

Evidence for rolling circle replication of tandem genes in *Drosophila*

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ABSTRACT

Extrachromosomal circular DNA (eccDNA) is one characteristic of the plasticity of the eukaryotic genome. It is found in various organisms and contains sequences derived primarily from repetitive chromosomal DNA. Using 2D gel electrophoresis, we have previously detected eccDNA composed of chromosomal tandem repeats throughout the life cycle of *Drosophila*. Here, we report for the first time evidence suggesting the occurrence of rolling circle replication of eccDNA in *Drosophila*. We show, on 2D gels, specific structures that can be enriched by benzoylated naphthoylated DEAE-cellulose chromatography and were identified in other systems as rolling circle intermediates (RCIs). These RCIs are homologous to histone genes, *Stellate* and *Suppressor of Stellate*, which are all organized in the chromosomes as tandem repeats. RCIs are detected throughout the life cycle of *Drosophila* and in cultured fly cells. These structures are found regardless of the expression of the replicated gene or of its chromosomal copy number.

INTRODUCTION

The eukaryotic genome is organized in linear chromosomes. Yet, a minor fraction of the DNA also exists as a population of small circular molecules named extrachromosomal circular DNA (eccDNA). This DNA was reported in many organisms including yeast, *Drosophila*, *Xenopus*, mice, hamster, monkeys and humans [reviewed in (1)]. It is homologous to a wide variety of chromosomal sequences, primarily to repetitive chromosomal DNA. Hence, it is believed to be derived from the chromosomes.

A neutral–neutral 2D gel electrophoresis method facilitates the characterization of eccDNA. In this technique, DNA

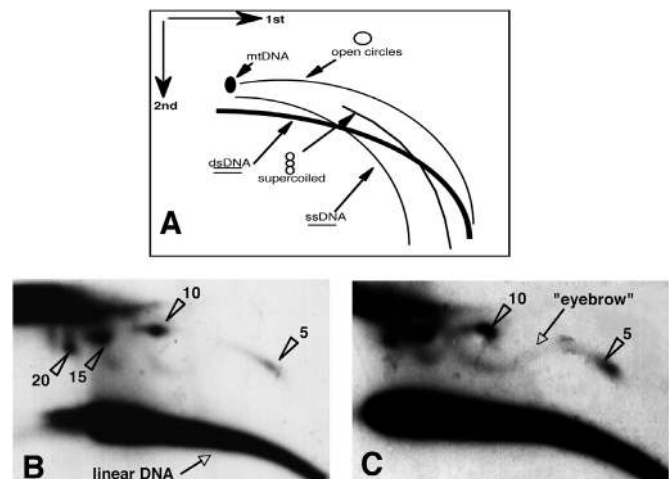


Figure 1. Migration pattern of eccDNA and RCIs of histone genes. (A) Schematic outline of the 2D gel electrophoretic patterns of genomic DNA generated by populations of linear and circular molecules (3). Each arc consists of molecules sharing the same structure, but differing in mass. This analysis identifies double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), relaxed (open) circular molecules, supercoiled molecules and mitochondrial DNA (mtDNA). (B-C) A 2D gel of wild-type (Canton-S) adult DNA was hybridized with a cloned *Drosophila* histone H3 gene probe. A short exposure (B) revealed distinct circular multimers of 5 kb (arrowheads) and a massive arc of double-stranded linear DNA. In longer exposure (C) specific arcs emerging from each spot were detected (arrow, 'eyebrow'). Similar results were obtained with histone H4 as a probe (data not shown).

molecules are separated according to both their size and structure (2,3). Thus, a population of molecules sharing the same structure, but of heterogeneous molecular mass, generates a continuous arc on such a gel. Arcs typical of supercoiled molecules, open circles and linear molecules can be distinguished following hybridization (Figure 1A).

eccDNA has been noted in *Drosophila* embryos and cultured cells (4,5), and we recently employed the 2D gel method to begin a systematic study of eccDNA in *Drosophila* (6).

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We found that in *Drosophila* eccDNA, ranging from <1 kb to >20 kb, comprises up to 10% of the total cellular repetitive DNA and is present throughout the fly's life cycle. We showed that the eccDNA population contains circular multimers of tandemly repeated coding genes, such as histones, rDNA, *Stellate* and the *Suppressor of Stellate*, and of tandemly repeated sequences from centromeric heterochromatin.

One of the primary questions regarding eccDNA is whether these molecules can replicate. In yeast, extrachromosomal ribosomal circles (ERCs) contain ARS elements (autonomously replicating sequences) that may function as an origin of replication permitting their extrachromosomal bi-directional replication (7). However, eccDNA is very heterogeneous, and sometimes consists of very short satellite repeats. Therefore, a specific initiation site that obeys the cell-cycle control is not likely to account for the entire eccDNA population.

Rolling circle replication (RCR) is an additional mode for replicating circular DNA. Several examples of RCR exist in viral genomes, such as the T4 bacteriophage (8) and the Geminivirus in plants (9,10). The mitochondrial DNA of the malaria parasite also replicates by a rolling circle mechanism (11). RCR of extrachromosomal telomere circles was proposed as a mechanism for telomerase-independent elongation of telomeres both in yeast and in human cells (12–16).

To date, the only example of RCR of chromosomal coding genes in higher eukaryotes occurs during the extrachromosomal amplification of the ribosomal genes in early oogenesis of amphibians (17–19). The amplified extrachromosomal rDNA accumulates in the egg and enables transcription of large amounts of ribosomal RNA required for early development of the young embryo.

Here, we report for the first time evidence suggesting the occurrence of RCR of eccDNA in *Drosophila*. We show, on 2D gels, intermediates of RCR that can be enriched on benzoylated naphthoylated DEAE-cellulose (BND-cellulose) chromatography. These rolling circle intermediates (RCIs) are found throughout the life cycle of *Drosophila* and in cultured fly cells and are homologous to several genes that are organized in tandem repeats. The presence of RCIs is independent of the number of chromosomal repeats of the replicated sequence and of its expression. Possible implications of this finding on the metabolism of tandem repeats are discussed.

METHODS

Preparation of genomic DNA

Total genomic DNA was prepared by rapid homogenization of 50–100 mg *Drosophila* embryos/larvae/adults and of one 25 cm² flask of cultured Kc cells in 30 mM EDTA, 1% SDS, 0.5% Triton X-100, 0.3 M NaCl, and subsequent incubation at 50°C for 90 min with 1 mg/ml proteinase K. The DNA was extracted with equal volumes of phenol and phenol:chloroform and precipitated with ethanol or isopropanol. The DNA was resuspended in 1× SSC and digested with 0.2 mg/ml RNase A for 1 h at 37°C followed by phenol:chloroform extraction and ethanol precipitation. The precipitated DNA was resuspended in TE and was ready for further manipulations. Usually the yield was sufficient for 5–10 2D gels.

Neutral–neutral 2D electrophoresis

Separation of DNA in neutral–neutral 2D gels was performed as described by Brewer and Fangman (20), with modifications as described previously (2,3). Briefly, the DNA was first separated on 0.4% agarose at 1 V/cm in 1× TBE and the gel was rinsed in 1× TBE containing 0.3 µg/ml ethidium bromide (EtBr). The lane of choice was cut and placed on a clean gel support at 90° to the direction of the first electrophoresis. The lane was cast with 1% agarose containing 0.3 µg/ml EtBr and was electrophoresed in 1× TBE in the presence of 0.3 µg/ml EtBr. The first dimension was run overnight at 1 V/cm, and the second dimension for 5 h at 4 V/cm, both at room temperature.

Blotting and hybridization

The gels were agitated in 0.25 M HCl for 30 min, rinsed in water and agitated again in 0.4 M NaOH for at least 30 min. Southern blotting was performed with Hybond N⁺ nylon membranes (Amersham). Probes were labeled by random priming kit (Biological Industries, Israel). As probes for histone H3 and 28S rDNA we used PCR fragments that were prepared according to the published corresponding sequences (accession numbers X14215 and M21017, respectively). The PCR products were sequenced and verified for accuracy. The *Stellate* probe was a 700 bp insert of p14Sa4 containing the *Stellate*-specific Sau3A I fragment. The *Suppressor of Stellate* [*Su(Ste)*] probe was the 350 bp insert of pBKS-Ysp containing the Y-chromosome-specific fragment of *Su(Ste)*.

Radiolabeled DNA was detected by autoradiography and the signal was quantified with a PhosphorImager (Fuji FLA 2000) using the Tina 2.10g software. Owing to the difficulties in accurately quantifying the signal of the replication intermediates or of RCIs, we quantified the linear DNA signal, and always compared DNA samples having similar levels of linear DNA, and looked for difference in the amount of RCIs or the Y-shape replication intermediates.

BND-cellulose chromatography

BND-cellulose enrichment of replicating DNA molecules was performed on total genomic DNA that was prepared from adult flies. DNA samples were digested with EcoRV prior to their separation on the BND-cellulose. EcoRV leaves the histone gene complex intact but cleaves rDNA within the coding region and generates a fragment of 3800 bp that is visible using 28S rDNA probe and serves as a positive control for the enrichment of Y-shaped chromosomal replication forks. For the analysis of '*Stellate*' RCIs, DNA was cleaved with BamHI, which leaves '*Stellate*' genes intact, and cleaves the histone gene complex into a linear fragment of 5 kb, whose Y-shaped replication intermediates can be analyzed as a control (data not shown).

Reactions were carried out exactly as described by J. Huberman, (<http://saturn.roswellpark.org/huberman/BNDCellulose.html>). The caffeine-eluted DNA that contains the replication intermediates was compared with the input DNA, to confirm the enrichment of the replication intermediates.

Fly and cell techniques

All fly strains were maintained, and crosses were performed, at 25°C in shell vials supplemented with cornmeal-molasses *Drosophila* medium.

The fly strains used were Canton-S and Oregon-R (21), females bearing the FM7 chromosome, which contains a high copy number of *Stellate* (22) and Attached-X (XXY) females C(1)DX, y^1, f^1 [to examine *Su(Ste)* DNA in females], *abo*¹/SM1 (Bloomington) and *abo*²/Cy (S. Pimpinelli) were crossed to each other to get the heteroalleles *abo*¹/*abo*². A strain carrying a deletion of the entire HIS-C Df(2L)-TW161, *cn bw*/CyO, abbreviated here as Df(2L)TW161/CyO, was crossed to *okra*^{A17-11} *cn bw*/CyO, GFP *cn pr* to obtain hemizygous and heterozygous offspring that share the same genetic background. All strains, mutations and balancer chromosomes are described in FlyBase (21). Embryos and larvae were staged as described previously (6).

Kc cells and Schneider 2 cells were grown in Schneider medium containing 10% fetal bovine serum (Hyclone), 100 U/ml penicillin and 0.1 mg/ml streptomycin in 25 cm² flasks at 25°C. Cells were harvested 3–4 days post-seeding for DNA preparation.

RESULTS

RCIs of histone genes are detected by 2D gels

The histone genes complex (HIS-C) in *Drosophila melanogaster* consists of five structural genes (His1, His2A, His2B, His3 and His4) for the five different histones (H1, H2A, H2B, H3 and H4) and is clustered on chromosome 2 in ~100 tandem copies. The repeating unit appears in two forms of either 5 kb (the predominant form) or 4.8 kb long (the minority form) and each form is clustered with its own kind (23).

We have previously found that, as for any chromosomal tandemly repeated sequence examined, eccDNA homologous to histone genes occurs primarily as multimers of the 5 kb unit (6) (Figure 1B). Our gel conditions cannot resolve circles of 4.8 kb from 5 kb, as was tested using plasmids of the corresponding sizes (data not shown). In addition, restriction enzyme analysis revealed that the 4.8 kb variants comprise <15% of the forms in two of the strains (Canton-S and Oregon-R) used throughout most of our analysis (data not shown). Hence, even if the 4.8 circular variant could be resolved on the 2D gel, it would probably be under-detected.

Long exposure of the membranes revealed specific continuous sigmoid arcs, emerging from each circular multimer and extending toward higher molecular weights (Figure 1C). This pattern resembles that seen for σ -structures, which were well documented by 2D gel analysis of plasmid replication products, and represent broken θ -replication intermediates whose mass ranges between 1 and 2X (where X is the size of the template) (24,25). However, the typical pattern of θ -replication intermediates (20) is missing from our gels. Furthermore, the arcs we detected extend beyond the 2X size and may consist of σ -structures harboring a longer tail. Hence, we suspected that the molecules comprising the sigmoid arcs are RCIs. Indeed, such arcs have been previously observed using 2D gels and were identified as RCIs in the following experimental systems.

The first example is the replication of 6 kb long circular molecules consisting of mitochondrial DNA of the malaria parasite *Plasmodium falciparum*. A 2D gel analysis revealed a sigmoidal pattern, designated ‘eyebrow’, starting at the spot of the relaxed circular 6 kb monomer and running toward the high-molecular weight region of the gel [see Figure 6 in (11)]. Analysis by electron microscope identified within the mtDNA ‘lariats’, i.e. linear molecules of various lengths with single terminal loops. The authors assert that the ‘eyebrow’ is a signature of lariat molecules engaged in the process of RCR. Second, RCR products of the *in vitro* system of bacteriophage T4 generated a specific ‘eyebrow’ arc on 2D gel [Figure 3 in (8)]. Furthermore, RCR can generate intermediates with a single-stranded or double-stranded tail. Belanger *et al.* (8) have created *in vitro* conditions that can yield either type of T4 RCIs, and their 2D gel analysis could discriminate between them. The ‘eyebrow’ arc of T4, which is similar to those we obtained with histone circles, is specific for RCR of both leading and lagging strands. A different migration pattern of the bacteriophage arcs was observed under conditions that allow only single-stranded RCR. Accordingly, we found that cleavage with enzymes that cut within the histone gene cluster (e.g. HindIII, XhoI, EcoRI and BamHI) completely abolished the histone-related RCIs. This further indicates that they are double stranded (data not shown).

Third, a cell-free system derived from *Xenopus* egg extract and a plasmid DNA template yielded RCIs in the presence of the topoisomerase II inhibitor VP16 [Figure 6 in (25)], whose migration is identical to the putative histone RCIs we observe.

Taken together, these reports strongly support our conclusion that the pattern of the sigmoid arcs we observe represents DNA structures of RCIs of eccDNA homologous to histone genes.

It is worth noting that our purification procedure of the DNA samples included extensive proteinase K digestion and phenol extractions, making it unlikely that the DNA ends would be held together by proteins and form an artifact that would migrate like RCIs.

RCIs can be enriched by BND-cellulose chromatography

To verify the presence of replication forks on the putative RCIs that we detected on 2D gels, we asked whether the molecules that comprise the sigmoid arcs could be enriched by BND-cellulose chromatography. Such enrichment is used for the analysis of replication intermediates in many eukaryotic systems (26,27). It is based on the binding of the single-stranded region that exists in any replication fork to the column, and its separation from the majority of the double-stranded non-replicating DNA. The replication forks can be released from the column by caffeine.

DNA samples from adult flies were cleaved with EcoRV prior to their separation on BND-cellulose. EcoRV leaves the histone gene complex intact, hence enabling the detection of histone RCIs. EcoRV also cleaves the repeating unit of rDNA within its coding region and generates a fragment of 3800 bp that can be detected using 28S rDNA probe. The Y-shaped replication intermediates of this linear fragment can be visualized on the same 2D gel. Thus, these Y shapes serve as a positive control for the enrichment of chromosomal replication forks in the BND-cellulose-bound DNA. Indeed 2D gel

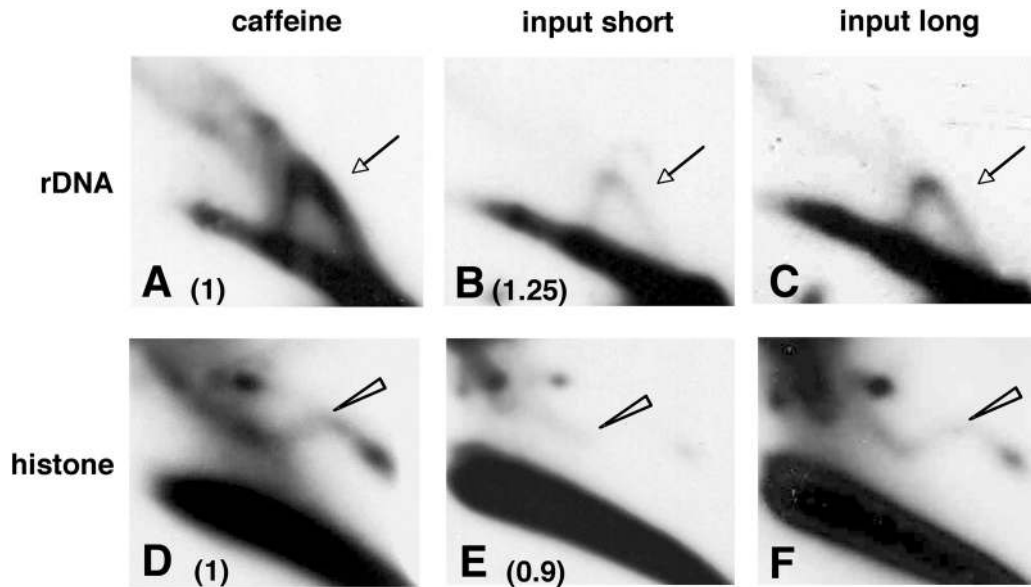


Figure 2. RCIs are enriched by BND-cellulose chromatography. Genomic DNA from *Drosophila* adults was cleaved with EcoRV and subjected to BND-cellulose chromatography. The BND-cellulose-bound DNA was eluted by caffeine (caffeine, **A**, **D**) and was analyzed on 2D gel in comparison to a non-enriched sample of the same DNA (input, **B**, **C**, **E**, **F**), and quantification was performed as described in Materials and Methods. Hybridization with 28S rDNA probe reveals Y-shaped chromosomal replication forks of a 3.8 kb EcoRV fragment (arrow). Enrichment of replication forks is observed in the caffeine fraction (**A**) in comparison with their level in a similar amount of the input DNA (**B**) or even after a longer exposure of the input (**C**). Similarly, hybridization with histone H3 probe clearly demonstrates the enrichment of the putative RCIs (arrowhead) in the caffeine fraction (**D**) compared with their level in the input DNA (**E**) even at a long exposure (**F**). The numbers in parentheses refer to the relative values of the linear DNA signals in each comparison of two samples hybridized with the same probe, where the signal of the caffeine eluted DNA is 1.

analysis revealed enrichment of the Y-shaped replication forks of 28S rDNA by BND-cellulose (Figure 2A) in comparison with their level in a sample of the same DNA prior to the chromatography, i.e. the input DNA (Figure 2B and C). Re-hybridization with histone H3 probe shows that the sigmoid arcs were also enriched by BND-cellulose (Figure 2D) compared with their level in the input DNA sample (Figure 2E and F). As shown below (Figure 4), sigmoidal arcs homologous to *Stellate* genes DNA could also be enriched by BND-cellulose. Quantification of the replication intermediates and of RCIs is not trivial mostly due to high variability in background levels. Nevertheless, we quantified the data obtained from several independent experiments to get an estimate of the enrichment of BND-cellulose-bound DNA. The estimated enrichment of chromosomal replication intermediates is 4- to 10-fold and that of RCIs is 3- to 6-fold.

This experiment demonstrates co-enrichment of chromosomal replication forks with the extrachromosomal molecules that generate the sigmoid arc on 2D gels. It suggests the presence of extrachromosomal replication forks in these molecules and further supports their identification as RCIs.

RCIs of eccDNA are abundant

We detected RCIs corresponding to the histone gene cluster in adult flies of every laboratory strain tested and asked whether they are found in other stages of the *Drosophila* life cycle. We found histone RCIs in DNA from early embryos, and in first, second and third instar larvae of the wild-type strains (Figure 3) (data not shown). Hence, RCIs accompany the wide distribution of eccDNA in many stages during development.

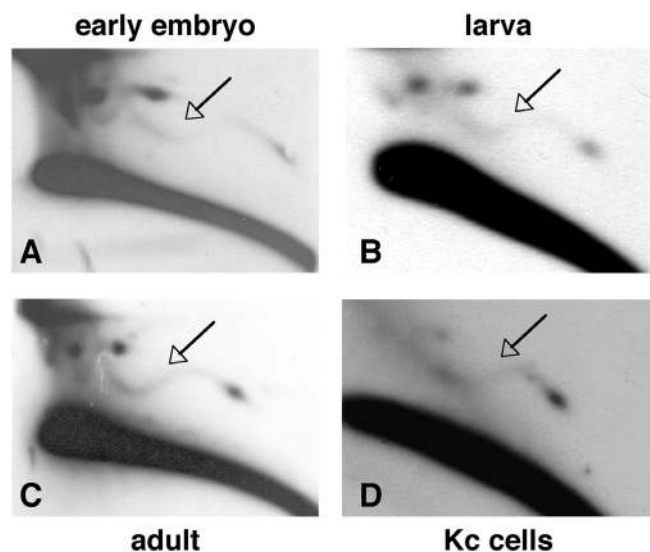


Figure 3. Histone genes RCIs exist throughout the life cycle of *Drosophila*. 2D gel blots of DNA samples from (A) early embryos, (B) 3rd instar larvae, (C) adult flies, and (D) Kc cells were probed with histone H3. Arrows indicate RCIs.

We next asked whether RCIs are also found in cultured *Drosophila* cells. eccDNA has been detected in embryonic Schneider (S2) and Kc cells (4,28) using different assays, but no replication intermediates of these molecules were ever reported. We analyzed genomic DNA from S2, S2R+ and Kc cells and found RCIs homologous to histone H3 forming the same pattern obtained *in vivo* (Figure 3D) (data not shown).

RCIs are not restricted to histone genes

An intriguing question is whether RCIs can occur on eccDNA of tandemly repeated sequences other than histone genes. RCIs are identified according to their typical migration pattern on a 2D gel, hence can be detected only between circular multimers that are well separated from each other on this gel. Therefore, to determine whether RCIs corresponding to additional sequences do exist we chose to use as probes sequences of relatively large tandem repeats, such as *Stellate* (*Ste*) and [*Su(Ste)*].

Copies of the *Stellate* gene are organized in euchromatic and heterochromatic clusters of tandemly repeated units of 1.25 or 1.15 kb, respectively, on the X chromosome of *Drosophila* (22,29). Their eccDNA appears as clearly spaced spots (6), and thus their RCIs, if they exist, should be detectable. Indeed, we identified RCIs corresponding to the *Stellate* genes upon long exposure of membranes carrying DNA from several laboratory strains, including the wild-type strains Oregon-R (data not shown) and Canton-S (Figure 4C), and females carrying the FM7 X-chromosome, which contain high copy number of *Stellate* genes (Figure 4D). As seen for histone RCIs, *Stellate*-homologous RCIs could also be enriched by BND-cellulose chromatography (Figure 4A and B).

To verify that the sigmoid patterns observed with *Stellate* probe are indeed RCIs as those observed for HIS-C, we re-hybridized the membrane of Figure 4D with histone H3 probe (Figure 4E). Figure 4F shows the merged image of Figure 4D and E. The 5 kb histone circular monomer co-migrated with one of the *Stellate* multimers, and the sigmoid arcs emerged from both co-migrated as well.

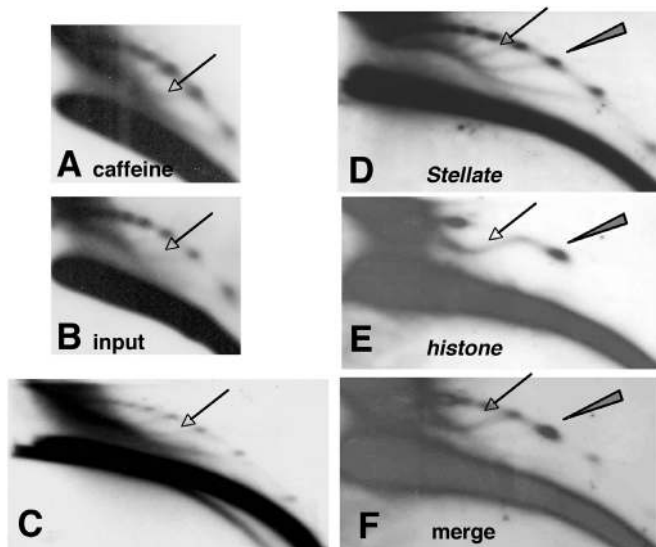


Figure 4. RCIs corresponding to *Stellate* genes can be enriched by BND-cellulose and they co-migrate with histone RCIs. (A-B), Genomic DNA cleaved with BamHI was analyzed before (B) and after (A) BND-cellulose chromatography and probed with *Stellate*. DNA from adult males (Canton-S, C) and females (FM7, D) was analyzed on 2D gel. The membrane from D was re-hybridized with histone probe (E), and the merge of D and E (F) demonstrates the co-migration of the histone RCIs emerging from the 5 kb monomer, with those emerging from one of the *Stellate* multimers. Arrows indicate RCIs and arrowheads indicate a circular monomer (histone) or multimer (*Stellate*).

RCIs corresponding to the Y-linked gene [*Su(Ste)*] were also detected, although in a less distinct pattern. This is because of the variability in the length of the *Su(Ste)* repeat, which can be either 2.5 or 2.8 kb (30) and their heterogeneous organization in the clusters (31). Therefore, its eccDNA probably consists of a mixture of both variants, generating a rod-like pattern on the arc of eccDNA rather than the distinct spots that are typical of exact multiples of a defined unit (6). Accordingly, the RCIs emerging from each spot of *Su(Ste)* are fuzzier (Figure 5).

Note that we did not observe a significant difference between males and females in the amount of RCIs homologous to histones (data not shown), *Ste* (Figure 4) and *Su(Ste)* (Figure 5, XXY females were used). The differences in the amounts of RCIs shown in Figure 4C and D is due to differences in the copy number of the tested gene and the fact that different strains tend to generate eccDNA at different levels.

The 18S and 28S rDNA genes appear in tandem arrays of 150–250 repeating units on chromosomes X and Y. However, their unit size is heterogeneous owing to the variation in the length of the intergenic spacer. Therefore, the eccDNA homologous to 28S rDNA (as a probe for the entire rDNA unit) typically migrates as a short arc of large circles (>10 kb), in which discrete spots representing multimers cannot be detected due to their length heterogeneity (6). Thus, the 2D gel method is not useful for identifying RCIs homologous to rDNA.

Similarly, because of the limitations of the 2D gels, it was impossible to test for the presence of RCIs emerging from eccDNA composed of multiples of short tandem repeats, such as the 5S rDNA (a unit of 375 bp) and some satellite DNA (repeating units of several bp to 359 bp). These eccDNAs appear as discrete spots, which are very close to each other or form a continuous arc, respectively (6). Therefore, if RCIs did emerge from these circles they would generate a uniform smear below the arc of eccDNA and might be difficult to identify. Yet, eccDNA homologous to the 5S rDNA did bind to the BND-cellulose, as was detected following hybridization of the membrane used in Figure 2A and D, that carries the caffeine eluted DNA, with 5S rDNA

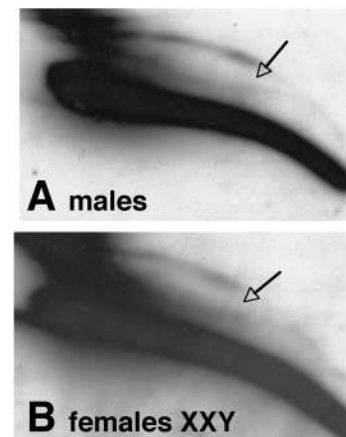


Figure 5. RCIs of *Suppressor of Stellate* genes. DNA from adult males (Canton-S) (A) and XXY females (B) was analyzed on 2D gel and probed with *Suppressor of Stellate*. Arrows indicate RCIs.

probe (data not shown). This finding suggests that extrachromosomal replication forks are also present on eccDNA of the 5S rDNA.

Our findings show that RCIs can occur on eccDNA of several different sequences from the *Drosophila* genome, suggesting that they may be a common feature of eccDNA, including those circles consisting of satellite DNA and rDNA repeats.

RCR and gene amplification

The finding of extrachromosomal replication raises the question of whether this is a new mode of gene amplification. The only known example of RCR in higher eukaryotes is the ribosomal gene amplification in the oocytes of amphibians (17–19). However, unlike such programmed gene amplification, which occurs in specific cells at a defined developmental stage, RCR in *Drosophila* is independent of the developmental stage.

We next asked whether the RCR of coding genes in *Drosophila* might play a similar role to that of ribosomal DNA amplification in amphibian oogenesis, i.e. providing extra copies of genes to be transcribed in large doses. The following evidence suggests that this is probably not the case.

RCIs occur independently of gene expression

We detected RCIs of the X-linked *Stellate* genes in both males and females (Figure 4C and D), although these genes are transcribed only in male testes (29). Interestingly, we detected RCIs of the Y-linked *Su(Ste)*, in reproductively normal females carrying a Y chromosome in addition to two X chromosomes (Figure 5B, XXY), although this gene too is expressed exclusively in the testes (31,32). These findings demonstrate that RCR of a gene occurs even where the gene is not expressed.

Mutants in the gene *abnormal oocyte* (*abo*) exhibit overproduction of histone proteins (33). If the role of RCR was to provide more of the protein encoded by the replicated gene (in addition to its chromosomal copies), one would expect a reduced level of RCIs of the histone gene cluster when the chromosomal histone genes are overexpressed as in *abo* mutants. However, in these mutants (*abo*¹/*abo*² heteroalleles), we found levels of histone RCIs similar to those observed in their heterozygous siblings (Figure 6).

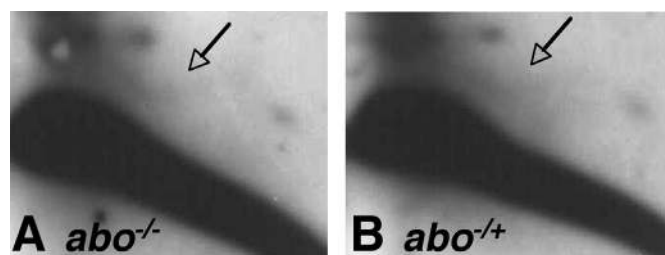


Figure 6. The *abo* mutation does not affect RCIs corresponding to histone genes. DNA from mutant *abo*¹/*abo*² flies (A) and their heterozygous sibling flies, which are a mixture of *abo*¹/*Cy* and *abo*²/*SM1* and are labeled *abo*^{+/+} (B) was analyzed on 2D gel and probed with histone H3. Arrows indicate RCIs. The samples in both A and B contain similar levels of linear DNA as was quantified by PhosphorImager, yet no clear difference in the level of RCIs was observed.

RCIs are independent of histone gene copy number

If the role of RCR was to amplify certain genes, one would expect the process to be more intense when the chromosomal copy number of the corresponding genes is below normal. For example, 'Compensation', an increase of the copy number of histone genes has been proposed to occur in hemizygous flies (34). This increase was reported to be a gradual process that takes place during several generations and is induced when replacing the balancer chromosome that contains the normal set of histone genes, with a new balancer.

To test this possibility, we asked whether hemizygous flies carrying a chromosomal deletion of the entire histone locus [Df(2L)TW161] display elevated levels of both eccDNA and RCIs corresponding to histone genes. Since differences in genetic background may affect the levels of eccDNA, we crossed the Df(2L)TW161/*CyO* flies to another laboratory stock (*okra*^{A17-11} *cn bw/CyO* GFP *cn pr*) that carries two normal sets of the histone gene clusters. This cross yielded three phenotypic groups that enabled the comparison of hemizygous flies with their siblings. We could not detect any clear difference in the levels of histone eccDNA and RCIs between the genotypes, indicating no compensation for the deficient histone clusters by extrachromosomal replication (data not shown). In addition, testing the flies carrying the deficiency and the newly crossed-in balancer (*CyO*, GFP) over several generations did not reveal any increase in the amount of eccDNA or RCIs homologous to histone genes, which could account for the acquisition of extra copies (data not shown). It should be noted that since eccDNAs (and RCIs) of HIS-C comprise a small fraction of the total content of HIS-C copies (~1–2%, according to some of our previous quantifications), we would expect their level to rise several-fold if they were the means for increasing the total cellular copy number of HIS-C. Such an increase in eccDNA and/or RCIs should have been clearly visible on the 2D gels.

We conclude that neither the expression level of histone genes nor their copy number in the chromosomes affects the level of RCIs corresponding to these genes.

DISCUSSION

In this work, we provide structural evidence for a RCR of several genes in *Drosophila*. It is based on the migration patterns of eccDNA on 2D gels, which revealed signals that were previously attributed to RCIs in other experimental systems: the *in vitro* systems of bacteriophage T4 and of *Xenopus* egg extracts, and the mtDNA of the malaria parasite. We further confirmed the identity of the RCIs as molecules that harbor replication forks by demonstrating their enrichment by BND-cellulose chromatography. The possibility that the molecules we refer to as RCIs were in fact some kind of recombination intermediate in the formation of eccDNA from a linear DNA substrate is unlikely, since such substrates have not been detected, and if existed they would have generated circular molecules mainly by non-homologous end joining (NHEJ). Yet, NHEJ is probably not the main mechanism of eccDNA formation as previously discussed in detail by us (6,35,36). Furthermore, our analysis of the kinetics of eccDNA formation in *Xenopus* egg extracts revealed open circles as the earliest detectable structure, whereas DNA molecules migrating as

RCIs have not been detected (36). This suggests that RCI-like molecules are not early intermediates of eccDNA formation.

The abundance of RCIs throughout the life cycle of *Drosophila* suggests that they reflect a normal process. Understanding the mechanism of RCR requires its biochemical and physiological characterization. While the data reported here is based on *in vivo* experiments, our finding that RCIs also occur in cultured cells opens the way for such characterization *in vitro*. While the role of RCR is still unclear, we gained some insight about it. First, it appears that RCR is not related to a functional gene amplification, i.e. RCR occurs independently of the chromosomal copy number of the amplified genes or the level of their expression. This was demonstrated by the fact that neither deletion of histone genes in hemizygous flies nor overexpression of histone genes in *abo* mutants affected the levels of histone genes RCIs. Second, RCR also occurs when the replicated sequences are not expressed. Such is the case of *Stellate* and *Su(Ste)*, which are specifically expressed in the testes, yet their RCIs were also detected in females (Figures 4 and 5).

A phenomenon of 'rDNA magnification' has been described in the past to account for the acquisition of extra-rDNA copies in *bobbed* mutant flies that carry deletions in the rDNA clusters (37). One of the mechanisms suggested to explain magnification (though not supported by later studies) included the extrachromosomal replication of rDNA circles and their subsequent integration to the chromosome (38,39). Although we have detected rDNA circles in *Drosophila* (6), this mechanism could not be tested using the 2D gel method because RCIs of rDNA cannot be resolved due to their size heterogeneity. In addition, while magnification occurs only under 'magnifying conditions', i.e. specific genetic combinations of the rDNA-deficient males (or females that harbor the Y chromosome), we observed eccDNA of rDNA and RCIs (of other genes) in wild-type males and females and throughout their life cycle. Thus it seems unlikely that this RCR is related to magnification.

The RCR we describe suggests that distinct genomic sequences may escape the tight control of replicating once and only once per cell cycle as occurs in gene amplification. The canonical cases of gene amplification occur in a programmed manner during development (e.g. chorion gene amplification in *Drosophila* or ribosomal gene amplification in *Xenopus*), or reflect genomic instability in cancerous cells, such as N-Myc amplification in human neuroblastoma [reviewed in (40)]. However, here we report for the first time that tandemly repeated genomic sequences, including coding sequences, might replicate outside the chromosomes irrespective of the developmental stage of the fly. Whether or not this process leads to a change in the total genomic content of the replicated sequences is not known. As described below, the formation of eccDNA may generate a deletion in the chromosomal clusters of tandem repeats while RCR of eccDNA may form extra copies of the very same repeats.

Our previous results from a *Xenopus in vitro* system indicate that the formation of eccDNA does not require DNA replication and can occur with any cluster of tandem repeats, most probably by homologous recombination within the chromosome (looping out) (35,36). Hence, excision of eccDNA should generate a deletion within the cluster of tandem repeats. Accordingly, although not required for the formation of

eccDNA, we detected DNA synthesis on the newly formed eccDNA in *Xenopus* egg extracts. This synthesis was independent of normal initiation of chromosomal replication but was sensitive to aphidicolin, indicating the involvement of the replicative polymerases α and δ (35,36).

RCR may contribute to the accumulation of extra copies of sequences that are included in the eccDNA, but it is not clear whether these replication products can re-integrate into the chromosome. RCR and a subsequent gene conversion or integration were proposed to explain telomerase-independent elongation of telomeres both in yeast (12–14) and in human ALT cells (15,16). We suggest that RCR may occur on any eccDNA, and if so, the linear products may re-integrate into the chromosome, thus contributing to the expansion of tandem repeats.

Expansion of tandem repeats occurs readily and is traditionally explained by the sloppiness of the replication and recombination machineries (41). Re-integration of RCR products into the genome may provide a novel mechanism for expansion of tandem repeats in eukaryotic genomes. Conversely, the excision of eccDNA from the chromosome may play a balancing role in controlling the number of chromosomal repeats and preventing their overexpansion.

Finding similar RCIs in other organisms will indicate whether this is a general constituent of the plasticity of the eukaryotic genome.

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