female offspring exhibited a strong bobbed phenotype, a hallmark of reduced rDNA. The phenotype was identical in penetrance and expressivity in both CTCF/+ and +/+ offspring, indicating the bobbed phenotype mapped to the Y chromosome common to all females, the location of the tDNA. We measured tDNA copy number using Real Time Polymerase Chain Reaction and discovered them to be 62.5% (±3.2% S.E.M.) the size of the wild-type controls. Despite the decrease in tDNA copy number, CTCF mutant animals dissected from pupal cases also showed increases in R1 and R2 expression, and 23S rRNA expression relative to heterozygous sibling animals (Figure 3H), similar to the increased expression of the tDNA we observed in S2 cells when CTCF mRNA and protein levels were reduced. We additionally detected increased abundance of unprocessed junctions (25S-ITS2 and ITS2-26S), which normally are efficiently processed and do not appear in the stable ribosome-bound pool of rRNA, indicating an increase in nascent transcription due to reduced CTCF activity.

In mammalian cells, both DNA binding and CTCF localization in the nucleolus require an active poly-ADP-ribosylation/glycosylation cycle [40], and mammalian nucleolar structure is affected by 3-aminobenzamide treatment [20] which inhibits this cycle. In Drosophila mutated for either enzyme responsible for the cycle (PARP or PARG), the nucleolus is seen to fragment [41,42,43] similar to what we describe for mutants of CTCF. Mutation of either component is expected to result in a similar phenotype, since PARP is itself poly-ADP-ribosylated, which leads to its inhibition. PARP requires PARG to be reactivated, and hence reduction of either gene product results in an inhibition of a robust poly-ADP-ribosylation cycle and a net decrease in this post-translational modification. Therefore, we reasoned that the disruption of nucleoli in PARP or PARG mutants may be a consequence of reduced nucleolar CTCF. We predicted that disrupting PARP and PARG would not only alter the structure of the nucleolus, but would (1) reduce CTCF in the nucleolus, and (2) reduce silencing of the tDNA. We tested these predictions by reducing PARP and PARG activities using double-stranded RNA treatment of S2 cells. Our results showed that reduction of PARP or PARG resulted in nucleolar disruption similar to that seen in cells with reduced CTCF (Figure 4A–D), although to a lesser degree. Genetic mutations of PARP resulted in disrupted localization of CTCF, including a loss from the nucleolus (Figure 4E–F). We did not detect obvious decreases in euchromatic localization of CTCF, indicating that non-nucleolar CTCF either does not require the poly-ADP-ribosylation cycle, or the maternally-supplied PARG is sufficient for proper localization of that subset of CTCF in salivary gland nuclei.

In populations of S2 cells treated with interfering RNAs directed at PARP and PARG (Figure 4G) we observed increased R1 expression, and increased R2 expression in the case of interfering RNA directed at PARG (Figure 4H). Disrupting the poly-ADP-ribosylation cycle, then, has the predicted effects of CTCF loss from nucleoli, disrupted nucleolar structure, and loss of tDNA silencing.

CTCF mutation is not known to affect position effect variegation of marker genes near new junctions of centric heterochromatin and euchromatin of inverted chromosomes or transpositions, and we did not observe effects of CTCF mutation on either w<sup>m4</sup> or w<sup>mut</sup>, two variegating alleles of white<sup>+</sup> (data not shown). This is not surprising, since CTCF is not seen to bind to interphase or condensed heterochromatin of Drosophila [16]. In addition to the transcriptionally-silent centric heterochromatin, the tDNA also induces position effect variegation, as has been detailed in yeast, plants, and Drosophila [39,44,45]. We reasoned that if CTCF is involved in regulation of the tDNA, then mutations in CTCF should act as modifier of variegation, but only if that variegation is induced by the tDNA. The Karpen laboratory generated a series of P-elements inserted in the 1 chromosome which variegate for both white<sup>+</sup> and yellow<sup>+</sup>, one of which mapped to the F-linked tDNA (line D285) [46]. We crossed males carrying this tDNA-linked P-element to females who were heterozygotes for either a CTCF mutation (CTCF[35.2]), or an unrelated chromosomal deficiency that removed CTCF (Df[3L]0465). In parallel, females bearing these CTCF alleles were crossed to non-rDNA inserts. We compared expression of the white<sup>+</sup> marker gene to genetically identical flies who did not have reduced maternal CTCF expression. We confirmed that non-rDNA variegating F-linked alleles of white<sup>+</sup> (Fig. 5A, B) were not affected by maternal heterozygosity of CTCF. However, variegation of the tDNA-linked transpositional insertion was strongly suppressed by maternal heterozygosity of CTCF (Figure 5C), indicating that CTCF acts to repress both the RNA Polymerase I-derived tDNA and these RNA Polymerase II reporter genes early in development.

**Discussion**

We have described the localization of the boundary element protein CCCTC-binding Factor (CTCF) in the nucleolus of Drosophila. We find it in cell types taken from different stages of development, and identify a specific binding site in the resident R1
transposable element using chromatin immunoprecipitation. Knockdown of CTCF activity using RNA interference or mutation resulted in disruption of nucleolar structure and derepression of the two rDNA-resident transposable elements (R1 and R2), a small increase in steady-state processed rRNA, and an increase in preprocessed rRNAs (indicating increased nascent transcription). Reduction of gene activity of either PARP or PARG, thought necessary for CTCF function, resulted in similar phenotypes. As we predicted, mutation of CTCF acted as a suppressor of variegation specifically for an rDNA-inserted marker gene. Our work extends our understanding of the repertoire of functions for the CTCF protein by demonstrating occupancy, binding, and regulation of rDNA by CTCF, and consequence of loss-of-function mutation on rDNA behavior.

Our data support a model for CTCF-mediated regulation of the rDNA consistent with CTCF acting as a direct transcriptional repressor. It is possible that the ability of CTCF to work over long distances, create chromosome interactions that can span hundreds of kilobases, and block both enhancer-promoter interactions and heterochromatin spreading, may contribute to the unique epigenetic regulation demanded by this repeated gene array, which has functional rRNA genes interspersed with silent transposon-inserted or uninterrupted copies. This juxtaposition leads to peculiar behaviors, such as induction of position effect variegation, epigenetic silencing, and nucleolar dominance.

In flies with reduced CTCF activity, we observed effects on R1, R2, and uninserted rRNA expression, despite finding binding only in the R1 element. These experimental results are consistent with genetic and cytological data from other laboratories. Eickbush and colleagues have proposed a hypothesis to explain chromosome-specific rDNA expression. In their model, clustering of R1 (and R2) elements affects expression of closely-linked cistrons [11,32]. Arrays with homogeneously interspersed R1 and R2 elements are therefore poised to be inactivated, while arrays with R1 and R2 elements near the array flanks are active and behave as dominant arrays in conditions which elicit nucleolar dominance. Our finding of a sole CTCF binding site in the R1 element might influence uninserted cistrons (or R2-containing cistrons) by nature of their proximity to CTCF-containing R1-inserted cistrons. This “domain” organization of the nucleolar rDNA is supported cytologically by the presence of intense discrete chromatin foci which contain R1 and R2 elements [12].

When measuring transcription in cells treated with interfering double-stranded RNAs directed at CTCF, we observed a moderate increase in the 35S transcription. This effect was not as dramatic as that seen on the R1 and R2 elements, despite those elements being co-linearly transcribed with the rest of the 35S pre-rRNA. This is consistent with a very long half-life and very high steady-state level of 18S, 5.8S/2S, and 28S rRNAs where changes in transcription affect the pool of rRNAs relatively little, and a low

Figure 5. Maternal heterozygosity for CTCF suppresses rDNA-induced position effect variegation. (A) Expression of white from P-element B486 in wild-type flies derived from wild-type (left), CTCF+/+ (middle), and Df(3L)0463+/+ (right) mothers. (B) Expression of white from P-element ROMA in wild-type flies derived from wild-type (left), CTCF+/+ (middle), and Df(3L)0463+/+ (right) mothers. (C) Expression of white from rDNA-inserted P-element D285 in wild-type flies derived from wild-type (left), CTCF+/+ (middle), and Df(3L)0463+/+ (right) mothers.

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abundance of R1 and R2 transcripts where even small changes in transcription are seen as a large "fold" increase in RNA.

Copies of the R1 element exist at the chromocenter, and a truncated R1-like element is found on chromosome 4 [12,47,48,49], and so it is conceivable that our chromatin immunoprecipitation signal is derived from these clusters. However, Drosophila CTCF is undetectable at either of these locations ([16] and our data), strongly suggesting that the chIP and Real-Time PCR signals we observe derive from the tDNA.

Despite being "unused," silent copies may serve some purpose in tDNA biology and evolution. The mechanism to maintain silent insertions within the arrays may be understood in light of experiments and hypotheses from Peng and Karpen, and work from our laboratory [34,50,51]. Normally the tDNA arrays are subject to very slow loss or magnification [52,53], however nucleoli are disrupted and the tDNA loss rate increases in animals bearing mutations in chromatin modifying enzymes (the Histone H3-K9 methyltransferase Su(var)-9 or the methylhistone binding protein Heterochromatin Protein 1) or those with experimentally shortened arrays [34,35]. We believe the disruption of nucleolar structure by reduction of CTCF, PARP, or PARG to be similar. In all of these cases, we envision that tDNA transcription is increased (by removal of transcriptional repression as in this study, by removal of silencing chromatin modifications in the Peng and Karpen studies, or by compensatory mechanisms to maximize tDNA transcription of short arrays in our previous work), resulting in increased intrachromosomal recombination and subsequent nucleolar fragmentation.

Our results demonstrate a clear role for CTCF in tDNA regulation, but formally we cannot show that this effect is direct, since disruption of CTCF or PARP/PARG are expected to have pleiotropic effects on other genes in the genome. However, our demonstration of CTCF occupancy in the nucleolus by immunofluorescence and chromatin immunoprecipitation both suggest the regulation is direct. This is supported by data from human cells, which shows CTCF to be in the nucleolus by immunofluorescence [20] and chromatin immunoprecipitation [24], and hence we do not believe that CTCF-dependent regulation of the 35S rRNA gene is unique to Drosophila, as the presence of consensus CTCF binding sites within the tDNA repeat unit is conserved in humans and Xenopus [24,31], conserved interactions have been shown between Myc, cohesin, and CTCF, all of which impact rRNA regulation [54,55,56], and overexpression of CTCF truncations affects nucleolar transcription in mammals [20]. Many proteins are facultative or transient members of the nucleolar protein fraction, and dynamic membership of CTCF might be a general mode of rRNA gene regulation.

Materials and Methods

Fly Husbandry and stocks

Fly stocks were maintained at 25°C on standard cornmeal-based medium, supplemented with yeast. Stock w1118 P[act-GFP]TM3, P[w+mut] = Act(GFP)TM3R was obtained from the Kyoto Stock Center. CTCF 107-2/TM6B, Tb and Df(3L)0463/TM6B, Tb were kindly provided by Dr. Pamela Geyer at the University of Iowa and was characterized in a previous study [16]. D2B6, B4B6 and ROMA2 are stocks containing P[Spe/P] transposons to the Y chromosome [44]. C1/DX is C1/DX, y/ b3.3.

Immunofluorescence and Confocal Microscopy

Salivary glands were dissected in phosphate-buffered saline (PBS), incubated for 20 seconds in PBS containing 1% Triton X-100 and 3.7% formaldehyde, then transferred to a solution of 3.7% formaldehyde and 50% acetic acid for 2 minutes and immediately washed. Slides were washed twice in PBS for 10 minutes, transferred to PBS containing 0.1% Triton X-100 for 10 minutes and blocked with PBS containing 1% BSA for one hour at room temperature. Primary antibody was added and slides were incubated overnight at 4°C. Rabbit anti-CTCF antibody was used at 1/500 dilution. Mouse anti-Fibillin1 antibody was purchased from Abcam and was used at 1/200 dilution. Slides were washed twice in PBS containing 1% BSA and incubated with secondary antibody at room temperature for 2 hours. Goat anti-rabbit conjugated to rhodamine and goat anti-mouse conjugated to DL488 (Jackson ImmunoResearch) were each used at 1/200 dilution. Slides were immersed in 4',6-diamidino-2-phenylindole (DAPI) at 1 ng/mL for 5 minutes, washed, and mounted in Vectashield (Vector Laboratories).

Brains were dissected in 0.7% (w/v) sodium chloride, incubated for 7 minutes in 0.5% (w/v) Sodium Citrate, transferred to a solution of Methanol/Formaldehyde/water (11/11/2 ratio) for 30 seconds, then transferred and squashed in 45% acetic acid. Thereafter, the slides were treated as above.

For whole mount salivary glands, dissection was performed in PBS containing 1% Triton X-100, then transferred to PBT (PBS containing 0.1% Tween-80) and fixed in PBS containing 1% Triton X-100 and 3.7% formaldehyde. The tissue was blocked for 1 hour in PBT supplemented with 1% BSA. Primary antibodies were diluted in PNB (PBT containing 1% BSA and 500 mM NaCl) and incubated overnight at 4°C. The tissue was then washed in PNB and incubated in secondary antibody for 2 hours, then washed and mounted in 70% glycerol.

For confocal microscopy, sequential excitation was performed at 488 nm (for DL488), 543 nm (for Rhodamine) and 405 nm (for DAPI) in an Olympus FV1000 confocal microscope. The images were processed using FV19-ASW 1.7 Viewer.

S2 cell immunofluorescence was performed as described [57]. Quantification of CTCF and fibrillarin immunofluorescence signal was done by independently capturing DAPI (for DNA), and rhodamine and fluorescein (for protein epitopes) channels and exporting to NIH Image-J. Entire fluorescence signals were integrated and divided by exposure time to determine intensity/time in arbitrary units. Individual nucleus measurements were normalized to DAPI signals to create datasets amenable to graphical and statistical comparison.

Chromatin Immunoprecipitation

ChIP experiments were carried out as described [58], with some modifications. Briefly, 200 uL of third instar larvae were used per immunoprecipitation reaction; chromatin was cross-linked for 10 minutes at room temperature with 1% formaldehyde. Sonication was performed for 8 minutes, with 20 second pulses followed by 40 seconds "cooling off" period. After confirming fragment size averaging approximately 500 base pairs, protein concentration was estimated using the Bradford assay. 500 g of chromatin was incubated with 3-4 uL of rabbit anti-CTCF antibody. 50 g of chromatin was set apart as input. For all buffers, PMSF and Complete Protease Inhibitor Cocktail tablets (Roche) were used as protease inhibitors. DNA was diluted in 1/20 for antibody and no antibody samples and 1/300 for input. Real Time PCR was used for quantification of precipitated DNA.

Primers used to amplify regions shown in Figure 2 are: 1 GGTGTGCAGAACAGTGCTGATC and CGAGGTGTGGTTGGCAGTC; 2 GCACAACACCTGTCATCA and GAGGTGTCGGCAACAC; 3 GAGTAGCCAAACCTCGTCGTCATC and CGAGGTGTGGCAACAC; 4 GTGGTTGTCTAGCAGAGGTTGCT and CAATATGAGGTGCACCA; 5
Reverse Transcriptase Real Time PCR (RT-Q PCR)

RNA from adult flies or S2 cells were extracted as described [59]. Primers used for the reverse transcriptase reaction were: 35S TCGCTAG, TAATTAGTGACGCGC and (d) CCCTTGGCTGTGGTTCTTGATTCGA, uninserted R1 and R2 (a) GCCTCGTCATCGC and (c) CCACGAGCGCAACGAAAACACG, R2 (e) CTCTAGCCGAACACTCCAAATAGG. Real-Time PCR: 35S 2S PCR amplified a fragment of the GTCCGATCACCGA were used as endogenous control which sequence AAGTTGTGGACGAGGCCAAC and CGGTTCTC- GGGAGTGATGGAGTTTCCG. Primers with sequence GAATAATTAGCGG and CCGAGGTGTAATATCTCCCAC, GAGCACATAAACC, 24 CAAGTAAGCGCGGGTCAACGG GG, 23 GCAGCTGGTCTCCAAGGTG and CCCAGAAC- GTGCACTCTAC and CCAGCAATCGTATGCTCGCTG, 29 GAATAATTAGCGG and CCGAGGTGTAATATCTCCCAC, GAGCACATAAACC, 24 CAAGTAAGCGCGGGTCAACGG GG, 23 GCAGCTGGTCTCCAAGGTG and CCCAGAAC- GTGCACTCTAC and CCAGCAATCGTATGCTCGCTG, 29

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